WATER BUFFALO MILK CHAIN IN BANGLADESH

Ph.D.

Dissertation

Department of Physiology
Sylhet Agricultural University
Sylhet

Department of Veterinary and Animal Sciences
Università degli Studi di Milano
Lodi, Italy

July 2023

WATER BUFFALO MILK CHAIN IN BANGLADESH

A Dissertation

Submitted in accordance with the requirements of the Sylhet Agricultural University, Sylhet, Bangladesh and Università degli Studi di Milano, Lodi, Italy

for the degree of

DOCTOR OF PHILOSOPHY

By

Shuvo Singha

ID: 20010201; Reg: 5185 Year 2019-20 (SAU)

Reg: R12775 Year 2019 (UNIMI)

Department of Physiology
Sylhet Agricultural University
Sylhet

Department of Veterinary and Animal Sciences
Università degli Studi di Milano
Lodi, Italy

July 2023

WATER BUFFALO MILK CHAIN IN BANGLADESH

A Dissertation

Submitted in accordance with the requirements of the Sylhet Agricultural University, Sylhet, Bangladesh and Università degli Studi di Milano, Lodi, Italy

for the degree of

DOCTOR OF PHILOSOPHY

By

ID: 20010201; Reg: 5185; Year 2019-20 (SAU)

Reg: R12775; Year 2019 (UNIMI)

Approved as to style and contents by

…….	…….	…….
Prof. Md. Matiar Rahman Howlader DVM, Ph.D. Sylhet Agricultural University Bangladesh	Prof. Fabrizio Ceciliani DVM, Ph.D. Università degli Studi di Milano Italy	Assoc. Prof. Ylva Persson DVM, Ph.D. State Veterinarian National Veterinary Institute Sweden

…….	…….
Assoc. Prof. Gerrit Koop DVM, Ph.D. Utrecht University The Netherlands	Prof. Md. Mizanur Rahman DVM, Ph.D. Chattogram Veterinary and Animal Sciences University Bangladesh

July 2023

This modest effort to accomplish this Ph.D. dissertation is dedicated to my Ph.D. supervisors, my parents, my younger sister, and my fellow colleagues who have supported me during the entirety of this journey.

DECLARATION

I, Shuvo Singha, declare that this thesis is submitted to fulfill the requirements for a dual (joint) Doctoral program between Sylhet Agricultural University, Bangladesh, and the University of Milano, Italy. I am the sole author of this thesis, and I also declare that, except where otherwise stated, this dissertation is based entirely on my work and has not been submitted in any form to any other university for any degree. All the contents of the thesis that are not referenced or acknowledged are the author's copyrighted material. The material may be used only with the author's permission, and the author reserves the right to make any changes to the original document.

Date: July 2023 Shuvo Singha

ACKNOWLEDGEMENTS

All praises go to the Almighty, Who enables the author to accomplish his thesis. The author expresses his most profound and heartfelt gratitude to his respected supervisor Prof. Dr. Md. Matiar Rahman Howlader, Department of Physiology, Faculty of Veterinary, Animal and Biomedical Sciences, Sylhet Agricultural University (SAU), Bangladesh, for his guidance. The author also wants to show his sincere gratitude to the supervisory team members Prof. Dr. Fabrizio Ceciliani, Department of Veterinary Medicine and Animal Sciences (DIVAS), University of Milano (UNIMI), Italy, Dr. Ylva Persson, State Veterinarian, and Associate Professor, National Veterinary Institute (SVA), Sweden, Dr. Gerrit Koop, Associate Professor, Sustainable Ruminant Health, Department of Farm Animal Health, Department of Population Health Sciences, Utrecht University (UU), the Netherlands, and Prof. Dr. Md. Mizanur Rahman, Department of Medicine and Surgery, Chattogram Veterinary and Animal Sciences University (CVASU) for their supervision and valuable guidance during the fieldwork, laboratory protocol development, data analysis, drafting of the manuscripts, and thesis writing. The author is grateful to Prof. Dr. Md. Ahasanul Hoque, Department of Medicine and Surgery, CVASU, Prof. Dr. Mohammed Kawser Hossain, Department of Surgery and Theriogenology, SAU, and Prof. Dr. Sofia Boqvist, Department of Biomedical Science and Veterinary Public Health, Swedish University of Agricultural Sciences (SLU), Uppsala, Sweden for their contribution in the study proposal development, manuscript preparation, and guidance to follow the regulations between the two host institutes. The author also acknowledges the support of Prof. Dr. Valerio Bronzo and Prof. Dr. Paolo Moroni, DIVAS, UNIMI, and Professor Dr. Syed Sayeem Uddin Ahmed, Department of Epidemiology and Public Health SAU, for their kind help for the shipment of the bacterial isolates from CVASU to DIVAS, UNIMI. The author thanks Dr. Domenico Vecchio, Istituto Zooprofilattico Sperimentale del Mezzogiorno- Italian National Reference Centre on Water Buffalo Farming and Productions Hygiene and Technologies (IZSM) facilitating several buffalo farm visits in Italy and his guidance in assessing body condition score of water buffalo. The author also wants to thank Prof. Dr. Cristina Lecchi, Prof. Dr. Maria Filippa Addis, Prof. Dr. Renata Piccinini, and Dr. Clara Locatelli, DIVAS, UNIMI, for their guidance and cooperation during the laboratory works. He also wants to give special thanks to Dr. Sanjib Chandra Nath, Dr. Ovirup Bhuson Paul, and Dr. Salma Chowdhury for their help during the whole research work and Dr. Ajay Deb

Nath and Dr. Mohammad Abdul Mannan for their partial support during their field activities. The author would like to express his deep sense of gratitude to Udder Health Bangladesh (UHB), Sylhet Agricultural University Research System (SAURES), and the Buffalo project "Climate change mitigation by a sustainable water buffalo dairy chain in Bangladesh" financed by the Swedish Research Council for providing necessary research funds. I thank the participating buffalo farmers for their cooperation. The author also thanks the UHB bacteriology laboratory, CVASU for their technical support, the Department of Livestock Services, Bangladesh, and a non-governmental organization named the "Palli Karma-Sahayak Foundation" working on buffalo in Bangladesh for sharing buffalo distribution data with the research team during the field visits. Finally, the author thanks his parents, seniors, juniors, and well-wishers.

The Author

July 2023

BIOGRAPHICAL SKETCH

Shuvo Singha was born on December 27th, 1993, in Jashore, Bangladesh. His father's name is Suvash Chandra Singha, and his mother's name is Sumitra Rani Sarker, who are currently teacher in two government primary schools in Jashore, Bangladesh. Shuvo completed his five-year primary education during 1998-2002 at Manirampur Government Primary School. Then he attended Manirampur Government Secondary School from 2003-2007 and Manirampur Degree College from 2008-2009. He then graduated in Veterinary Medicine (DVM) in 2016 and his MS in Epidemiology in 2019 under the Department of Medicine and Surgery Chattogram Veterinary and Animal Sciences University, Bangladesh. During his master, he served 18 months as a research assistant in "Udder Health Bangladesh" funded by Swedish Research Council. He did his master's research on "Incidence rate of clinical mastitis, determination of antimicrobial resistance using minimum inhibitory concentration, and identify the risk factors of clinical mastitis in dairy cows in intensive rearing system in Chattogram, Bangladesh". In 2019-2020, he started his journey as a joint doctoral Ph.D. fellow and research assistant in "The water buffalo milk chain in Bangladesh" between the two host institutes: Sylhet Agricultural University, Bangladesh and University of Milano, Italy. A Swedish Government funded the Ph.D. project, "Climate change mitigation by a sustainable water buffalo dairy chain in Bangladesh", jointly collaborating between Bangladesh, Italy, Sweden, and the Netherlands. During his Ph.D., he was given the opportunity to train and guide four masters students from Bangladesh, one from Sweden, and another from Italy on cultural identification of mastitis-causing bacteria in water buffalo, bulk milk somatic cell count, and aerobic bacterial counts on milk and milk product samples and finally drafting their thesis. As part of their Ph.D. studies, he spent one year in DIVAS, UNIMI, and another three months at Utrecht University. During this oversea stay, he was trained in Real-time PCR and 16s rRNA sequencing. He received one month of laboratory training on testing milk quality, identifying zoonotic pathogens, and screening genetically modified feed in Istituto Zooprofilattico Sperimentale del Mezzogiorno, Portici, Italy, which is the reference laboratory for investigating animal diseases in Italy. He also had the opportunity to undertake several epidemiological courses during his stay, and he had the opportunity to visit several organized water buffalo farms in Italy and the Netherlands. During his secondment in the EU, he had a chance to experience travel to five EU countries: Italy, France, Germany, Switzerland, and the Netherlands.

LIST OF PUBLICATIONS

Peer-reviewed publications published within the Ph.D. frame time.

From Ph.D. work

1. **Singha S**, Ericsson CD, Chowdhury S, Nath SC, Paul OB, Hoque MA, Boqvist S, Persson Y, Rahman MM. *Occurrence and etiology of subclinical mastitis in water buffalo in Bangladesh*. Journal of Dairy Research. 2021;88(3):314-320. doi: 10.1017/S0022029921000698. PMID: 34412714 **(Published** on 20.08.2021**)**.
2. **Singha S**, Koop G, Ceciliani F, Derks M, Hoque MA, Hossain MK, Howlader MMR, Rahman MM, Khatun M, Boqvist S, Persson Y. *The prevalence and risk factors of subclinical mastitis in water buffalo (Bubalis bubalis) in Bangladesh*. Research in Veterinary Science. 2023;158:17-25. doi: 10.1016/j.rvsc.2023.03.004. PMID: 36907020 **(Published** on 08.03.2023**)**.
3. **Singha S,** Koop G, Rahman MM, Ceciliani F, Addis MF, Howlader MMR, Hossain MK, Piccinini R, Locatelli C, Persson Y, Bronzo V. Pathogen group-specific risk factors for intramammary infection in water buffalo **(Under review on PLOS One).**
4. **Singha S**, Persson Y, Dutta, PK, Ceciliani F, Rahman MM. *Traditional buffalo milk chain in Bangladesh*. Animal Health Report Nº 15. International Dairy Federation. 2021; 24-25.
5. **Singha S,** Ceciliani F, Rahman MM, Mannan MA, Chowdhury S, Nath SC, Paul OB, Persson Y, Boqvist S. *Water buffalo milk chain and associated factors affecting bulk milk somatic cell count, and bacterial counts in Bangladesh*. Tropical Animal Health and Production. 2023, 55(4), 242. https://doi.org/10.1007/s11250-023-03644-x **(Published** on 16.06.2023**).**
6. **Singha S,** Koop G, Rahman MM, Ceciliani F, Howlader MMR, Boqvist S, Cremonesi P, Hoque MN, Persson Y, Leechi C. Prevalence of foodborne zoonotic pathogens in milk and milk products along the buffalo milk value chain in Bangladesh (**Manuscript preparation in progress for submission**).

Other publications

1. **Singha S**, Koop G, Persson Y, Hossain D, Scanlon L, Derks M, Hoque MA, Rahman MM. Incidence, *Etiology, and Risk Factors of Clinical Mastitis in Dairy Cows under Semi-Tropical Circumstances in Chattogram, Bangladesh*. Animals (Basel). 2021;11(8):2255. doi: 10.3390/ani11082255. PMID: 34438713; PMCID: PMC8388477. **(Published** on 30.07.2021**).**
2. Bari MS, Rahman MM, Persson Y, Derks M, Sayeed MA, Hossain D, **Singha S**, Hoque MA, Sivaraman S, Fernando P, Ahmad I, Samad A, Koop G. *Subclinical mastitis in dairy cows in south-Asian countries: a review of risk factors and etiology to prioritize control measures*.

Veterinary Research Communication. 2022;46(3):621-640. doi: 10.1007/s11259-022-09948-x. PMID: 35701569. **(Published** on 14.06.2022**).**

3. Islam MF, Rudra PG, **Singha S**, Das T, Gebrekidan H, Uddin MB, Chowdhury MYE. *Molecular Epidemiology and Characterization of Theileria in Goats*. Protist. 2021;172(2):125804. doi: 10.1016/j.protis.2021.125804. PMID: 33964594. **(Published** on 26.03.2021**).**
4. Nath C, Das T, Islam MS, Hasib FMY**, Singha S,** Dutta A, Barua H, Islam MZ. *Colistin resistance in multidrug-resistant Escherichia coli isolated from retail broiler meat in Bangladesh*. Microbial Drug Resistance. 2023 Sep 12. doi: 10.1089/mdr.2023.0026. Epub ahead of print. PMID: 37699212. **(Published** on 12.09.2023**).**

LIST OF OTHER ACTIVITIES

LIST OF COURSES AND ACTIVITIES (2019-2023)

SL.	Course name	Hosting institute	Duration
1.	The animal microbiome: basic concepts and implications for veterinary medicine and animal productions	Tutor: Prof Filippa Addis, Department of Veterinary and Animal Sciences, University of Milano, Milan, Italy	3 Credits (15th May to 5th June 2020)
2.	Laboratory rotation at reference Veterinary Research Centre	IZSM National Reference Centre for Hygiene and Technologies of Water Buffalo, Salerno, Campania, Italy	15 days (16th-3rd March 2022)
3.	Intensive buffalo farm visit in Italy	IZSM National Reference Centre for Hygiene and Technologies of Water Buffalo, Salerno, Campania, Italy	3 days (19th, 21th, and 23th March 2022)
4.	Workshop on how to prepare a research data dissemination plan	University of Milano, Milan, Italy	2 days (15th February and 8th July 2022)
5.	Business and Value creation in University's spin-off	University of Milano, Milan, Italy	2 days (8th June and 16th July 2022)
6.	Empirical surveys and causal experiments in consumer Research	Tutor: Assoc. Prof Demartini Eugenio, Department of Agricultural and Environmental Sciences, University of Milano, Milan, Italy	3 Credits (12th to 26th May 2022)
7.	Fundamentals of statistics (for veterinary and animal science)	Tutor: Prof Paola Crepaldi, Department of Agricultural and Environmental Sciences, University of Milano, Milan, Italy	4 Credits (12th April to 11th May 2022)
8.	Digital Imaging in scientific publications	Tutor: Prof Valentina Lodde, Dept of Veterinary and Animal Sciences, University of Milano, Milan, Italy	3 Credits (15th to 28th February 2022)
9.	Introduction to statistical analysis of ecological and environmental data	Tutor: Assoc. Prof Ambrosini Roberto, Department of Environmental Science and Policies, University of Milano, Milan, Italy	5 Credits (1st to 26th January 2022)
10.	Modern Methods in Data Analysis	Tutor: Drs. C.L.J.J. Kruitwagen, Julius Center for Health Sciences and Primary Care, UMC Utrecht, Utrecht University	4.5 Credits (9th – 27th January 2023)
11.	Generalized Linear Models	Tutor: Ms. R.K. Stellato, Julius Center for Health Sciences and Primary Care, UMC Utrecht, Utrecht University	1.5 Credits (27th February-3rd March, 2023)
12.	Study Design in Veterinary Epidemiological Research	Tutor: Dr Egil A.J. Fischer, UU - Faculty of Veterinary Sciences - Department of Farm Animal Health	1.5 Credits (27th February-31st March 2023)

PARTICIPATION IN INTERNATIONAL SCIENTIFIC CONFERENCES

1. Oral presentation on "***Herd, buffalo, and quarter specific prevalence and risk factors of subclinical mastitis in water buffaloes of Bangladesh***" held on 4-8th of September 2022, 31st World Buiatrics Congress, Madrid, Spain. https://www.wbc-madrid2022.com
2. Oral presentation on "***Subclinical mastitis in water buffaloes in Bangladesh: prevalence and risk factors***" virtually held on 3-5th of June 2021, 10th Asian Buffalo Congress. https://www.abc2021.org/
3. Oral presentation on ***"Subclinical mastitis in water buffalo: Understanding causes"*** virtually held on 3-6th of November 2020, 8th International Symposium on Sheep, Goat and other non-Cow Milk. https://www.fil-idf.org/sheepandgoat2020/

PARTICIPATION IN CONFERENCES IN UNIMI, ITALY, AND SAU, BANGLADESH

1. Presented thesis outputs in 2nd Ph.D. Seminar held on 10th May 2023 organized by Sylhet Agricultural University, Sylhet, Bangladesh.
2. Presented thesis methodology in 1st Ph.D. Seminar held on 30th January 2023 organized by Sylhet Agricultural University, Sylhet, Bangladesh.
3. Oral presentation on "***The buffalo milk value chain in Bangladesh***" held on 26-28th of September 2022, VAS Days organized by the Department of Veterinary Medicine and Animal Sciences, Università degli Studi di Milano, Lodi, Italy.
4. Oral presentation on "***Prevalence, etiology and risk factors of mastitis in buffaloes: a systematic review and meta-analysis***" held on 8-10th and 12th of February 2021, Winter Presentation and Lectures organized by Department of Veterinary Medicine and Animal Sciences, Università degli Studi di Milano, Lodi, Italy.
5. Participated in the 2nd Ph.D. welcome day held on 27th November 2020 organized by Università degli Studi di Milano, Italy.

AWARDS

Awarded as a non-paying guest at "Uppsala Health Summit 2022" on "HEALTHY LIVES FROM SUSTAINABLE FOOD SYSTEMS" held on 25-26th October, Uppsala, Sweden.

WATER BUFFALO MILK CHAIN IN BANGLADESH

S SINGHA

ABSTRACT

The present study aimed to assess udder health by estimating the prevalence of subclinical mastitis (SCM) and identifying intramammary infection (IMI) causing pathogens. Furthermore, milk quality was evaluated by measuring bulk milk somatic cell count (BMSCC) on buffalo farms and total bacterial count (TBC), total non-aureus staphylococci count (TNAS), total *Staphylococcus* (*S.*) *aureus* count (TSA), total Enterobacteriaceae count (TEC), and the presence of zoonotic pathogens along the water buffalo milk chain in Bangladesh. A total of 248 water buffalo farms from nine buffalo concentration districts, comprising 51 free-range, 106 semi-free-range, 33 households, 54 semi-intensive, and four intensive buffalo farms, were visited to collect individual quarter milk to diagnose SCM using CMT; BMSCC was also measured on-farms. One hundred twenty-two farm bulk milk samples, 109 middlemen milk samples, 111 milk collection centers, and 35 milk product samples were collected and assessed for TBC, TNAS, TSA, and TEC. A subset of these samples, comprising farm bulk milk (n = 34), the middleman (n = 37), the milk collection center (n = 37), and milk products from shops (n = 35), were analyzed using real-time PCR to estimate the prevalence of seven important zoonotic pathogens: *S. aureus*, *Escherichia* (*E.*) *coli*, Shiga toxin-producing *E. coli* O157:H7, *Campylobacter* (*C.*) *jejuni*, *Listeria* (*L.*) *monocytogenes*, *Salmonella* (*S.*) *enterica*, and *Yersinia* (*Y.*) *enterocolitica*. Quarter, buffalo, farm level, and milk value chain-related data was collected using questionnaires and observations. The overall SCM prevalence was high at the quarter level (27.9 %) and the buffalo level (51.5 %). Non-aureus staphylococci were the predominant IMI-associated bacterial species. The proportion of non-aureus staphylococci or *Mammaliicoccus sciuri* (NASM), *S. aureus*, and other bacterial species identified in the buffalo quarter samples varied between buffalo farms. An intensive buffalo-rearing system, teats on the left, funnel-shaped teats, udder asymmetry, single milker, and a lack of quarantine facility were all associated with poor buffalo udder health. The geometric mean of BMSCC was 217,000 cells per mL of milk, which was low on average, but some farms could improve substantially. Farm-hygiene-related risk factors for IMI were poor milking hygiene and cleanliness of the hind quarters. The likelihood of IMI from any pathogen and NASM were higher in buffalo herds with poor milking hygiene. Poor cleanliness of the hind quarters increased

the chances of IMI caused by any pathogen, NASM, and *S. aureus*. Farm management-related risk factors for IMI were the milking frequency and buffalo source. Farms with buffalo purchased from another herd were associated with IMI from any pathogen. Udder symmetry and body condition score (BCS) were risk factors related to buffalo breeding and nutrition. Asymmetrical udders were associated with IMI caused by any bacteria and with IMI caused by *S. aureus*. Poor BCS showed higher odds of IMI from any pathogen and by NASM. Progressively increasing TBC, TNAS, and TEC levels were observed along the milk value chain ($P < 0.05$). High TBC was associated with the winter season, cleaning the milk containers using pond water, a mix of buffalo and cow milk, and a poor cleanliness score for the milk containers. Farms located in coastal areas were associated with high levels of TNAS. Zoonotic pathogens' prevalence increased over the water buffalo milk value chain. Three classical enterotoxin-encoded genes for *E. coli O157:H7* were tested, *eae*, *stx1*, and *stx2,* of which the *stx2* genotype was the most prevalent in milk products (74 %). *L. monocytogenes* and *Y. enterocolitica* were more prevalent in the farm bulk milk (65-79 %) than in the later milk value chain nodes. The prevalence of *S. enterica* was low (0 - 2.9 %) in all the milk chain nodes, and all the samples were negative for *C. jejuni*. Using plastic containers and dirty clothes to clean milk containers were the risk factors associated with contamination by zoonotic pathogens in the buffalo milk value chain.

There was a high prevalence of animal and quarter level SCM, but the overall mean BMSCC was satisfactory. A higher prevalence of SCM and BMSCC was observed in intensive farms than in free-range or semi-intensive farms. Non-aureus staphylococci were the most prevalent-IMI-causing pathogen. Poor milking hygiene and cleanliness of hind quarters were associated with a higher prevalence of NASM. Overall, the prevalence of most zoonotic pathogens in milk and milk products was very high. Our findings indicate that improving udder health and hygiene practices during milk handling could help ensure the safety and quality of buffalo milk and milk products consumed in Bangladesh.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

Abbreviation	Elaboration
BBA	Bovine blood agar
BCS	Body condition score
BMSCC	Bulk milk somatic cell count per mL of milk
BPA	Baird Parker Agar
C. jejuni	*Campylobacter jejuni*
CFU	Colony forming unit
CM	Clinical mastitis
CMT	California mastitis test
CVASU	Chattogram Veterinary and Animal Sciences University
DIVAS	Department of Veterinary Medicine and Animal Sciences
DLS	Department of Livestock Services
E. coli	*Escherichia coli*
EMB	Eosin Methylene Blue
GDP	Gross domestic product
IMI	Intra-mammary infection
L. monocytogenes	*Listeria monocytogenes*
LBMSCC	Log10-transformed BMSCC
LMIC	Low and middle-income countries
LRT	Likelihood ratio test
MAC	MacConkey agar
MALDI-TOF MS	Matrix-assisted laser desorption/ionization mass spectrometry
MCMT	Modified California mastitis test
MSA	Mannitol salt agar
NAS	Non-aureus staphylococci
NASM	Non-aureus staphylococci or *Mammaliicoccus sciuri*
NHLBI	National Heart, Lung, and Blood Institute
NMC	National mastitis council
PCA	Plate count agar
PKSF	Palli Karma-Sahayak Foundation
PRISMA	Preferred Reporting Items for Systematic Reviews and Meta-Analyses
RT-PCR	Real-time polymerase chain reaction
S.	*Staphylococcus*

SAU	Sylhet Agricultural University
SCC	Somatic cell count
SCM	Subclinical mastitis
SFMT	Surf field mastitis test
Strep.	*Streptococcus*
TBC	Total bacterial count per mL of milk
TEC	Total Enterobacteriaceae count per mL of milk
TNAS	Total non-aureus staphylococci count per mL of milk
TSA	Total *Staphylococcus aureus* count per mL of milk
UHB	Udder Health Bangladesh
UNIMI	Università degli Studi di Milano
UVH	Upazila veterinary hospital
VIF	Variance inflation factor
VRBG	Violet red bile glucose agar
WST	White slide test
Y. enterocolitica	*Yersinia enterocolitica*

CHAPTER-1

INTRODUCTION

CHAPTER 1

GENERAL INTRODUCTION

Livestock is an integral component of Bangladesh's agro-based farming system and is the primary protein, draught power, and manure-based organic fertilizer source (Uddin et al., 2011). It also serves as a principal source of employment. The livestock sector contributes 20 % of the employment for the entire population and provides partial employment for another 50 % (DLS, 2022). It is estimated that in 2021-2022, livestock made up 1.9 % of the Gross Domestic Product (GDP), and the GDP growth rate was 3.1 %, higher than in any year since 2007-2008. The Upazila veterinary hospitals (UVH), a part of the government livestock services, collect buffalo population data at the sub-district level in Bangladesh. Data collection on the number of farms is managed through a census by the UVH every year. The current dairy production in Bangladesh is dominated by 24.7 million head of cattle and 1.5 million head of buffalo (DLS, 2022). The annual milk output has grown from 0.72 million metric tons in 2006 - 07 to 13.1 million metric tons in 2021 - 22, ensuring milk availability of 200 mL per person per day, failing to meet the requirement of 250 mL (DLS, 2021). Milk demand is increasing faster than supply, and production is still insufficient.

In recent years, urbanization, education growth, a drop in poverty, and a rise in purchasing power have indeed changed people's food preferences, causing them to demand more animal-derived food products such as milk, meat, butter, cheese, ice cream, and locally made traditional sweets, which are processed from milk (Uddin et al., 2011; Nahar et al., 2022). Therefore, the Bangladesh government has enforced a priority on dairy development in recent years by launching large projects like the "Livestock and Dairy Development Project" (Worldbank, 2022) with 500 million USD in financial support from the World Bank for the development of livestock value chain and food safety; providing the free distribution of vaccines from the Bangladesh Livestock Research institute since 1998; offering cash incentives to the farmers; and exempting pasteurized milk from value added tax to encourage dairy farmers and meet the needs of the current milk deficit (Uddin et al., 2011; Hamid et al., 2016b).

Water buffalo (*Bubalus bubalis*) is the second-highest contributor to global milk production and is South Asia's primary milk-producing dairy species (Minervino et al., 2020). Water buffalo are known for their better ruminal fermentation and nitrogen

utilization capacity than dairy cows, resulting in higher efficiency in their ability to digest low-quality feed, convert it into high-quality meat and milk, and resist many diseases (Jabari et al., 2014; Deb et al., 2016; Samad, 2020). Water buffalo contributes significantly to the total national milk production in neighboring Asian countries, e.g., India (56 %), Pakistan (61 %), and Nepal (65 %), whereas water buffalo contribute only 3 - 4 % of the total milk production in Bangladesh (Hamid et al., 2016a; Hamid et al., 2016b). Although water buffalo are an important livestock species in Bangladesh, they have never been prioritized or used to improve milk and meat production potential. However, water buffalo are essential to the national economy (Hamid et al., 2016a). Therefore, there is a substantial potential for an increase in water buffalo farming in Bangladesh. Large coastal areas and numerous existing and developing islands could provide feed resources. This species could be exploited by improving buffalo husbandry to meet an additional source of milk to close the existing demand and supply gap (Samad, 2020).

Water buffalo are sporadically distributed across the country, mainly in the free-range based buffalo-rearing system in the saline coastal area or household subsistence in river basin areas of Bangladesh. A few intensive buffalo farms are managed under government or private initiatives, mainly used for breeding (Faruque et al., 1990; Hamid et al., 2016a; Samad, 2020). The buffalo population in Bangladesh is primarily the indigenous riverine type, except for a few swamp-type buffalo in the eastern part of the country. However, some high-yield crossbreeds of Murrah, Nili-Ravi, Surti, and Jafarabadi are also farmed in the area surrounding the Indian border (Hamid et al., 2016a; Hamid et al., 2016b). Buffalo are primarily reared for milk and meat production and partially to overcome draught issues. Bangladesh's coastal and semi-coastal districts, surrounded by the Meghna-Ganga and the Jamuna-Brahmaputra river floodplains, are the critical areas of buffalo concentration, where 40 % of the buffalo population in Bangladesh is kept. This is due to the availability of fallow pasture land and green forages (Faruque et al., 1990; Hamid et al., 2016b). The daily milk production per buffalo (2.0 - 3.5 Liters) is higher than the milk yield from indigenous cattle, and the milk has a high-fat percentage, ranging from 6.8 - 13.2 % (Faruque et al., 1990; Hamid et al., 2016b; Habib et al., 2017). Therefore, this milk has a higher nutritional potential, can be sold at a better price, and contributes to minimizing the current milk deficit in the country. Buffalo milk production and sale are carried out directly by producers, vendors, and commercial milk processors

(Hamid et al., 2016a). However, mastitis is considered a significant economic concern in water buffalo, especially in developing countries where the animals are farmed in conditions that sometimes do not meet the basic hygiene requirements.

Mastitis is an inflammation of the mammary gland that causes detrimental effects on the quality and quantity of milk in dairy animals, especially cows, buffalo, and goats (Hussein et al., 2020; Krishnamoorthy et al., 2021a; Bari et al., 2022), resulting significant economic losses, increasing antibiotic use, and impairing animal welfare (Sinha et al., 2014; Ahmed et al., 2018; Badua et al., 2020). Buffalo mastitis is notably caused by a bacterial intra-mammary infection (IMI) (Moroni et al., 2006; Badua et al., 2020), leading to pathological changes in milk, the udder, and/or the animal. The changes can be clinical or subclinical. Clinical mastitis (CM) in water buffalo can be diagnosed through visible changes in the milk or the udder. When it cannot be determined through manual palpation or visual examination, it is defined as subclinical mastitis (SCM) (Baloch et al., 2016; Javed et al., 2022). Subclinical mastitis is three times more prevalent than CM in buffalo and is responsible for milk quality deterioration and processability (Ali et al., 2014; Pasquini et al., 2018; Moradi et al., 2021). Subclinical mastitis in water buffalo can be diagnosed by directly measuring the milk somatic cell count (SCC) using a somatic cell counter (Jhambh et al., 2017; Kaur et al., 2018). In contrast, the California mastitis test (CMT) is a qualitative test that measures the SCC indirectly; it is mainly used to detect SCM in individual animals or quarters (Baloch et al., 2018; Islam et al., 2019).

Bacteriological culture is a reliable test for determining IMI for CM and SCM in dairy animals (Constable et al., 2016; Smistad et al., 2022). Still, it is not a routine practice in Bangladesh due to the lack of availability of technical facilities, the lack of awareness of farmers, and the inadequate numbers of field practitioners (Hamid et al., 2016b). IMI identification in water buffalo is critically important, particularly for SCM, as the clinical signs remain unnoticed, and affected animals may act as reservoirs for other healthy animals in the herd (Ali et al., 2014; Pizauro et al., 2019). Water buffalo are known to be less susceptible to mastitis compared to dairy cows because of anatomical differences in the mammary gland. For example, water buffalo have a long narrow teat canal and a thicker epithelium and keratin layer, which may prevent easy invasion of microorganisms. The high milk fat is also hypothesized to overlay and protect the epithelial layer of the teat lining (Thomas et al., 2004).

Data on the prevalence of SCM in water buffalo in Bangladesh is not robust. A few previous studies have demonstrated a varied prevalence of 11 - 28 % in the Bhola district in Bangladesh (Islam et al., 2019; Biswas et al., 2020; Aliul et al., 2021). However, the actual prevalence is probably much higher and more variable in the larger buffalo population because of the diversification of buffalo-rearing systems, buffalo management, and farm-related factors, which could result in a wide variability of IMI-causing pathogen exposure. Previous studies identifying the risk factors of SCM in water buffalo are limited in Bangladesh, if not absent. A previous study showed that SCM prevalence was higher in buffalo < 5 years of age and in early lactations (1^{st} - 2^{nd} parity) (Biswas et al., 2020). However, no further information is available, for example, reporting the association of quarter level factors (e.g., teat shape or teat lesions), animal level factors (e.g., milk yield, previous history of CM and pregnancy status), and farm level factors (e.g., type of buffalo-rearing system, type of hand milking, pre-milking stimulation, and milkers' and udder hygiene). Therefore, a nationwide cross-sectional study could help estimate the prevalence of SCM, identify IMI-causing pathogens, and determine associated risk factors to identify and implement the most optimal preventive measures.

It is known in dairy cows that milk includes minimal organisms inside the mammary gland unless the animal has a systemic illness or IMI (Rainard and Riollet, 2006; Addis et al., 2016). Still, milk may be contaminated by bacteria, including *Staphylococcus*, *Streptococcus*, *Bacillus*, *Micrococcus*, *Corynebacterium*, and occasionally coliforms when excretes via the teat canal and touches the teat skin (White et al., 1989; Svennesen et al., 2019). Milk may also be contaminated when the animal suffers from a systemic infection, from the udder as a result of IMI, or through contamination from the environment during the collection and storage of milk (De Buyser et al., 2001; Verraes et al., 2015; De Silva et al., 2016). Most buffalo farmers are rural small-holder farmers who usually keep the animals in a traditional crop-livestock integrated system with little technical understanding of buffalo production or milk hygiene (Hamid et al., 2016b; Habib et al., 2017). There is also a lack of government support and training facilities to upgrade their knowledge (Habib et al., 2017). Therefore, milk can also be contaminated by various types of microorganisms mainly because of inadequate technical knowledge related to water buffalo husbandry, for example, poor udder health and unhygienic handling of raw

milk during transportation and processing along the water buffalo milk production chain in Bangladesh (Hamid et al., 2016b; Samad, 2020).

Bulk milk somatic cell count is a marketability threshold for milk quality in many developed countries (Troendle et al., 2017). Milk somatic cells in dairy animals mainly include neutrophils, lymphocytes, macrophages, and some epithelial cells from the epithelium lining of the alveoli and ducts (Alhussien and Dang, 2018). Levels of somatic cells in milk usually increase in water buffalo with the onset of inflammation in the mammary gland, mainly in response to an IMI (Moroni et al., 2006). Previous studies on BMSCC in Bangladesh are scarce due to a lack of awareness of its use and the lack of somatic cell counters. In international standards for drinkable milk, a BMSCC below 200,000 cells per mL indicates healthy milk in dairy cows (Barkema et al., 2013; Norman et al., 2013), with many European countries considering 400,000 cells per mL acceptable. The acceptable BMSCC limit in Canada was reported to be 500,000 cells per mL (Barkema et al., 1998; Sargeant et al., 1998). In other countries, like the USA, the standard legal limit for SCC in milk was reported to be 750,000 cells per mL (Norman et al., 2019), but the limit varies between states. However, there is no acknowledged threshold for BMSCC in water buffalo yet.

In previous studies, milk quality in dairy cows and small ruminants has been assessed through different bacterial count parameters, e.g., total bacterial count (TBC), total NAS count (TNAS), and total Enterobacteriaceae count (TEC) (Ndahetuye et al., 2020a; Berhanu et al., 2021; Khaldi et al., 2022). Total bacterial count in dairy cows involves quantifying bacteria in milk from different contamination sources, such as milking equipment, milk handlers, and environmental niches (Verraes et al., 2015; Berhanu et al., 2021). The legal limit for TBC of consumable raw milk suggested in the European Union is less than 1×10^5 CFU for cow milk and 1.5×10^6 CFU per mL in milk from other species (European Commission, No 853/2004). On the other hand, manufacturing cheese from non-cow raw milk should meet a TBC count lower than 5×10^5 CFU per mL (Pasquini et al., 2018). According to the New South Wales food authority standards, the legal limit for TEC count should be 10^2 to $< 10^4$, and the *E. coli* should be 3 to 10^2 CFU per mL.

Consequently, identifying factors associated with elevated bacterial counts is necessary to identify measures to maintain the hygienic quality of the milk and milk products. The milk value chain in Bangladesh is non-structured, with the essential infrastructure for

production input being insufficient for the transportation and processing of milk and lacking proper milk hygiene practices (Uddin et al., 2011; Nahar et al., 2022). Microbial contamination of the raw milk may vary depending on the education status and attitude of the milk handlers and milk hygiene during milk handling from production on the farm to further processing (Islam et al., 2018; Berhanu et al., 2021). Additionally, the traditionally (raw) fermented yogurt prepared from raw milk with no pasteurization is Bangladesh's most popular buffalo milk product. It can be a source of human pathogenic organisms (Khaton et al., 2014; Habib et al., 2021). A previous study on dairy cows showed that milkers' hands, udder, teat cups, utensils, and water used during milking and cleaning the containers were potential sources for the contamination of raw milk (Hamiroune et al., 2016). Long storage times and improper storage temperatures could also result in bacterial multiplication and increase consumer health risks (Tiwari et al., 2015). Better animal health, improved milking hygiene, proper cleaning of the milk containers, maintaining a cold chain during transportation, and pasteurization of milk before further processing could therefore be necessary to reduce the bacterial contamination from pre-harvesting (milk excretion) to post-harvesting (collection, storage, processing, and distribution) of buffalo milk and milk products. To the authors' knowledge, no previous studies have reported on bacterial contamination and associated risks along the milk value chain in raw buffalo milk and dairy products in Bangladesh. Therefore, assessment of udder health and milk quality and identification of the control measures are needed to improve buffalo milk production in Bangladesh.

1.1 General objectives

The present Ph.D. project, therefore, aims to fulfill the following objectives:

i) Conduct a systematic review and meta-analysis on the prevalence, etiology, and risk factors for SCM in water buffalo,

ii) Estimate the prevalence of SCM, identify risk factors associated with SCM and farm level risk factors related to BMSCC in water buffalo in Bangladesh,

iii) Estimate the prevalence and identify risk factors associated with IMI-causing pathogens in water buffalo in Bangladesh,

iv) Explore the water buffalo milk chain and identify factors affecting BMSCC and bacterial counts by measuring TBC, TNAS, TSA, and TEC in Bangladesh, and

v) Estimate the prevalence of zoonotic pathogens in the milk and milk products along Bangladesh's water buffalo milk value chain.

CHAPTER-2

REVIEW OF LITERATURE

CHAPTER 2

REVIEW OF THE LITERATURE

Water buffalo are important to the agricultural economies of South Asian countries with low and middle incomes as they serve as primary sources of milk, meat, and meat products. They are the second most significant dairy species after cows. Importantly, in India, Pakistan, and Nepal in southeast Asia, water buffalo are the principal contributor of milk and meat. Despite sharing the same geographic location and topographical environment, Bangladesh trails its neighbors in buffalo production. Moreover, Bangladesh is at risk of not having enough feed sources to produce dairy due to ongoing global climate change. In these circumstances, buffalo may serve as a potential animal in terms of its adaptability to harsh conditions with limited feed resources, disease resistance, and better quality milk and meat production. The purpose of this chapter is to review the pertinent research and describe the features of the buffalo population, the pathophysiology of the mammary gland, buffalo milk quality, and buffalo milk microbiota. The final aim of this chapter is to find out the knowledge gaps and demonstrate the relevance of conducting the present study.

2.1 Water buffalo are an important livestock species

Water buffalo (*Bubalus bubalis*) are members of the family "Bovidae" and the sub-family of "Bovinae". Water buffalo are believed to have received their name because they prefer riverine habitats. Two types of water buffalo exist: the swamp type and the river type, both known to be descended from a wild Asian water buffalo (*Bubalus arnee*) (Presicce et al., 2017). River-type buffalo were probably domesticated around 6,300 years ago in northwestern India. From there, they traveled west, passing through Egypt, Anatolia, and southwestern Asia before arriving in the Balkans and the Italian peninsula (Morgan et al., 2007). The genetic makeup and external appearance of water buffalo of the river type differ from those of the swamp type. The chromosomal count of river-type buffalo is higher than that of swamp-type buffalo (50 vs. 48). In contrast to swamp-type buffalo, which prefer to mud-wallow, river-type buffalo prefer to wallow in fresh water. Their horn anatomy demonstrates the primary anatomical difference. Swamp-type buffalo has larger horns oriented laterally or horizontally, and the horns of river-type water buffalo are shorter and firmly curled in all directions (Fig. 2.1). China has the highest total swamp buffalo population, and India with the most river-type buffalo. China contains 63 %

swamp-type buffalo, and India 69 % river-type (Zhang et al., 2020). There are 123 known buffalo breeds, 90 in Asia alone, many of which are local, and 15 breeds have been recorded as transboundary (Mason, 1951). The Nili-Ravi, Kundi, Murrah, Jafarabadi, and Mediterranean breeds are the most known among the river-type buffalo (Khan, 2002; Minervino et al., 2020). All European buffalo are believed to be of the same breed, known as the Mediterranean: in Italy, the Mediterranean type was specially developed and is known as the Mediterranean Italian breed; in Turkey, there is the Anatolian breed; in Egypt, the Egyptian breed; in Iraq, the Khuzestani or Iraqi breed; in Azerbaijan, the Azeri or Caucasian breed; and in Iran, there are the Azeri and Khuzestani breeds. China has recognized 18 known breeds/strains of swamp buffalo, while Indonesia has identified around seven breeds/strains (Triwulanningsih and Praharani, 2006; Yang and Zhang, 2006; Hamid et al., 2016a). In Bangladesh the buffalo population comprises of indigenous non-descriptive river-type buffalo, and the cross breeds of indigenous non-descriptive breeds either with migrated river-type buffalo from India or swamp-type buffalo migrated from Myanmar (Hamid et al., 2016a).

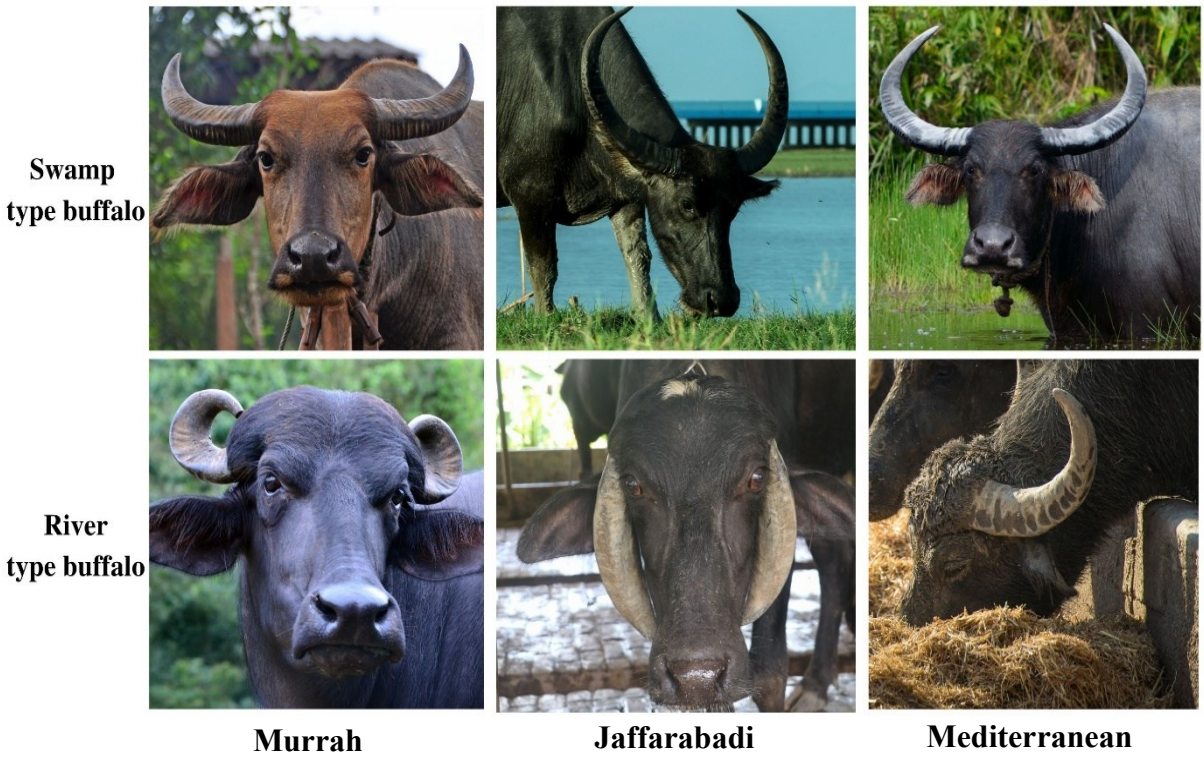


Fig. 2.1 Differences in horn structure between swamp-type and river-type buffalo

Photo source: UHB team and Canava graphic design platform.

2.2 Water buffalo population in the world and Bangladesh

An estimated 208 million water buffalo are found in 77 nations across five continents (Minervino et al., 2020). The evolution of the global water buffalo herd is shown in Fig. 2.2 using data from 1968 to 2018; over these 50 years, the population of buffalo herds has increased by 97.9 % worldwide. The global buffalo population has increased by about 40 % from 1968 to 2018.

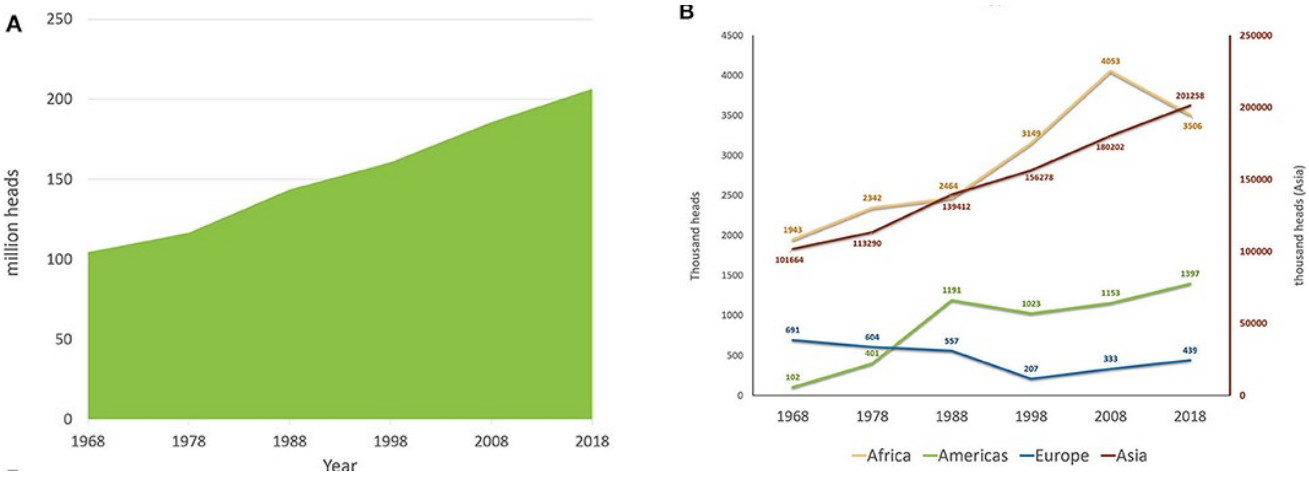


Fig. 2.2 Trends in the global buffalo population during 1968-2018

(A) The global buffalo population over the previous 50 years (1968–2018); (B) The global buffalo population over the previous 50 years (1968-2018), displayed by continent. The count for Asia is shown on the right red axis. The left axis depicts Europe, Africa, and America (Minervino et al., 2020).

About 197 million buffalo heads of water buffalo are found in Asia (97 %). South Asia accounts for 71 % of the global buffalo population in Asia. It should be emphasized that the buffalo population in Asia is steadily growing, but Europe's population declined until 1998 (Minervino et al., 2021). Due to the water buffalo breed development, and expansion of the cheese industry, only Italy has consistently increased its buffalo population. In contrast, in Eastern European countries where buffalo were used as draft animals for land work or carriages and had low milk yields, they were probably replaced by high-yielding cows and mechanization (Minervino et al., 2020). Therefore, water buffalo can be considered an economically important livestock species in South Asian countries and Italy.

Bangladesh only has 1.5 million buffalo (DLS, 2021), with roughly 40 % concentrated in the coastal areas. There are also a few buffalo pockets, including the coastal region, the Sylhet haor region, the sugar cane belt of Jamalpur, and the Kanihari buffalo pocket

in Trishal upazila of Mymensingh district of Bangladesh, which has been used exclusively for milk production (Sohel et al., 2015). In Fig 2.3, the district-level buffalo population in Bangladesh is shown.

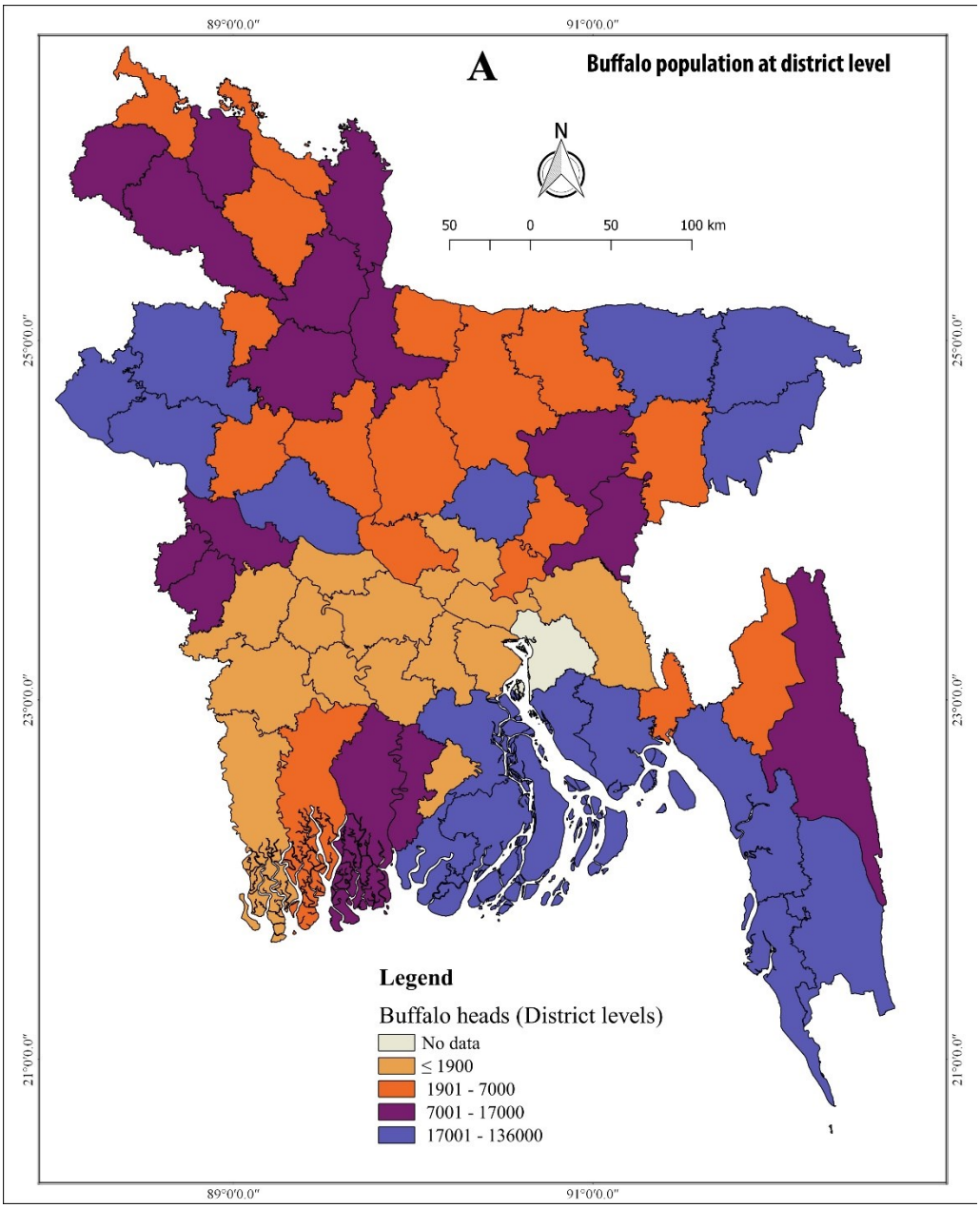


Fig. 2.3 District-level buffalo heads in Bangladesh. Data source: LDDP (2019)

Buffalo in Bangladesh can primarily be split into two categories (i) indigenous non-descriptive river-type buffalo located in the country's coastal regions and marshlands, and (ii) migratory buffalo from India and Myanmar found in the sugarcane belt and Cox's Bazar district, respectively (Saadullah, 2012). The migratory buffalo from India are primarily of the river type, and those from Myanmar are of the swamp type. However, some crossbred between the two species can be seen in the coastal area. In 1960, a small

number of Nili-Ravi buffalo were brought into Bangladesh from Pakistan to provide local farmers with breeding bulls. After that, in 1990, the Department of Livestock Services (DLS) brought in one hundred pregnant heifers of the Nili-Ravi breed from Pakistan. These buffalo were raised in the Bagherhat district on a newly formed government farm specializing in buffalo breeding. However, at present, the genetic merit level of the subsequent progeny of these purebreds has been reduced due to indiscriminate inbreeding. In addition to this, artificial insemination has been carried out in 39 Upazila (sub-districts) throughout 13 districts in Bangladesh between indigenous buffalo and Italian Mediterranean semen since the year 2010. These crossbreed water buffalo are now sporadically distributed throughout the country. They are concentrated in the Brahammaputra-Jamuna floodplain and the Meghna-Ganga floodplain (Faruque and Hossain, 2007). Around the Indian border, some crossbreeds of the non-descriptive breed with Murrah, Nili-Ravi, Surti, and Jafarabadi are also known (Faruque et al., 1990; Borghese, 2005). In general, indigenous non-descriptive varieties of water buffalo are the only recognized native breed in Bangladesh (Faruque et al., 1990).

2.3 Global features of the buffalo milk production

Buffalo milk production reached 128 million tons in 2018, accounting for 15 % of global total milk production (Minervino et al., 2020). It's interesting to note that between 2011 and 2018, buffalo milk production has grown by 32 %. About 98 percent of the world's buffalo milk is produced in only ten nations (India, Pakistan, China, Egypt, Nepal, Italy, Myanmar, Iran, Colombia, and Brazil) (Minervino et al., 2020). South Asian SAARC countries produce 93 % of the world's buffalo milk, with India and Pakistan contributing 68 % and 24 %, respectively (Hamid et al., 2016a). The buffalo is the most important milk-producing animal in India and Pakistan, with a much higher population than cows (Minervino et al., 2020). Due to a lack of data on buffalo milk production, the amount of milk produced globally is probably underestimated. Currently, 50 of the 77 nations with buffalo populations do not report any information on buffalo milk production; these 50 countries account for 7.5 million buffalo, approximately 6 % of all known buffalo populations worldwide (FAOSTAT, 2019). Among the buffalo-producing countries, only Italy has so far built a buffalo milk traceability system that collects real-time data on buffalo milk production at the national level (Cappelli et al., 2021).

In Bangladesh, buffalo are farmed in household subsistence and extensive farming in saline coastal regions as a draft animal and partially for milk and meat production (Hamid et al., 2016a). Buffalo milk production in Bangladesh is 0.04 million tonnes, which shares only a negligible amount of total national milk (4 %) compared to neighboring countries. The average annual buffalo milk production is 407 liters per animal, lower than the neighboring countries. A comparative milk production scenario from SAARC countries is given in Table 2.1.

Table 2.1 Buffalo milk production (million tons) in the world and SAARC countries (Hamid et al., 2016a; Minervino et al., 2020)

Region / Country	Buffalo population (million heads) (%)	Milk production (million tons) (%)	Annual milk production (L/animal)
World	208.1 (100)	95.8 (100)	-
Asia	201.4 (97)	93.0 (97)	-
SAARC countries	160.1 (77)	89.3 (93)	-
India	114.2 (55)	65.1 (68.0)	1,407
Pakistan	38.8 (19)	23.0 (24.0)	1,909
Nepal	5.3 (0.03)	1.1 (0.01)	842
Bangladesh	1.5 (0.007)	0.04 (0.004)	407
Sri Lanka	0.3 (0.001)	0.05 (0.0005)	648
Bhutan	0.0005 (0.000002)	0.00008	-

2.4 Buffalo production system in Bangladesh

2.4.1 Buffalo-rearing systems

Buffalo are raised across Bangladesh, with concentrations in coastal salty, plain, marshy, and hilly areas, depending on feed availability. Buffalo are raised under four production systems based on land area and type: free-range or extensive, household subsistence, semi-intensive, and intensive (Fig. 2.4). In the lower coastal area, buffalo are reared using the free-range rearing system locally known as "bathan". Bathan system comprises offshore islands, mudflats, and newer accretions. Coastal regions feature extensive vegetation for grazing, and farmers utilize buffalo for milk and meat. The herd size ranges from 50 to 600 animals and is raised on natural grazing with little or no supplemental feed (Deb et al., 2016; Habib et al., 2017). The animals rely only on grazing on fallow land in remote islands, and buffalo are also moved to various islands depending on green roughage availability. Household buffalo are raised in stalls with 6-7 hours of grazing in the backyard or on public property with little or no additional feed

supplementation. Herd size averages 1-3, with a maximum of 10. Semi-intensive herd size averages 4-15 animals, and buffalo are shifted from bathans close to the households, mainly in coastal or semi-costal locations in rice harvesting season. In intensive rearing, buffalo herd sizes range from 20 - 200 animals are always kept in the shed and bathed and fed. In winter, buffalo are bathed twice a day and three times in summer (Hamid et al., 2016a; Rahman et al., 2019a).

Fig. 2.4 Five different buffalo-rearing systems in Bangladesh

Photo source: UHB team.

2.4.2 Buffalo diseases

Hemorrhagic septicemia, calf pneumonia, helminthiasis, enteritis, and mastitis are the primary infectious diseases in water buffalo in Bangladesh (Islam et al., 2016a). Islam et al. (2016a) found that 64 % of buffalo had gastrointestinal parasites. Some other infectious diseases are bubaline herpesvirus (Caruso et al., 2016) and various zoonotic diseases, such as cryptosporidiosis, leptospirosis, giardiasis, salmonellosis, and tuberculosis (De Barros et al., 2020; Minervino et al., 2021). Prolonged acyclicity, postpartum anestrus, and losing embryos or fetuses are the few most known reproductive issues in water buffalo (El-Wishy, 2007; Qayyum et al., 2018). Buffalo is also affected by several metabolic diseases, e.g., ketosis and hypocalcemia, mainly during the early

post-partum period (Krishna et al., 2014). However, prior global research indicates that mastitis is one of water buffalo's most important infectious diseases (Ahmad, 2001; Abd-Elrahman, 2013; Pizauro et al., 2014).

2.5 Mastitis in water buffalo

Mastitis is an inflammation of the mammary gland, mainly resulting from the penetration of pathogens into the gland. Clinical mastitis is defined as inflammation with visible symptoms like changes in milk, local symptoms of the udder, and systemic symptoms (Fig. 2.5). Subclinical mastitis is defined as inflammation without visible symptoms and can only be identified by measuring inflammatory indicators in the milk. (Fig. 2.6). Currently, SCM in water buffalo are diagnosed either by direct measurement of the somatic cells by the somatic cell count (SCC) or indirect assessment by multiple screening tests, for example, the California mastitis test (CMT) or Electrical conductivity test (Dhakal et al., 2008; Hussain et al., 2018; Aldujaily et al., 2019). Diagnosis of SCM can be performed at the quarter level by testing individual quarter milk samples or at the animal level by testing the composite milk samples taken from four quarters of an animal (Table 2.2). Research on mastitis in water buffalo has mainly focused on assessing the prevalence of CM or SCM, identifying IMI-causing pathogens, and determining underlying risk factors (Moroni et al., 2006; Sharma and Sindhu, 2007; Salvador et al., 2012). Particularly IMI-causing pathogens were studied for antimicrobial resistance and biofilm-forming ability during episodes of SCM in water buffalo (Hoque et al., 2022; Selim et al., 2022; Urer et al., 2023). Recently, changes in lipidome or peptidomics profiles have been described in water buffalo, which could explore possibilities to identify the biomarkers of IMI in water buffalo (Addis et al., 2022; Ceciliani et al., 2022). Importance has also been given to developing a somatic cell count threshold in water buffalo (Dhakal, 2006). Also, previous studies attempted to explore the relationship of somatic cell count measurement with different influencing factors, such as IMI (Moroni et al., 2006; Zhang et al., 2022), effects of diseases other than mastitis, epidermal growth factor, and genetic influences (Sahoo et al., 2010; Kazak et al., 2022; Shaban et al., 2022). Several other studies aimed to evaluate mastitis treatment and prevention strategies by implementing dry cow therapy (Guccione et al., 2020), improving milking hygiene (Sahin et al., 2016; Sannino et al., 2018), or enhancing immunity through additional dietary supplementation, e.g., Selenium, vitamin E and Zinc, antioxidants, probiotics,

and herbal or medicinal plants (Shailja and Singh, 2002; Chandra et al., 2016; Yigit et al., 2018). Overall, scientists worldwide tried various creative approaches to enhance udder health, but mastitis remained an immense challenge in water buffalo which needs more research.

Table 2.2 Grading of subclinical mastitis using California mastitis test concerning somatic cell count and electrical conductivity test of quarter milk sample

Type of test	Scores	Definition	Somatic cell count	EC
California mastitis test	Score-1	No thickening (Healthy)	< 200,000 cells per mL	< 3.7 mS/cm
	Score-2	Slight thickening (Suspicious)	≥ 200,000 cells per mL	≥ 3.7 mS/cm
	Score-3	Distinct thickening (SCM)		
	Score-4	Immediate thickening with slight gel formation (SCM)		
	Score-5	Strong gel formation and the surface of the milk mixture elevates (SCM)		

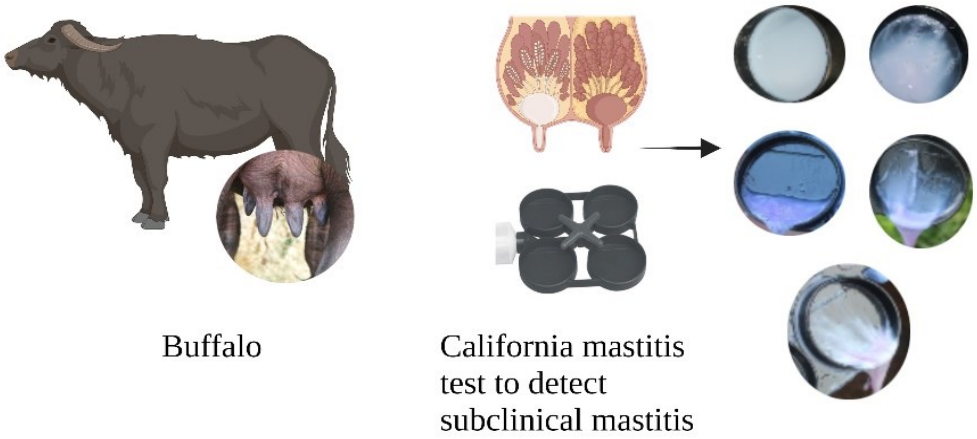


Fig. 2.5 Diagnosis of subclinical mastitis by performing California mastitis test on healthy individual quarter milk samples in water buffalo

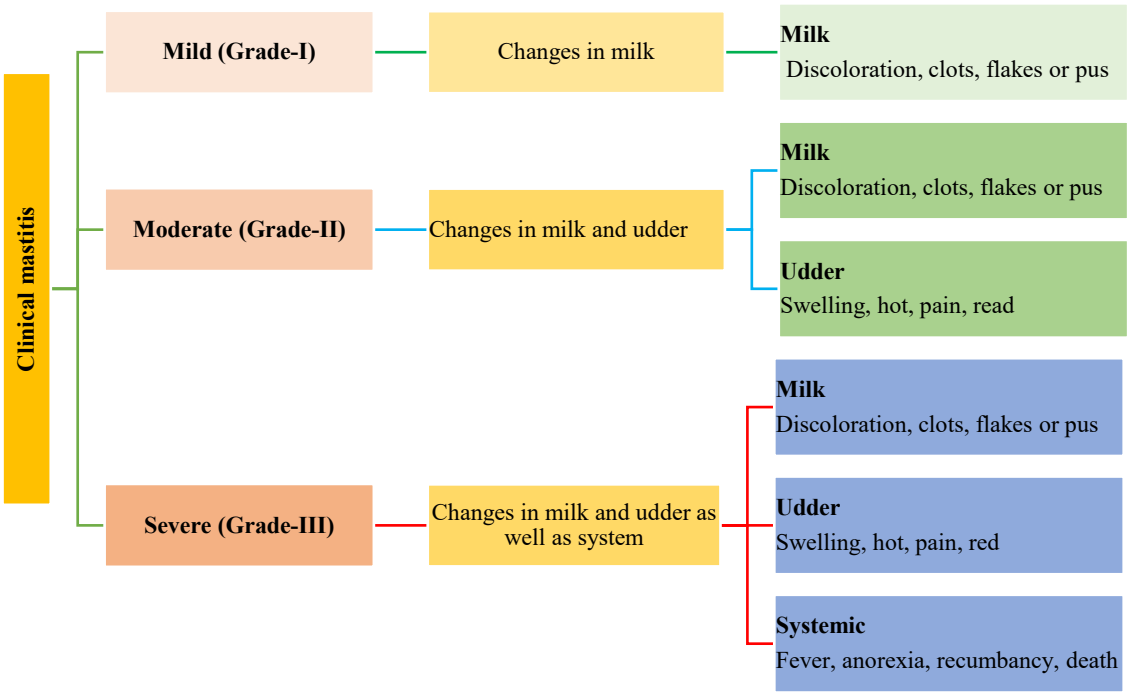


Fig. 2.6 Grading of clinical mastitis based on the presentation of the clinical signs

2.6 Prevalence of subclinical mastitis in water buffalo

In previous studies, SCM was mainly diagnosed directly using somatic cell count (SCC) and indirectly by CMT or surf field mastitis test (SFMT). The prevalence estimation was primarily based on only a few buffalo farms, and most cases were diagnosed using a test whose definition was uncertain. Given this, the assessment may have underestimated or overestimated the actual prevalence of SCM in water buffalo. A general summary of the prevalence of SCM in four South Asian countries is given in Table 2.3.

Table 2.3 Overview of the Prevalence of subclinical mastitis in South Asian Countries

Country	References	The number of buffalo tested	Number of herds	Animal level prevalence (%)	Quarter level prevalence (%)	Threshold
Bangladesh	Biswas et al. (2020)[a]	70	2	20.0	NA	Not Defined
	Islam et al. (2019)[a]	40	1	33.0	NA	Not Defined
India	Kaur et al. (2018)[b]	81	1	28.6	10.6	> 400,000 cells per mL
	Jhambh et al. (2017)[a, b]	217	1	31.8	17.1	Pos scores 2 to 5, and SCC > 50,000 cells per mL
Pakistan	Baloch et al. (2016) [a]	210	NA	54.3	39.3	Pos scores 2 to 5
	Ali et al. (2014) [a]	390		41.8	21.1	Pos scores 2 to 5
	Hussain et al. (2013) [a]	592	10	NA	15.2	Pos scores 2 to 5
	Hameed et al. (2012) [d]	382	NA	36.4	16.0	Pos Slime/gel form
Nepal	Tiwari et al. (2022) [a]	50	NA	70.0	NA	Pos scores 2 to 5
	Dhakal and Nagahata (2018)[a, b, c]	355	NA	NA	15.0	Pos scores 1 to 3, SCC > 200,000 cells per mL, EC < 3.7 mS/cm

Different diagnostic tests were used to detect subclinical mastitis: a = CMT; b= SCC; c= EC; d= SFMT; NA was noted when no data was given.

2.7 Risk factors of subclinical mastitis in water buffalo

Previous studies reported various farm, animal, and quarter-specific variables that increased the prevalence of SCM in water buffalo. Risk factors associated with SCM in water buffalo are briefly discussed below.

2.7.1 Breed

Previous studies showed (Hussain et al., 2013; Islam et al., 2019; Aliul et al., 2021) that the prevalence of SCM was comparatively higher in Murrah or Nili-Ravi than in indigenous non-descriptive buffalo breeds. Compared to the Murrah buffalo, the Nili-Ravi buffalo demonstrated a greater prevalence of SCM (Kaur et al., 2018). However, the biological reasons for protecting against SCM in buffalo breeds are unknown. It's known that indigenous cows are more immune to infections than crossbred cows, so that they might have a decreased incidence of SCM. Regarding cows, local breeds are adapted

to the environment and often produce less milk than crossbred cows, which may help explain why crossbred cows are more susceptible to SCM than local varieties.

2.7.2 Age, parity, and lactation stage

Younger buffalo (≤ 3 years) had a higher likelihood of having SCM than older buffalo (> 3 years) (Salvador et al., 2012). In dairy cows, it was shown that older cows (> 3 years) in the later lactation stage (> 6 months of days in milk) had a higher risk of SCM (Olde Riekerink et al., 2007). The gradual degeneration of the body's immune system with age and anatomical alterations in the udder and the teats play a role in mastitis development proportional to the animal age (Petrovski et al., 2009). Buffalo, with 7th parity or higher, exhibited a substantial influence of parity on the prevalence (40 %) of SCM (Baloch et al., 2018). *Staphylococcus* spp. is one of the most common causes of SCM in water buffalo (Sharma and Sindhu, 2007; Kumar et al., 2008; Joshi et al., 2013). In most cases, staphylococci are responsible for chronic infections; a small percentage of these cases will never be treated (Smith et al., 2005). As a result, older animals are likely to accumulate more IMI than younger animals, which may lead to a higher prevalence of SCM (Bari et al., 2022). In the early lactation stage, the prevalence of SCM is significantly higher than in late lactation in water buffalo (Salvador et al., 2012). Because in the early stages of lactation, animals may frequently experience more stress due to parturition and postpartum events such as colostrum production, resulting in a high SCM prevalence (Bari et al., 2022).

2.7.3 Udder and teat conformation

Water buffalo have a distinct morphological difference in udder and teat shapes. The udder may be a bowl, globular, or pendulous shaped, whereas the teat may be cylindrical, conical, or funnel-shaped (Kaur et al., 2018). There is a correlation between the udder and teat conformation and a higher prevalence of SCM. Prasad et al. (2010) showed that Murrah buffalo with longer, wider teats placed closer to the floor had a higher prevalence of SCM. In terms of the shape of the teat-end, SCM was observed more commonly in the round (14 %) teat-ends as opposed to pointed (9 %) teat-ends (Kaur et al., 2018). When protecting against IMI, the teat is the first line of protection. Therefore, the morphometric characteristics of the udder and the teats (Fig. 2.7) are among the potential risk factors that may predispose the animal to IMI (Okano et al., 2015). The udder and teat shape

may influence milking techniques, particularly hand milking. It was evidenced that cows and buffalo that are easy to milk have a lower risk for CM. The high prevalence of CM in hard milkers may be caused by the increased pressure applied during the milking process, which might cause injury to the mammary tissue (Hameed et al., 2012).

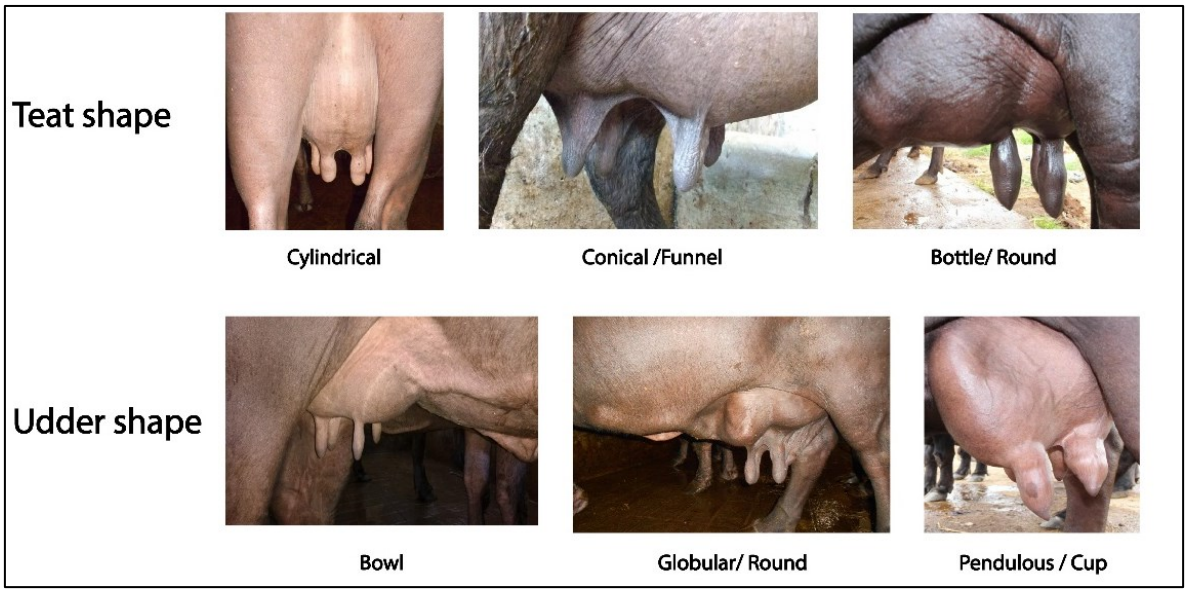


Fig. 2.7 Morphological differences in teat and udder shapes observed in water buffalo in Bangladesh

Photo source: UHB team

2.7.4 Season

The climatic conditions of a region may influence the SCM prevalence in water buffalo (Dhakal et al., 2007). In Nepal, buffalo CM cases peaked in summer (37 %), followed by autumn (32 %) and spring (February-April) (8 %) (Dhakal et al., 2007). In Punjab, India, a higher incidence of CM cases (35 %) was observed in monsoons than in other seasons (Bhutia et al., 2019). From early July to September in Punjab, India, monsoon weather remains hot and humid (above 80 % humidity). With frequent rain, floors in the dairy shed get muddy. These conditions may increase the proliferation of IMI-causing bacteria, such as *E. coli,* in the environment, exposing dairy buffalo to them (Sharma et al., 2012; Purohit et al., 2014; Gao et al., 2017). In dairy buffalo, heat and dietary stress in response to the season may also influence immunity and predispose an IMI resulting in a high prevalence of SCM (Keviletsu and Yadav, 2010).

2.7.5 Housing and herd size

A previous study found that backyard-housed Nili-Ravi buffalo had a higher CM prevalence than those in free-range areas. Clinical mastitis was most elevated in animals kept on bricks, followed by those on cement. Earthen-floored animals had the lowest prevalence (Hameed et al., 2012). Mastitis is common in animals confined on brick and cement floors, possibly due to repeated lying and standing on the hard surface, which may cause teat injury. Poor hygienic and managemental conditions may be accounted for a high prevalence of mastitis (Ali et al., 2011). A prior study found that large herds (40 %) had the highest SCM prevalence, followed by medium-sized (32 %) and small (28 %) herds. The management of a large herd, if not mechanized, is more laborious and challenging than that of a small or medium herd and may predispose to a high SCM prevalence (Ali et al., 2014).

2.7.6 Milking management

The buffalo farms use either machine or hand milking practices. However, hand milking is typical in South Asian countries like Bangladesh, India, Pakistan, and Nepal (Ali et al., 2014; Singha et al., 2023). For the hand milking method, a complete hand or folded thumb is often used, for example, either the stripping or knuckling technique is applied (Fig. 2.8). Buffalo that were milked using the complete hand milking had a lower prevalence when compared to buffalo that were milked using a folded thumb. It is possible that the trauma caused by folding one thumb while milking buffalo could be responsible for the high prevalence of CM, as previously seen in cows and water buffalo (Hameed et al., 2012). Another possible contributor to teat injury is the pre-milking teat stimulation procedures. Calf sucking, concentrate supplementation, and oxytocin are all preferred methods to boost milk ejection in water buffalo. The prevalence of SCM was substantially greater when calve suckling was used for stimulation than when concentrate was given before milking (Bilal et al., 2004; Hameed et al., 2012). During hand milking, it is standard practice to drag the calf away from the teat while still suckling, which can cause teat injury and predispose the calf to IMI.

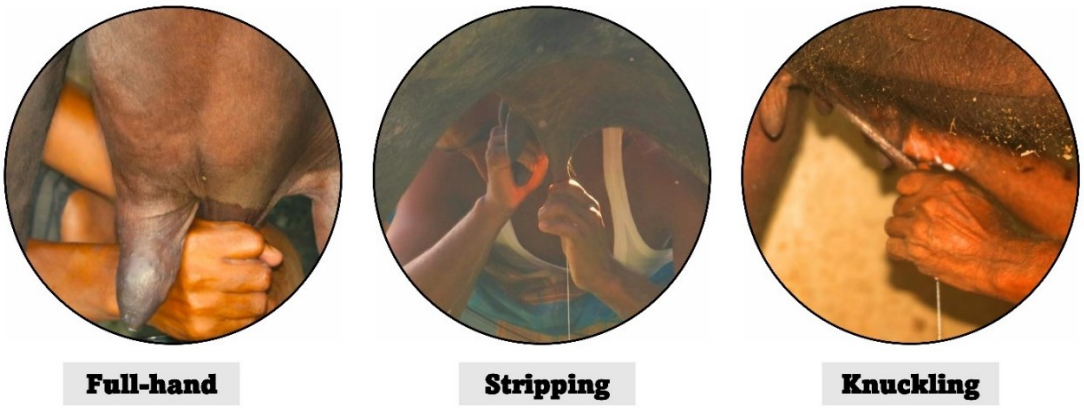


Fig. 2.8 Different types of hand milking systems used in water buffalo in Bangladesh

2.7.7 Hygiene, sanitation, and biosecurity

SCM prevalence in water buffalo is greatly affected by herd hygiene. The largest prevalence of SCM (47%) was found on farms with poor hygienic practices. SCM was found on 31% and 22% of farms with reasonable and good hygiene, respectively (Ali et al., 2014). Farms with poor drainage systems had a higher prevalence of CM than farms with better drainage systems. Dung removal frequency correlates with CM in buffalo (Hameed et al., 2012). Lack of proper hygiene and sanitation allows for the spread of bacteria, which may increase cases of mastitis. Some potential biosecurity precautions against SCM include routinely removing manure from the sheds, limiting interaction with other animals, installing footbaths at the entry, and providing access to handwashing facilities before handling the animals (Bari et al., 2022).

Generally, risk factors associated with SCM in water buffalo are frequently reported based on univariable analysis. However, only limited studies (Salvador et al., 2013) are known to estimate the strength of the association of the herd or animal level risk factors on SCM in water buffalo. Therefore, further investigation is necessary to assess the risk factors and help identify effective control measures to reduce SCM in water buffalo.

2.8 Intramammary infection causing pathogens in water buffalo

Intramammary infections can be found in CM and SCM, mainly in response to bacterial invasion, although many other pathogens are known to be linked to IMI (Keane, 2019). Based on the transmission mode, IMI-causing pathogens are classified into i) contagious

pathogens and ii) environmental pathogens. Contagious mastitis pathogens spread from animal to animal (via the milking machine or the milker), whereas environmental mastitis pathogens spread from the environment (Cobirka et al., 2020). Previous studies reported that water buffalo IMIs are caused mainly by *Staphylococcus* spp., *Streptococcus* spp., and different Gram-negative bacteria at the genus level and *S. aureus*, *Strep. agalactiae*, *S. epidermidis*, and *E. coli* at the species level (Moroni et al., 2006; Joshi et al., 2013; Ali et al., 2014). In contrast to *S. aureus* and *Strep. uberis*, where persistent infections with relatively low bacterial counts are more common, *E. coli* infections tend to last only a few days and produce a high bacterial count. *E. coli* infections are more likely to induce severe clinical symptoms than *S. aureus* infections in dairy cows (Schukken et al., 2011). After the bacterial invasion, the host immune response can be affected following disease progression (Keane, 2019). Therefore, identifying IMI-causing pathogen species and transmission routes is vital for effective mastitis treatment and preventive strategies.

2.9 Identification of mastitis pathogens

The National mastitis council (NMC) guidelines provide the instruction to perform a bacteriological culture to identify IMI-causing pathogens from the milk samples (Adkins et al., 2017c). In earlier investigations, IMI-causing microorganisms in quarter milk samples from healthy or SCM-affected water buffalo have generally been detected using bacteriological culture alone or in combination with biochemical or molecular assays utilizing PCR (Preethirani et al., 2015; Jhambh et al., 2017). In recent years, Matrix-assisted laser desorption/ionization mass spectrometry (MALDI-TOF MS) has become the standard approach for the fast, accurate identification of cultivated bacterial species (Borriello et al., 2012; Nonnemann et al., 2019). However, there are several pros and cons associated with each of these techniques. PCR-based diagnosis of IMI pathogens cannot differentiate between live and dead bacteria. On the other hand, culture-based methods make the species-level determination using bacterial culture difficult and time-consuming. Live IMI-causing bacterial species can be determined with better accuracy, followed by a combination of bacterial culture and MALDI-TOF MS (Ngassam Tchamba et al., 2019; Nonnemann et al., 2019).

2.10 Treatment strategy

No guidelines on treating SCM or CM in water buffalo are available. NMC (2022) guideline suggests antibiotic treatment of cows with CM should wait until the bacteriological culture results are available to ascertain the type of organisms responsible for CM. Whether the samples are analyzed on-farm or sent to a lab, the culture results will be forwarded within 24 hours, and antibiotics will be selected accordingly. Antibiotics will not be given to cows afflicted with Gram-negative bacteria. Still, they will be given other medications, such as oxytocin to improve milk let-down, intravenous or oral fluids, and non-steroidal anti-inflammatory drugs (Landin et al., 2011). Antibiotic treatment for a confirmed Gram-positive bacterial infection will be selected following antibiotic susceptibility test results. For the first 24 hours, while waiting for the bacteriological culturing, except for cases of severe CM needing prompt treatment, the cows will only receive supportive medication, such as oral or systemic fluid and anti-inflammatory drugs (Adkins et al., 2017c). Udder Health Bangladesh has recently developed a guideline for treating acute and chronic forms of CM in dairy cows in developing countries (Paul et al., 2019). The UHB guidelines specify the suggestions for delayed antimicrobial therapy, the cases needing immediate therapy, and where antimicrobial therapy may remain ineffective.

2.11 Antimicrobial susceptibility of intramammary infection-causing pathogens in water buffalo

Antibiotics are the principal therapies for CM in dairy cows and water buffalo. Several research in Bangladesh and neighboring countries have looked at the state of antibiotic susceptibility against bacteria that cause mastitis in water buffalo. A recent study in Bangladesh (Hoque et al., 2022) aimed to use antibiogram and virulence gene profiling to characterize *S. aureus* in SCM milk samples from riverine buffalo in Bangladesh. Antibiogram testing showed that 96 % of the *S. aureus* isolates were multidrug-resistant. Another 29 and 16 isolates had methicillin-resistant and panton-valentine leucocidin genes, respectively. Another study in Turkey recently reported that 14 % of NAS isolates showed multidrug resistance (Gurler et al., 2022). Therefore, addressing the issue of the dissemination of antimicrobial resistance is critically important to protect water buffalo health and public health safety.

2.12 Prevention and control of mastitis

So far, no guidelines for mastitis prevention and control strategies in water buffalo have been established. Therefore, the recognized strategies for dairy cows could be adapted or adjusted to develop precise guidelines for water buffalo. The National Mastitis Council (NMC, 2022) has advised 10-point mastitis control strategies for dairy cows, which recommends setting an udder health goal by targeting periodic SCC and monitoring improvement (Fig. 2.9). However, all these points may not be applicable in the situation where awareness about SCC is largely lacking, or laboratory facilities could be barely reachable by the buffalo farmers at the remote area.

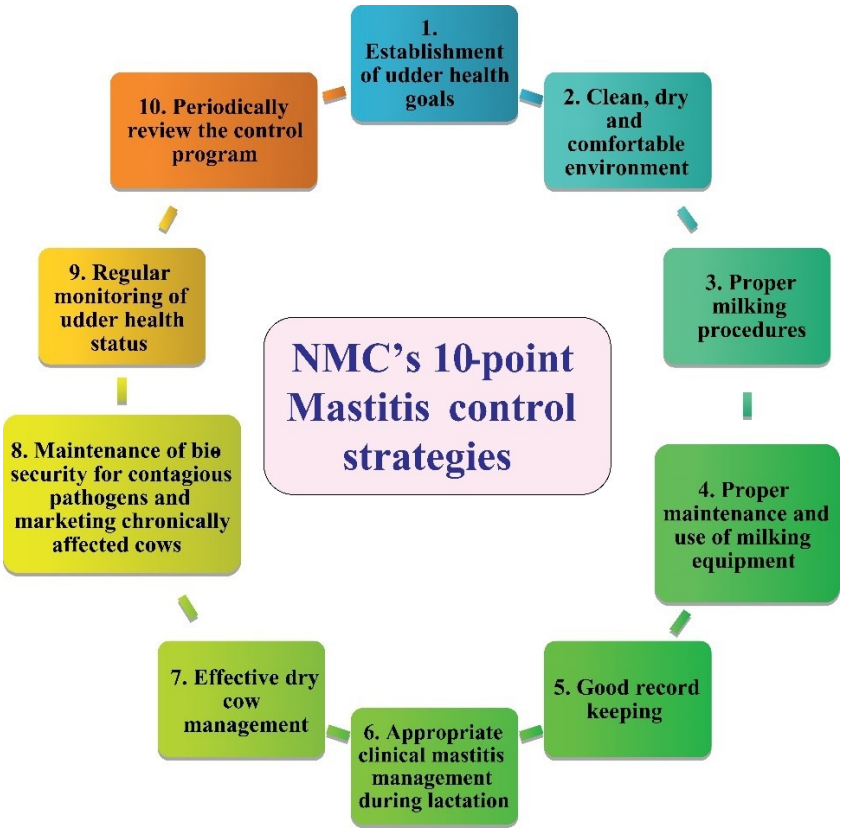


Fig. 2.9 Recommended 10-point mastitis control program by the national mastitis council.

2.13 Buffalo milk value chain in Bangladesh

In Bangladesh, there is a growing interest in buffalo milk production because of the popularity of buffalo milk products. Due to cheaper management and feeding expenses, traditional buffalo-rearing in a mixed-crop livestock system is growing in Bangladesh (Deb et al., 2016; Hamid et al., 2016b). However, 1.5 million water buffalo in Bangladesh

produce barely 4 % of the country's milk (Hamid et al., 2016a). The milk production is reasonably low, ranging from 1-3 L per animal per day (Samad, 2020). Farmers sell their milk directly to neighborhood markets, collecting centers, or sweet shops or indirectly through middlemen in this informal milk value chain. Mainly milk from different areas is collected at milk collection centers or sweet shops. The fixed middleman collects the milk from the remote farms on a yearly contract basis, which is transported without any cooling facility. Island milk is collected from several bathans in each killa (several bathans make a killa), and the middleman mixes all the milk from the entire island. Then, water vehicles (usually boats) convey mixed milk from many islands to the main island and sell it to the adjacent market, collection center, sweet shop, or even other middlemen. Milk pasteurization facilities are unavailable, and producers use raw or boiled milk to process the milk product, depending on traditional preferences in different locations in the country. Consumers buy milk or products, e.g., curd, milk drink, and sweets made from blended milk. Buffalo milk products, notably raw milk curd, and blended drinks, are more popular in southern Bangladesh. In the milk collection centers, the milk fat percentage is determined, and the middlemen are paid depending on the fat % and total volume of milk. Since bathan-based farming is generally done in rural places, long transport times are needed to reach collecting centers or neighboring markets for selling. Thus, milk quality may be compromised by long transportation without any cooling facility and can easily be contaminated with bacteria during the handling of the milk. According to the authors' knowledge, milk quality, for example, bacterial contamination between production and consumption along the buffalo milk chain, has not been studied.

2.14 Quality of buffalo milk

Buffalo milk may play an important role in contributing to the human diet, especially in low- and middle-income nations. Buffalo milk has a higher nutritional value than cow milk in almost all categories. Moreover, milk products, e.g., mozzarella cheese, ghee, and other items, are specialties of buffalo milk (Abd El-Salam and El-Shibiny, 2011). Many previous research worldwide tried to assess the quality of water buffalo milk in terms of nutrient composition or public health safety perspective, as discussed below.

2.14.1 Nutritional composition

Buffalo milk is enriched with high fat, lactose, protein, casein, and ash. The most critical part of buffalo milk is milk fat, which can vary between 6 to 9 g per 100 g. Lactose is the second most crucial part of buffalo milk, ranging from 3 to 5 g per 100 g. The amount of protein reported in buffalo milk ranged from 3 to 5 g per 100 g. Based on these numbers, buffalo milk is more enriched than cow milk (Table 2.4) (El-Salam et al., 2011; Medhammar et al., 2012). Compared to cow milk, buffalo fat globules are larger but less stable. Saturated fatty acid levels in buffalo milk fat are slightly higher than in cows, and the distribution of triglycerides and their physical properties are also somewhat different. Buffalo milk casein micelles are larger, more mineral-rich, and more susceptible to alkali disruption at higher pH values than cows (El-Salam et al., 2011). Due to its high-fat content (on average, 8.3 %), buffalo milk could produce fat-enriched dairy products, e.g., butter and ghee (Mehta, 2009). The proteins in buffalo milk (β-lactoglobulin and casein) were less allergenic than those in cow milk. Cow milk can significantly increase protein-specific immunoglobulin E sensitization and lymphocyte proliferation index and, consequently, cause allergies in consumers, as pointed out by (Kapila et al., 2013).

Table 2.4 Proximate nutrient composition of milk from different dairy species

Species	Energy kJ (kcal)	Moisture (%)	Total protein (g)	Total fat (g)	Lactose (g)	Ash (g)
Buffalo[a]	412	83.2	4.0	7.4	4.4	0.8
Cow[a]	263	88.1	3.2	3.3	5.1	0.7
Goat[b]	-	-	3.4	3.8	4.1	0.8
Sheep[b]	-	-	6.2	7.9	4.9	0.9
Yak[a]	417	82.6	5.2	6.8	4.8	0.8
Dromedary camel[a]	234	89.0	3.1	3.2	4.3	0.8
Bactrian camel[a]	319	84.8	3.9	5.0	4.2	0.9
Llama[a]	326	84.8	4.1	4.2	6.3	0.7

[a] Medhammar E, Wijesinha-Bettoni R, Stadlmayr B, Nilsson E, Charrondiere UR, Burlingame B 2012: Composition of milk from minor dairy animals and buffalo breeds: a biodiversity perspective. Journal of the Science of Food and Agriculture 92 445-474. http://dx.doi.org/10.1002/jsfa.4690.

[b] Wendorff W, Haenlein GF 2017: Sheep milk–composition and nutrition: Handbook of milk of non-bovine mammals.

2.14.2 Udder health and hygienic quality of buffalo milk

Bulk milk somatic cell count (BMSCC) and bacterial counts of milk, for example, total bacteria count (TBC), staphylococci count, and Enterobacteriaceae count (TEC), allows for the evaluation of udder health status and hygienic quality of buffalo milk (Costa et

al., 2020). The following section provides information regarding the various indicators of hygienic quality assessment and the presence of zoonotic pathogens in buffalo milk.

2.14.2.1 Bulk milk somatic cell count

The SCC is an intriguing and valuable marker of buffalo udder health (Costa et al., 2020). Since IMI is the primary factor in determining the SCC, this parameter is an excellent surrogate for assessing the prevalence of IMI, regardless of whether CM or SCM is present. Values of SCC can also be easily collected either from bulk milk or as a herd average of individual cow measurements obtained from dairy herd improvement programs (Dufour et al., 2011). The report on BMSCC in Bangladesh is scarce due to unawareness of its use and the unavailability of somatic cell counters. In international standards in dairy cows, the range of BMSCC below 200,000 cells per mL of milk is indicated as a safety margin (Barkema et al., 2013; Norman et al., 2013), where many countries in Europe consider the acceptable value of 400,000 cells per mL and in Canada the exceeding limit was reported to be 500,000 cells per mL (Sargeant et al., 1998; Barkema et al., 2013) in drinkable milk. In other countries like the USA, the standard legal limit for SCC in milk is 750,000 cells per mL (Norman et al., 2019), which varies between states. Naturally, there is a slight increase of BMSCC at early and late lactation, so the average of BMSCC at three lactation stages gives a reasonable value for BMSCC. Although it is impossible to use BMSCC estimates to calculate the exact number of affected cows in a herd, this approximates the number of affected quarters at the herd level (Constable et al., 2016). Quarter milk with an SCC of over 100,000 cells per mL is deemed mastitis in Murrah buffalo, recently proposed by the National Dairy Research Institute (NDRI) of India (Personal Communication: Prof. Abdul Samad, 2020). There are currently no international thresholds for the acceptable level of BMSCC in water buffalo. An overview of the SCC threshold of buffalo milk used in different studies is displayed in Table 2.5.

Table 2.5 Bulk milk somatic cell count threshold used as an indicator of milk quality in water buffalo in different countries of the world

Country	Level	The threshold used (cells per mL of milk)	Measurement method	Study period	Number of farms	Reference
Italy	Farm	175,000	CombiFoss FT+	36 months	1	Pasquini et al. (2018)
Philippines	Buffalo	200,000	Fossomatic Minor	6 months	1	Salvador et al. (2013)
China	Quarter	200,000	CombiFoss FT+ FOSS	11 months	1	Zhang et al. (2022)
India	Quarter	200,000	Analytical Nucleocounter	-	2	Preethirani et al. (2015)
Italy	Quarter	200,000	Fossomatic apparatus	2 months	14	Catozzi et al. (2017)
Italy	Quarter	200,000	Bentley Somacount 150	14 months	2	Moroni et al. (2006)
Nepal	Quarter	200,000	Microscopic	-	3	Dhakal (2006)
Nepal	Quarter	200,000	Microscopic	36 months	-	Dhakal and Nagahata (2018)
Egypt	Quarter	250,000	PortaSCC® Quick Test	-	1	Abd Al-Galil and Khalil (2016)
India	Quarter	300,000	Microscopic	12 months	-	Srinivasan et al. (2013)
India	Quarter	400,000	SomaScope Smart	-	-	Kaur et al. (2018)
Italy	Quarter	400,000	MilkoScan FT6000	60 months	121	Costa et al. (2020)
Brazil	Quarter	400,000	SomaCount 300	-	4	Medeiros et al. (2011)

2.14.2.2 Aerobic bacteria count

Total bacterial count, total staphylococcal count, total Enterobacteriaceae count, and total coliform count are a few of the bacteriological criteria that can be used to assess milk quality. It is standard practice to employ TBC as markers to evaluate the overall cleanliness of the production facility. Regulation (EC) No 853/2004 establishes the limit values for TBC as follows: 100,000 CFU per mL for raw cow milk, 1,500,000 CFU per mL for raw milk from other species, and 500,000 CFU per mL for raw milk from species

other than cows used to manufacture cheese products without heat treatments. A study in Brazil reported that the microbiological parameters in buffalo bulk milk, including the TBC and thermotolerant coliforms, were 9.0×10^4 CFU per mL and 1.6×10^2 MPN per mL, respectively. Another Italian study reported that raw milk environmental contaminating microorganisms were between 6.5 and 23.4×10^1 CFU per mL of buffalo milk over three years. This study showed that the milk was clean due to adequate hygiene when milking and handling raw milk (Pasquini et al., 2018). However, warm temperatures in summer may favor rapid bacterial proliferation and can readily raise milk's microbe concentration.

2.14.2.3 Zoonotic bacteria in buffalo milk and milk products

Pathogenic organisms spread from animal to human through animal-derived food are known as food-borne zoonotic pathogens (Tassew et al., 2010; Assefa and Bihon, 2018). Some bacteria that cause foodborne illness are particularly relevant regarding disease frequency and/or severity. Toxins produced by many bacteria (both Gram-positive and Gram-negative) cause food poisoning, with symptoms ranging from gastrointestinal ailments to paralysis and death. Gram-negative bacteria have reportedly responsible for around 69 % of bacterial food-borne illness cases (Le Loir et al., 2003; Kebede et al., 2014). Few previous studies attempted to identify zoonotic pathogens from buffalo milk. Previous studies identified the presence of *Listeria monocytogenes*, *Pseudomonas* spp., and *Coxiella burnetii* in buffalo milk and milk products (Khademi et al., 2019; Lauer Cruz and de Souza da Motta, 2019; Terzi Gulel et al., 2020). In Turkey, buffalo bulk milk and cheese had 4 % *Listeria monocytogenes* and 70 - 90 % penicillin, oxacillin, and erythromycin resistance (Gulel et al., 2020). A study in Romania identified that all the dairy product samples from buffalo milk were contaminated with high coliform, yeast, and mold contamination but Salmonella-free (Stetca et al., 2015). A retrospective study in Brazilian states identified a high seroprevalence of *Brucella* spp. (36 %) and *Mycobacterium tuberculosis* (41 %) from buffalo milk during 2012-2019. This indicates that transmitting zoonotic pathogens through water buffalo milk may also threaten buffalo farm personnel and milk consumers (Schwarz et al., 2021). Consequently, various zoonotic pathogens were identified in buffalo milk in different countries. Despite many published studies, little is known about zoonotic infections in the buffalo milk chain in low- and middle-income countries like Bangladesh.

2.15 Risk factors of bulk milk somatic cell count in buffalo milk

Breed, age, parity, lactation stage, season, food, udder illnesses like mastitis, and genetic polymorphism of milk proteins are just some factors that might affect the SCC of buffalo milk (El-Salam et al., 2011). A previous study highlighted that BMSCC increased in water buffalo milk in winter compared to summer (Pasquini et al., 2018). However, many herd-level risk factors in dairy cows are known to be associated with BMSCC. Wearing gloves during milking, post-milking teat dipping, milking sick cows at last, yearly inspection of the milking equipment, and adopting techniques to keep cows standing after milking were all associated with lower BMSCC. A free stall system, sand bedding, cleaning the calving area after each calving, monitoring dry-cow udders for mastitis, blanket dry-cow therapy, parenteral selenium supplementation, udder health care, and regular use of the CMT were related to lower SCC (Dufour et al., 2011). However, although they have not been studied, milking technique, housing circumstances, drying-off strategies, disease prevalence, and breed effects on dairy buffalo farms may be linked to BMSCC. To our knowledge, there are no studies on buffalo milk BMSCC in Bangladesh.

2.16 Risk factors of bacterial contamination along the milk chain

Risk factors of bacterial contamination in water buffalo milk could vary by the type of bacteria involved. The TBC in milk indicates overall hygiene standards during milk production, and thus it may not be possible to pinpoint individual contamination points with the different types of bacteria involved (Chambers, 2002). At inadequate refrigeration temperatures, psychotropic bacteria can grow and multiplicate. Psychotrophs are commonly found in untreated water, soil, and plants. They enter the milk through contamination of milking equipment or the exterior of the udder and teats (Murphy, 1997). Udders and equipment that have not been adequately cleaned and sterilized may also be the primary sources of thermoduric bacterial contamination in milk (Murphy, 1997). Cow manure, bedding, soil, and contaminated water contain coliforms from their intestinal tracts and may contaminate raw milk through udder and teat surfaces and contaminated milking equipment (Elmoslemany et al., 2009).

2.16.1 Contamination from udder infection

Pathogenic bacteria responsible for mastitis can also spread from cows to humans through direct contact during the handling of the animal or through indirect routes like milk and meat, through zoonotic bacterial transmission (Enger and Middleton, 2021). Disease dynamics in the reservoir host (e.g., cow affected by SCM for a chronic period), pathogen exposure through unhygienic bedding, parturition, flushing of keratin lining or teat canal injury, and within-human factors that affect susceptibility to infections, for example, improper dressing, open wounds, and/or proximity to animals determine the probability of zoonotic pathogens spillover (Alexander et al., 2018). The most known infectious organisms that have led to spillover and persistent IMI are *S. aureus* and *Strep. agalactiae*, with occasional *Strep. uberis*, *Mycoplasma* spp., and *Corynebacterium* (Schröder et al., 2012; Käppeli et al., 2019). Molecular characterization has indicated that MRSA lineages are also dynamically shared between farm animals and humans within and between farm facilities through direct contact or the environment (Locatelli et al., 2017).

2.17 Opportunities for buffalo production in Bangladesh

Buffalo farming in Bangladesh is currently facing several challenges, such as limited knowledge of buffalo udder health, insufficient feed sources, lack of primary buffalo management education, poor milk production, inadequate milking hygiene, hygienic handling of milk from farm to the processing of milk products, lack of monitoring milk quality, and insufficient milk marketing opportunities. The milk production of dairy buffalo can be increased through a planned breeding program. Using imported buffalo bull semen to inseminate semi-intensive farm-raised buffalo can increase the genetic potentials of local low-milk-yielding buffalo varieties in Bangladesh. A best-to-best progeny improvement program can be achieved by exchanging the elite Bangladeshi buffalo bull or artificial insemination. In the intensive and semi-intensive system, buffalo farmers have access to feed resources and have high milk production potential. However, there is a knowledge gap in buffalo nutrition which can probably be mitigated through training the farmers with the scientific knowledge on buffalo nutrition requirements. Udder and milkers' hygiene can be enhanced through hands-on training for buffalo farmers. In addition, udder health advice and preventive approaches based on the routine diagnosis of SCM and BMSCC testing need to be established and disseminated to

veterinarians to provide technical support to the buffalo farmers. The price of buffalo milk is regulated based on fat percentages and total milk volume. Buffalo milk quality monitoring, for example, routine BMSCC and TBC, can be incorporated into a reward and penalty system to promote farms with better hygiene and milk quality. Buffalo farmers must be encouraged and regularly trained in scientific buffalo management and hygienic milk handling. Finally, a real-time updated buffalo farm and population database, along with a milk traceability system, can be implemented to provide a complete picture of the buffalo supply chain in Bangladesh. This traceability system can monitor milk production and quality improvement across the supply chain.

2.18 Conclusion

This review discussed the water buffalo population and milk production to highlight the potential of water buffalo as an economically valuable livestock species in Bangladesh. Water buffalo SCM prevalence, associated risk factors, IMI-causing bacteria, and antimicrobial resistance were delicately reviewed. In addition, the zoonotic pathogens found in buffalo milk chains and the associated risk factors were closely reviewed. The prevalence of SCM in water buffalo nationally is one of the knowledge gaps. Other knowledge gaps include detecting IMI-causing bacteria species and zoonotic pathogens along the water buffalo milk chain (Fig. 2.10). The following knowledge gaps were the primary focus of this study in light of the possible improvement of the water buffalo milk chain in Bangladesh.

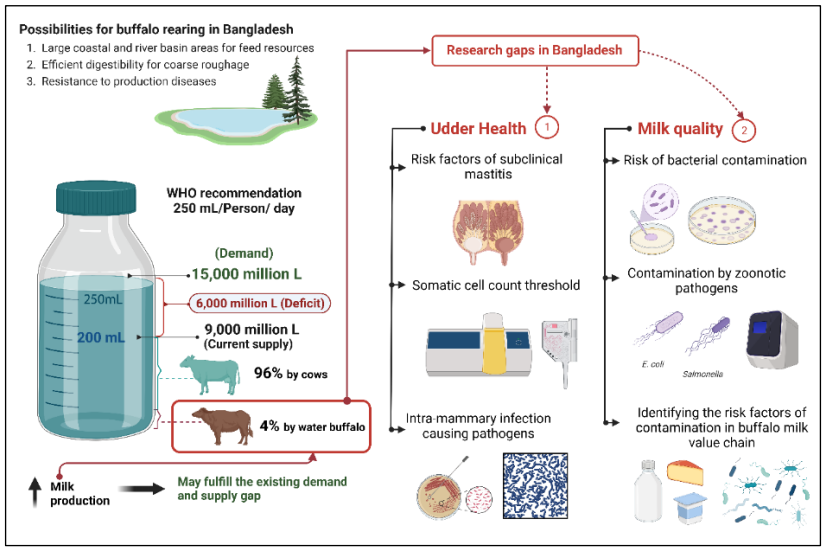


Fig. 2.10 Graphical illustration of the research gaps to improve udder health and milk quality in water buffalo in Bangladesh

CHAPTER-3

GENERAL MATERIALS AND METHODS

CHAPTER 3

GENERAL MATERIALS AND METHODS

3.1 Description of the study site

Bangladesh's coastal and semi-coastal areas have the largest densities of water buffalo population due to the availability of fallow pastureland and green forages (Faruque et al., 1990; Hamid et al., 2016a). From there, water buffalo farms and individual lactating water buffalo belonging to nine concentrated buffalo districts were included in the study to represent the buffalo population in Bangladesh effectively (Fig. 3.1). The buffalo farms included in this study belonged to five different rearing systems: free-range, semi-free-range, household, semi-intensive, and intensive. Free-range buffalo depend on grazing on fallow land in coastal or semi-coastal islands with the supplementation of straw or, at times, small amounts of concentrates. In free-range systems, buffalo are moved from one island to another based on the availability of green roughage. In the semi-free-range system, buffalo are transferred to the inlands during the dry season (October to March) due to feed scarcity on the islands and stay there for 3-6 months, depending on roughage availability. The household rearing system allows 5-7 hours of grazing, combining a supply of a minimal amount of straw, grass, and concentrates. In river-basin areas, the semi-intensive system allows buffalo to stay in sheds at night, graze in nearby pasturelands, and return daily. In an intensive system, buffalo are tied in sheds, stall-fed, and never allowed to graze (Hamid et al., 2016a; Hamid et al., 2016b).

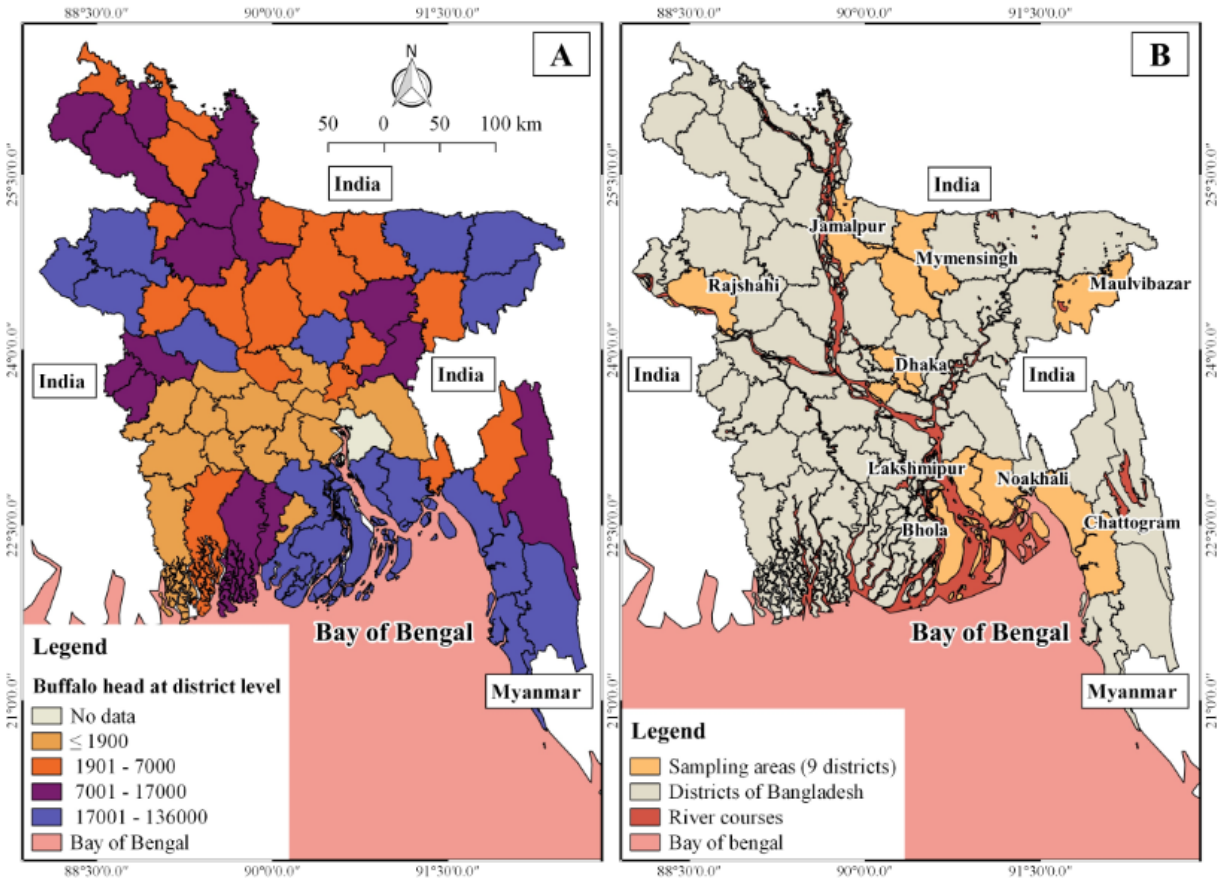


Fig. 3.1 Location of nine buffalo-concentrated districts in Bangladesh where buffalo farms were enrolled in the present study

A. shows the district-level buffalo heads in Bangladesh (Data source: LDDP (2019)). B. The orange indicates the location of the study's nine districts recruited for sampling (Rajshahi, Jamalpur, Mymensingh, Maulvibazar, Dhaka, Lakshmipur, Bhola, Noakhali, and Chattogram).

3.2 Selection of the farms

Buffalo-concentrated locations were identified based on data given by the researchers with the best knowledge about the buffalo population in Bangladesh. Based on their information, one buffalo-concentrated sub-district was selected from each district for sampling. Given that no sampling frame was available, lists of buffalo farms were collected from the buffalo farmers' database of the upazila veterinary hospital (UVH) located at each sub-district level or Palli Karma-Sahayak Foundation (PKSF), a non-governmental organization supporting the buffalo farmers. A total of 200 farms to be sampled was estimated according to an expected 50 % prevalence with 5 % absolute

precision, assuming an intraclass correlation coefficient of 0.25 for buffalo within farms. The listed buffalo farmers were contacted to get information on the number of lactating buffalo and the farm location. Additionally, four intensive farms were recruited for sampling because of the limited number of intensive buffalo farms in Bangladesh. Two of the four intensive farms were government-owned, and two more were private buffalo farms. A total of 248 water buffalo farms from free-range (n = 51), semi-free-range (n = 106), household (n = 33), semi-intensive (n = 54), and intensive (n = 4) buffalo farms were recruited for sampling, as presented in Table 3.1. Sampling was performed on 1 to 5 lactating buffalo on each farm. All the animals were sampled when a farm had ≤ 5 lactating buffalo, and five were randomly sampled when a farm had ≥ 5 lactating buffalo. However, because of non-cooperative animals, only one lactating buffalo could be sampled on nine farms (five semi-free-range, three households, and one semi-intensive farm). Because of the small number of intensive farms, all the lactating buffalo from these four farms were sampled. Milk sampling and possible potential risk factor data recording were conducted between February 2020 and April 2021.

Table 3.1 Distribution of 880 buffalo sampled from 248 buffalo farms over five different buffalo-rearing systems in nine buffalo-concentrated districts in Bangladesh

District	Total buffalo population	Number of buffalo sampled	Number of farms from other buffalo-rearing systems				
			Free-range	Semi-free-range	Household	Semi-intensive	Intensive
Noakhali	50,680	182	9	113	20	40	-
Jamalpur	11,955	145	17	84	9	35	-
Chattogram	44,494	135	85	38	12	-	-
Rajshahi	18,550	118	-	-	2	116	-
Moulvibazar	55,363	105	-	103	-	2	-
Bhola	52,190	100	48	13	30	7	2
Laxmipur	29,971	52	-	-	-	-	52
Mymensingh	3,087	28	5	15	4	4	-
Dhaka	1,621	15	-	-	-	-	15
Overall	**267,911**	**880**	**164**	**366**	**77**	**204**	**69**

3.3 Animals and ethical issues

The study was approved and performed in line with the guidelines of the SAU research system (SAU/Ethical Committee/AUP/21/06) of Sylhet Agricultural University, Bangladesh. The buffalo farmers signed a written informed consent before participating in this study.

3.4 Detection of subclinical mastitis using the California mastitis test and bulk milk somatic cell count

The SCM status of each buffalo quarter was measured and classified on a scale from 1 to 5 using CMT, as described by Baloch et al. (2016). California mastitis test can indirectly assess the presence of SCM where score 1 indicates a negative result (no gel formation, no indicative color change), score 2 indicates traceable (possible inflammation), score 3 or above indicates a strong positive result where 5 has the most gel formation and deep blue/violet color change (Barnum and Newbould, 1961). Then CMT was performed on each of the functional quarters of the buffalo before milking was started. A quarter was considered positive for SCM when the CMT score was ≥ 2 with no visible signs of CM (any changes in milk or swelling, and/or redness, or painfulness of the mammary gland). A buffalo was considered SCM-positive when one or more of its four functional quarters had SCM. Bulk milk SCC was measured in thoroughly mixed morning bulk milk containing milk from all the lactating buffalo at each farm using a DeLaval somatic cell counter (DCC) (DeLaval Group, Stockholm, Sweden) (Adkins et al., 2017a). Analyses were performed according to the manufacturer's instructions. A small amount of milk (around 60 µL) was loaded into the cassette and inserted into the DCC. The DCC cassette stained the somatic cells in milk with a DNA-specific fluorescent reagent. The results were then displayed immediately on the screen. The DCC showed the BMSCC results as cells perµL of milk and were multiplied by 1000 to estimate the cells per mL of milk. The measurement was done immediately at the farm following the collection of the farm bulk milk samples.

3.5 Sample collection, preservation, and transportation

After CMT, one healthy quarter (CMT score 1) milk sample was collected per animal, while all SCM positive quarter (CMT score 2 to 5) milk samples were collected separately per animal. After milking all the buffalo at each farm, the bulk milk was thoroughly mixed for 5 minutes, and samples were collected from the top of the bulk tank using a clean, sanitized dipper. 25-30 mL of the farm bulk milk sample was collected aseptically in a sterile 50 mL screw-capped falcon tube. Two representative aliquots from the same farm bulk milk were collected, of which one aliquot was used to perform the BMSCC analysis, and another was preserved aseptically for bacteriological culture. A 10 mL bulk milk samples were collected aseptically from the middlemen nodes after 1

hour from the collection of bulk milk samples on the farms. Another 10 mL of mixed bulk milk samples were collected at the milk collection centers one hour after the milk delivery by the middlemen. Approximately 30-35 g of milk products (yogurt, cheese, and buttermilk) were collected aseptically from each milk product shop in a 50 mL sterile screw-cap bottle. Samples taken from the middlemen, milk collection centers, and milk product shops were not linked with the source buffalo farm. All collected samples were transferred to an ice box immediately. Upon collection of all samples each day, the samples were frozen and temporarily stored in a deep freezer (-10 ºC to -15 ºC) available close to the field visit area. Samples were transferred within seven days to the Udder Health Bangladesh (UHB) bacteriological laboratory of Chattogram Veterinary and Animal Sciences University (CVASU) and stored at -20 ºC. Then the samples were undertaken for bacteriological culture after 24 hours.

3.6 Epidemiological data collection

Two structured questionnaires containing both open- or close-ended questions were developed in English for data collection; one provided potential information about water buffalo SCM, and the other included possible information about the water buffalo milk chain in Bangladesh. This questionnaire on water buffalo SCM comprised two subsections; section A included quarter and animal-related information, and section B included water buffalo farm-related information (questionnaires can be found in subsections 12.1 and 12.2 under chapter 12 Appendices). This milk chain questionnaire was divided into four subsections to describe the buffalo milk chain and determine factors associated with farm BMSCC and bacteria from the buffalo milk chain nodes. Section A captured data at the farm level and included 45 questions. Sections B and C contained 20 questions and collected information from the middlemen and collection centers. Section D included eight questions to gather information on milk products, such as the nature of milk, storage time, and the type of containers used (questionnaires found in subsection 12.3, 12.4, 12.5, and 12.6 under Chapter 12 Appendices). Written informed consent was obtained from the buffalo farmers before administering the questionnaire. The middleman and milk product shop owners also gave written or oral informed consent. The participation of the farmers, middlemen, and milk product shop owners was voluntary. Data were collected in face-to-face interviews with the farmers (farm owner

or acting manager) and through on-farm observations. All interviews were conducted in Bengali, but the questionnaire was recorded in English.

3.7 Isolation and identification of bacteria

Milk samples were subjected to bacteriological culture following the NMC 2017 guidelines (Adkins et al., 2017b), with some modifications noted below (Singha et al., 2021a). Quarter milk samples were inoculated (10µL) on a 5 % bovine blood agar base (BBA). At least three morphologically similar colonies on BBA were defined as positive growth. Gram-positive and Gram-negative bacteria were differentiated based on growth characteristics, followed by inoculation of selective agar media: Mannitol Salt agar (MSA), MacConkey agar (MAC), and Eosin Methylene Blue (EMB) agar. White, yellow, or golden colonies on BBA, a positive catalase test, yellow or pink color formation on MSA, and a negative coagulase test were considered NAS. Alternatively, white, yellow, or golden colonies with hemolysis on BBA, a positive catalase test, strong yellow color formation on MSA, and a positive coagulase test were considered *S. aureus*. *E. coli* was identified based on opaque colonies with fecal odor on BBA, a pink color formation on MAC, and a greenish metallic sheen on EMB agar. Isolates were preserved at -80 °C using Brain Heart Infusion Broth and 50 % buffered glycerol for future use. All the bacterial culture media and reagents were manufactured by Oxoid, Oxoid Ltd, Basingstoke, United Kingdom.

3.7.1 Matrix-Assisted Laser Desorption/Ionization Time of Flight (MALDI-DOF)

Frozen bacterial isolates were shipped to the Mastitis Laboratory, Department of Veterinary and Animal Sciences (DIVAS), Università degli Studi di Milano (UNIMI) for confirmation of the bacterial species, using MALDI-TOF MS. For MALDI identification, frozen bacterial isolates were inoculated (10 µL) on BBA and incubated for 24 hours. One or two colonies were deposited on the target plate using a toothpick overlaid with 1 µL of α-cyano-4-hydroxycinnamic acid matrix solution (Bruker Daltonics, GmbH, Bremen, Germany) and were dried. The spectra were acquired with a microFlex™ mass spectrometer (Bruker Daltonics, GmbH). The results were interpreted against the MBT Compass® 4.1 database (Addis et al., 2022). Log scores of ≥ 2.0 were the thresholds used for species confirmation of *S. aureus,* while log scores of ≥ 1.7 were used to confirm the species identification of remaining bacteria (Cameron et al., 2017).

3.8 Determination of antimicrobial susceptibility using penicillinase test

A subset of *Staphylococci* was tested for β-lactamase production using the cloverleaf method described by Mee'aad et al. (2018). The strains *S. aureus* ATCC 29213 and *S. aureus* ATCC 25923 were used for quality control.

3.9 Quantification of bacteria

Bulk milk samples from farms, middlemen, milk collection centers, and milk products underwent bacteriological quantification following the NMC protocol (Adkins et al., 2017b). The total bacteria count, *Staphylococcal* count, and *Enterobacteriaceae* count were performed on a Plate Count Agar (PCA), Baird Parker Agar with egg yolk tellurite (BPA), and Violet Red Bile Glucose Agar (VRBG), respectively. All agar used was manufactured by Oxoid, Basingstoke, UK.

3.9.1 Total bacteria count, Staphylococci count, and Enterobacteriaceae count

To perform bacteria count, 1 mL of the milk samples was mixed with 9 mL of diluent (sterile 0.9 % NaCl). Samples were serially diluted 10-fold up to 10^{-7}. To estimate the total number of aerobic bacteria in the samples, the pour plate technique was carried out following ISO:4833-1 (2013). From each 10-fold dilution prepared, 1 mL of the sample was mixed with 15 - 20 mL of molten PCA in a sterile petri dish and incubated aerobically at 30 ºC for 72 hours. *Staphylococci* count was determined using the surface plate technique, following Viçosa et al. (2010). 0.1 mL of the serially diluted sample was taken from each tube after vertexing and plated on the surface onto a solidified sterile BPA plate. The plate was incubated at 37 ºC for 48 hours, and then *S. aureus* and *non-aureus staphylococci* were enumerated. To confirm *S. aureus* colonies, five colonies were randomly chosen and tested for a positive coagulase test. Determination of *Enterobacteriaceae* was performed using the pour plate technique following 5th ed. NMKL-144 (Nordic Committee on food analysis) standards. A 1 mL vortexed sample was mixed with 10 - 15 mL of VRBG. Then, a second overlay of 5 - 10 mL of VRBG was added after solidification. Culture plates were incubated at 37 ºC for 48 hours. A further oxidase test was conducted on five randomly selected colonies to differentiate *Enterobacteriaceae* from non-*Enterobacteriaceae*. Bacteria enumerations were done considering the countable dilution containing < 300 colonies.

3.10 Genomic DNA extraction and purification

DNA extraction was performed in the Microbial genetics and bioinformatics laboratory under the Department of Microbiology, University of Dhaka, Bangladesh. DNA extraction was performed using Maxwell® 16 Cell DNA Purification Kit (Promega, UK) with the Maxwell® 16 Instrument platform (Promega, UK). One mL of milk sample was centrifuged at 16,000 rcf for 10 minutes (Oikonomou et al., 2014). Then the supernatant was discarded, and the remaining pellet was used for DNA extraction according to the manufacturer's instructions. The DNA samples were eluted in 100 µL elution buffer (Promega, UK) and were stored at -20 ºC until further possessing. DNA concentration and purity were analyzed using NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific, Waltham, Massachusetts, USA) at a wavelength of 260nm and A260/A280, respectively. DNA samples were appropriate for downstream those yielded DNA concentration (≥ 9 ng/ µL) and 260/280 absorbance ratios (≤ 1.6 to ≤ 2). The DNA samples were delivered with dry ice to the Molecular Pathology Laboratory, Department of Veterinary and Animal Sciences, Università degli Studi di Milano.

3.11 Real-time PCR

Real-time (RT) PCR was performed in 15 µL reaction volume using the CFX 96 System (Bio-Rad Laboratories, USA). Each reaction contained 7.5 µL of 2x Mix EVA Green (SsoFast EvaGreen® Supermix, Bio-Rad Laboratories, USA) and primers specific for target genes (Table 3.2). The PCR reaction was carried out using the same thermal profile for all the targets (2 min at 50 ºC, 3 min at 95 ºC, and 39 cycles of 10s at 95 ºC and 30s at 60 ºC). To assess melting curves, PCR products were incubated at 55 ºC for the 60s then the temperature was increased to 95 ºC at 0.5 ºC increments for 10s following the minimum information for publication of quantitative real-time PCR experiments (MIQE) guidelines (Bustin et al., 2009). The PCR efficiencies were determined using four-fold serial dilutions of DNA prepared from ATCC strains of the bacteria of interest, such as *S. aureus* ATCC 19048, *E. coli* ATCC 11229, *E. coli* O157:H7 ATCC 35150, *Listeria monocytogenes* ATCC 13932, *Yersinia enterocollitica* DSM 4780, *Salmonella enterica* ATCC 13076, and *Campylobacter jejuni* ATCC 33291. ATCC and DSM bacterial strains were obtained from the American type of culture type collection (MD, USA) and German collection of microorganisms and cell cultures (Braunschweig, Germany), respectively. No template controls were included in the assays. The results were analyzed using the

software Bio-Rad CFX Maestro 1.0 (Bio-Rad Laboratories, USA). The samples with a threshold of CT < 35 cycles were considered positive for the presence of the targeted genes.

Table 3.2 Primer sets used for qPCR to identify the presence of the nine target genes of seven zoonotic bacteria from the extracted DNA samples

Primers were selected from previously published studies (Cremonesi et al., 2014; Cremonesi et al., 2016)

Bacteria	Gene	Sequences (5'-3')	Accession number	Amplicon size
Staphylococcus aureus	htrA	GAAGTAATATCAGACA AATCAAATCAGTACC TCTTCCGGTAAAGTTAATGGCTTCTG	NC_009782	92bp
Escherichia coli	yccT	GCAGCGTGGTGGCAAAA CGTGACCACCTTGATTGCAT	CP10315	56bp
Escherichia coli O157:H7	eae	GTAACAATGTCAGAGG CGAGTTG CCACCGCTTGCTTTCAGTTTAA	AE005174	72bp
	stx1	GGATTTCGTACAACACTGGATGATC GATCAACATCTTCAGCA GTCATTACA	M16625	67bp
	stx2	ACCCCACCGGGCAGTT CGCGCCTGATAGACATCAAG	X07865	59bp
Listeria monocytogenes	inlA	TAACAGACACGGTCTCG CAAA TCCCTAATCTATCCGCCTGAAG	CP013288	66bp
Yersinia enterocollitica	yst	TGGAGCATTCGGCCAA GAA ATTGGTGTCGATAATG CATCACTGA	X65999	60bp
Salmonella enterica	invA	TGGAAAGGGAAAGCC AGCTT AATAGCGTCACCTTTG ATAAACTTCA	M90846	68bp
Campylobacter Jejuni	cadF	TGAACCAAGAGAAGG TGCTTTGT AAAACCAAAATGACCTTCCAAAGAAATAGTT	FJ946061	76bp

3.12 Statistical analysis

Data from the questionnaire-based survey and the bacteriological identification, quantification, and RT-PCR analysis were entered in an MS Excel 2016 spreadsheet. The data was cleaned, and coding and integrity were checked before statistical analysis.

3.12.1 Descriptive statistics

Descriptive statistical analysis was carried out to calculate the prevalence of SCM (at the quarter and buffalo levels) and the summary of BMSCC (at the farm level). To calculate the sampling-corrected prevalence, sampling weights were created based on the total buffalo population in an area, the number of buffalo, and the sampled quarters (Dohoo et al., 2010b). Bulk milk SCC and bacteria count (TBC, TNAS, TSA, and TEC) data were log10 transformed to achieve normal data distributions. Descriptive statistics were performed using a boxplot for BMSCC at the farm level and bacteria count (TBC, TNAS, TSA, and TEC) at each point of the buffalo milk chains (farm, middleman, milk collection center, and milk product). The summary (mean and range) was presented for the quantitative variables, such as the number of lactating animals, average milk yield at the farm level, and frequency, with percentages presented for the generic data, such as problems faced during transportation, storage, and farmers getting the right milk price.

3.12.2 Logistic regression model

Generalized linear mixed-effects logistic regression models were constructed to identify SCM-associated risk factors. For generalized linear mixed-effects logistic regression models, farm and buffalo ID within farms were used as random effects in these models. Subject-specific effects were used to calculate the odds ratio, meaning that fixed effects in a mixed model represent the effects of that factor within the cluster. The significance of the random effect terms was checked with the likelihood ratio test (LRT) using the latent variable approach (Dohoo et al., 2010a). Then, fixed variables that were significant at $P \leq 0.2$ in the univariable logistic regression were selected for the multivariable logistic regression. The model was manually constructed using a forward selection procedure, applying the maximum likelihood estimation procedure. The statistical significance of the contribution of individual predictors was determined using Wald's test. The presence of confounders was assessed by adding one of the variables in order of significance (the variables with the lowest *P*-value were added first) and, in each step, determining whether the beta coefficient of other variables in the model changed by more than 30 %, which was deemed confounding. Simultaneously, we set the collinearity based on significant changes (> 30 %) in the standard error. If a variable was found to be non-significant ($P > 0.05$), was not a confounder, and no indications of collinearity were seen,

the variable was removed from the model in the next step. Finally, significant ($P \leq 0.05$) variables were presented regarding odds ratio and 95 % C.I.

3.12.3 Linear regression model

A t-test or one-way ANOVA was performed for univariable analysis to identify possible factors ($P \leq 0.20$) affecting the BMSCC or bacteria contamination. Univariable analysis was performed for BMSCC and all four classes of bacteria count, TBC, TNAS, TSA, and TEC. Using the fixed variables of $P < 0.20$ in the univariable analysis, separate multivariable regression models were constructed at the farm level to identify the significant factors. During the construction of the multivariable regression models, a maximum likelihood estimation procedure was applied using a manual stepwise forward selection procedure. The presence of any confounding was assessed by removing one variable from the model and evaluating whether the coefficients were changed by 30 % and whether the confounding was biologically meaningful. The interaction was evaluated by constructing two-interaction product terms for any significant main effect, adding them to the model, and examining changes in P values of the main effects. The final model included variables with a $P \leq 0.05$. A variation inflation factor (VIF) and Cook Weisberg test were performed for model fit to identify multi-collinearity and heteroskedasticity, respectively.

CHAPTER-4

Prevalence, etiology, and risk factors for subclinical mastitis in water buffalo (*Bubalus bubalis*): A systematic review and meta-analysis

CHAPTER 4

Prevalence, etiology, and risk factors for subclinical mastitis in water buffalo (*Bubalus bubalis*): A systematic review and meta-analysis

4.1 Abstract

Subclinical mastitis (SCM) in water buffalo is an important production disease causing reduced milk yield and poor milk quality, ultimately leading to economic losses. It may also involve public health risks due to the spread of zoonotic infections and antimicrobial resistance. Water buffalo are the second most crucial dairy animal species worldwide regarding milk yield. Studies have examined SCM's prevalence or risk factors in water buffalo, but specific systematic reviews and meta-analyses are lacking. To close this research gap, a systematic review and meta-analysis were performed to determine i) quarter level pooled prevalence of SCM in water buffalo, ii) the distribution of pathogens causing intra-mammary infection (IMI), and iii) associations between herd, animal, and quarter-related risk factors and prevalence of SCM. Publications on SCM in water buffalo were retrieved from four electronic databases (PubMed, Scopus, Embase, and Web of Science). A total of 53 studies were deemed eligible for the study, providing data on 8,958 buffalo and 34,125 quarters. The pooled prevalence of SCM was 21 % (95 % CI 16 - 26 %) at the quarter level. *Staphylococcus* (*S.*) *aureus* was the most frequently reported bacterial species causing IMI (23 %; 95 % CI 14 - 33 %), followed by *Escherichia coli* (13 %; 95 % CI 8 - 19 %). SCM risk factors in water buffalo were assessed by aggregating data from publications in the systematic review. This showed that soil-based flooring, absence of dry cow therapy, young age, early lactation stage, and bottle-shaped teats are associated with increased SCM prevalence. Our analysis revealed substantial variation in SCM prevalence between studies and countries, with *S. aureus* causing more IMI in water buffalo than other bacterial species. However, risk factors for SCM in water buffalo are mainly unknown, indicating a need for further studies to identify effective managemental practices to improve water buffalo udder health.

4.2 Introduction

Water buffalo (*Bubalus bubalis*) is the second most crucial dairy species globally regarding milk yield, contributing about 15 % of global milk production (Minervino et al., 2020). Around 97 % of the water buffalo population is concentrated in Asia (Hegde, 2019). Besides their importance as dairy animals, water buffalo provide energy as draft and transport animals and are important sources of meat and hides. Due to their ability to digest low-quality roughage (Vega et al., 2010; Wang et al., 2020), water buffalo are very well adapted to smallholding (Ahmed et al., 2018) and free-range (Islam et al., 2019) farming systems with minimum supplementary feed in low-income countries.

As in dairy cows, subclinical mastitis (SCM) is one of the highly prevalent diseases in water buffalo (Elsayed et al., 2015; Ahmed et al., 2018). Subclinical mastitis has significant effects on both food security and safety by reducing milk yield, impairing the quality and safety of milk and milk products (Piccinini et al., 2006; Costa et al., 2020), culling productive animals, and creating a risk of development and spread of antimicrobial resistance, mainly in response to the use of antimicrobials (Elahi et al., 2018; El-Ashker et al., 2020). From an economic perspective, inputs from buffalo milk are integral to the rural-based livestock economy in Asian countries with a large buffalo population (e.g., India, Pakistan, Nepal, Bangladesh) (Nanda and Nakao, 2003). Economic analysis of mastitis has been performed for dairy cows but is lacking for water buffalo. An Irish study examining economic losses caused by mastitis in dairy cows estimated that total farm costs increased to 215,071 USD at BMSCC > 400,000 cells per mL (Geary et al., 2012). However, economic analyses vary between different studies in calculating the costs and benefits of mastitis management (Halasa et al., 2007).

The reported prevalence of SCM in water buffalo varies widely between countries and studies, depending on the detection test employed, study design, water buffalo characteristics (e.g., age, parity, lactation stage), and management practices (e.g., milking system, pre-milking stimulation) (Srinivasan et al., 2013; Hussain et al., 2018; Islam et al., 2019). In previous studies, intra-mammary infection (IMI) causing pathogens in samples from healthy or SCM-affected quarter milk samples in water buffalo have primarily been identified based solely on bacteriological culture or bacteriological culture in combination with biochemical or molecular tests using PCR (Preethirani et al., 2015; Jhambh et al., 2017). The most frequently reported IMI-causing pathogens in water

buffalo are *Staphylococcus* (*S.*) spp., *Streptococcus* (*Strep.*) spp., and various gram-negative bacteria at genus-level (Joshi et al., 2013; Pizauro et al., 2014; Singh et al., 2014) and *S. aureus*, *Strep. agalactiae*, *S. epidermidis,* and *Escherichia* (*E.*) *coli* at the species level (Chavoshi and Husaini, 2012; El-Razik et al., 2017; Jhambh et al., 2017). However, IMI-causing pathogens may also vary substantially within and between countries.

Previous research on SCM in water buffalo has mainly focused on identifying disease-causing pathogens and associated risk factors in different geographical regions. Examples of herd-related risk factors identified to date for water buffalo are bedding materials, rearing system, and type of milking system (Sharif and Ahmad, 2007; Sindhu et al., 2009). Examples of animal-related risk factors include age, breed, parity, and lactation stage (Sharif and Ahmad, 2007; Srinivasan et al., 2013; Hussain et al., 2018), while examples of quarter-related risk factors include teat position and teat shape (Sharif et al., 2007; Sharma and Sindhu, 2007; Kaur et al., 2018). Combining such epidemiological knowledge has been critical in developing strategies to control mastitis in, e.g., dairy cows and goats (Bergonier et al., 2003; Ruegg, 2017), but similar integration of existing information is still lacking for water buffalo. Knowledge of the prevalence of SCM, associated risk factors, and the prevalence of frequently known IMI-causing pathogens could contribute to a better understanding of the epidemiology of SCM in water buffalo. A systematic review and meta-analysis study can be one approach to collate existing information, as has been done, e.g., for bovine mastitis (Francoz et al., 2017; Getaneh and Gebremedhin, 2017; Jamali et al., 2018). Two recently published systematic reviews on mastitis (Krishnamoorthy et al., 2021a; Krishnamoorthy et al., 2021b) describe the overall global prevalence of mastitis and the distribution of essential pathogens in dairy cows and water buffalo. However, those reviews did not differentiate between SCM and clinical mastitis and present combined estimates for cows and water buffalo. Therefore, in the present study, a systematic review and meta-analysis focusing specifically on SCM in water buffalo were conducted with the aims of determining: i) quarter level pooled prevalence of SCM in water buffalo, ii) the distribution of pathogens causing IMI, and iii) the association between herd, animal and quarter-related risk factors and prevalence of SCM.

4.3 Materials and Methods

Guidelines on Meta-analyses of Observational Studies in Epidemiology (MOOSE guidelines) (Stroup et al., 2000), Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) statements, and the checklists provided in PRISMA 2015 (Moher et al., 2015) were followed. The quality of the individual publications reviewed was evaluated using the quality assessment tool for observational studies provided by the National Heart, Lung, and Blood Institute (NHLBI) (https://www.nhlbi.nih.gov/health-topics/study-quality-assessment-tools).

4.3.1 Search strategy

A systematic literature search was performed in four electronic databases (PubMed, Scopus, Embase, and Web of Science) to identify publications reporting the prevalence of SCM in water buffalo and/or associated risk factors and/or IMI pathogens. The Boolean operator "AND" was used to combine the terms, while "OR" was used to join the search keywords within each term. A broad search term was applied to include the maximum possible number of relevant publications. Two different types of search terms were considered. The keywords "buffalo" OR "bubaline" were used to identify the population, and "mastitis" OR MeSH term "mastitis, bovine" were used to indicate the outcome. No language and time restrictions were applied during the search. The database search was performed on August 4, 2021.

4.3.2 Inclusion and exclusion criteria

First, two authors independently screened the title and abstract of each hit to identify peer-reviewed original research publications to be included in the analysis. All full-text publications from the selected abstracts were further reviewed. Studies were included based on a list of inclusion and exclusion criteria (Table 4.1). The corresponding author was contacted by e-mail to retrieve full-text publications that could not be obtained in the databases. Duplicate studies were removed manually from the list. Another author with buffalo expertise screened and reviewed the relevant references from the eligible full-text publications missing in the initial database search, considering the eligibility criteria set, following the procedure described by Jamali et al. (2018).

Data extraction spreadsheets (one for "title and abstract screening" and another for "full-text publications screening") were developed, pre-tested, and further refined by another author. Two authors, in parallel, independently extracted the data from the selected full-text publications. All the authors resolved disagreements on the extracted data by cross-checking the full-text publications.

Table 4.1 List of inclusion and exclusion criteria used for identifying eligible studies for data synthesis in the meta-analysis

Inclusion criteria	Exclusion criteria
1. Quarter level prevalence and/or IMI pathogen distribution and/or risk factors for SCM are reported. [a]	1. The full-text publication is not available in English. [d]
2. When the same population group was used in multiple published studies, only one study was considered for inclusion. [a]	2. Unpublished studies, conference papers, experimental trials, reviews, case reports, or case series. [d]
3. The total number of quarters and numbers of SCM-positive quarters are reported. [b]	3. Repeated samples tested from the same animal are tested. [d]
4. The total number of animals and the number of SCM-positive animals are reported. [b]	4. Pooled samples (bulk milk) are analyzed, not individual quarter milk samples. [d]
5. SCM is detected at the animal or quarter level and diagnosed using CMT, MCMT, SFMT, WST, or SCC. A buffalo/buffalo quarter is defined as healthy when no clinical signs are present and are tested negative for SCC/CMT/MCMT/SFMT/WST, and SCM-affected buffalo/buffalo quarter is defined as no clinical signs but tested positive by any of the following tests. [b]	5. Composite milk samples from four-quarters of an animal are analyzed. [d]
6. The number of total quarter samples used for bacteriological examination and the number of positive samples for any IMI pathogen genus and/or species is reported.[c]	
7. Bacteriological culture or PCR is used for the identification of the IMI pathogen. [c]	

[a]Relevant to all study objectives: (i) quarter level pooled prevalence of SCM, (ii) distribution of pathogens causing intra-mammary infection (IMI), and (iii) association between management-, animal-, and quarter-related risk factors. [b]Only relevant to objectives (i) and (iii). [c]Only relevant to objective (ii). [d]Relevant to all objectives (i-iii).

4.3.3 Article coding and data extraction strategy

Data was extracted on: the first author, year of publication, country of study, SCM detection method, number of buffalo and/or quarters examined, number of positive quarters of SCM, number of samples used for microbiological examinations, pathogen distribution at genus or species level (number of samples tested, number of positive samples for each genus or species), and risk factors (number of buffalo or quarters tested, number of SCM-positive buffalo or quarters, *P* value, odds ratio, and 95 % CI). Finally, six datasets were prepared from the extracted data, one on quarter level SCM prevalence, two on IMI-causing pathogen prevalence (one dataset at genus-level and one at species-level), and three on risk factors (one dataset for herd-related risk factor data, one for animal-related risk factor data, and one for quarter-related risk factor data).

4.4 Data analysis

The data were analyzed using R (version 4.1.2; R Foundation for Statistical Computing, Vienna, Austria). The proportions were transformed by double-arcsine transformation (Freeman and Tukey, 1950) to approximate better the normal distribution to meet the meta-analytic model assumption (Jackson and White, 2018) before performing the meta-analysis. A meta-analytic model was fitted to estimate the weighted average proportion by employing the *escalc*, *rma*, and *predict* functions using the R package 'metaphor' version 2.4-0 (Viechtbauer, 2010). The final pooled proportion and 95 % CI were back-transformed to the original values for interpretation. Overall, study effects were assessed with random effect models using the DerSimonian-Laird estimator (DerSimonian and Laird, 1986). The test for heterogeneity (Q), the estimate of between-study variance (τ^2), and the estimate for the proportion of observed variability reflecting between-study heterogeneity (I^2) were determined. The pooled prevalence estimates were evaluated by country, SCM detection test employed, and year of publication. Publication bias was assessed by visual inspection of a funnel plot in which the double-arcsine-transformed proportion of SCM cases was plotted on the x-axis, as a measure of effect size, and the standard error on the y-axis, as a measure of study size. The funnel plot asymmetry was statistically tested by Egger's regression test. Buffalo population per region (country level) was visualized in R using published global water buffalo population data (Minervino et al., 2020).

4.5 Results

4.5.1 Study selection

A total of 797 publications were initially identified, combining 790 publications in the initial search with seven added manually (Dhakal, 2006; Elhaig and Selim, 2015; Patbandha et al., 2015; Preethirani et al., 2015; El-Razik et al., 2017; Islam et al., 2019; Algammal et al., 2020). The title and abstract screening revealed 219 publications eligible for review of the full-text publications based on the inclusion and exclusion criteria. A total of 124 full-text publications were reviewed, of which 71 were excluded based on the exclusion criteria listed in Table 4.1. The excluded full-text publications, with reasons, are listed in Supplementary Table 4.1. The various steps in the selection process included a large number (n = 744) of publications being excluded with reasons, as shown in Supplementary Fig. 4.1. At the end of the selection process, 53 publications were deemed eligible for data extraction and meta-analysis (Figure 4.1). Most eligible publications (n=50; 94 %) were classified as "fair" and only three publications as "good", based on the NHLBI quality assessment criteria (Supplementary Table 4.2).

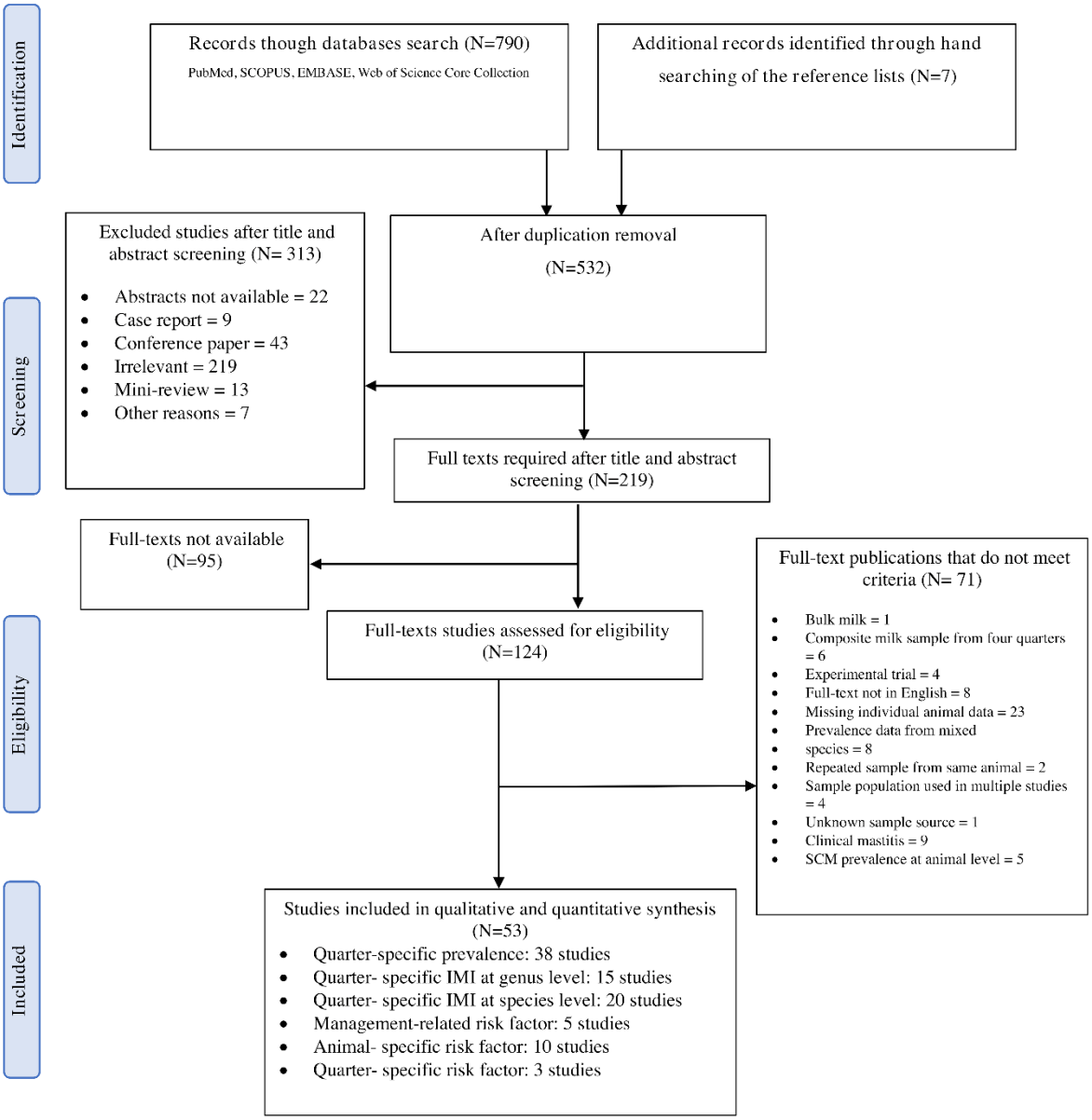


Fig. 4.1 Flow diagram of selection of publications for meta-analysis of subclinical mastitis in water buffalo

4.5.2 Study characteristics

A detailed description of the 53 eligible full-text publications can be found in Supplementary Table 4.3. These publications represented a total of 34,125 quarters and 8,958 buffalo from 10 different countries. Most of the publications (n = 20) were from India, followed by 14 publications from Pakistan, seven publications from Egypt, three publications each from Bangladesh and Brazil, two publications from Iran, and one publication each from Iraq, Italy, Nepal, and the Philippines. The eligible, but also the

ineligible, publications were published mainly between 2001 and August 2021, indicating that buffalo mastitis-related topics have been more frequently studied in recent years (Fig. 4.2).

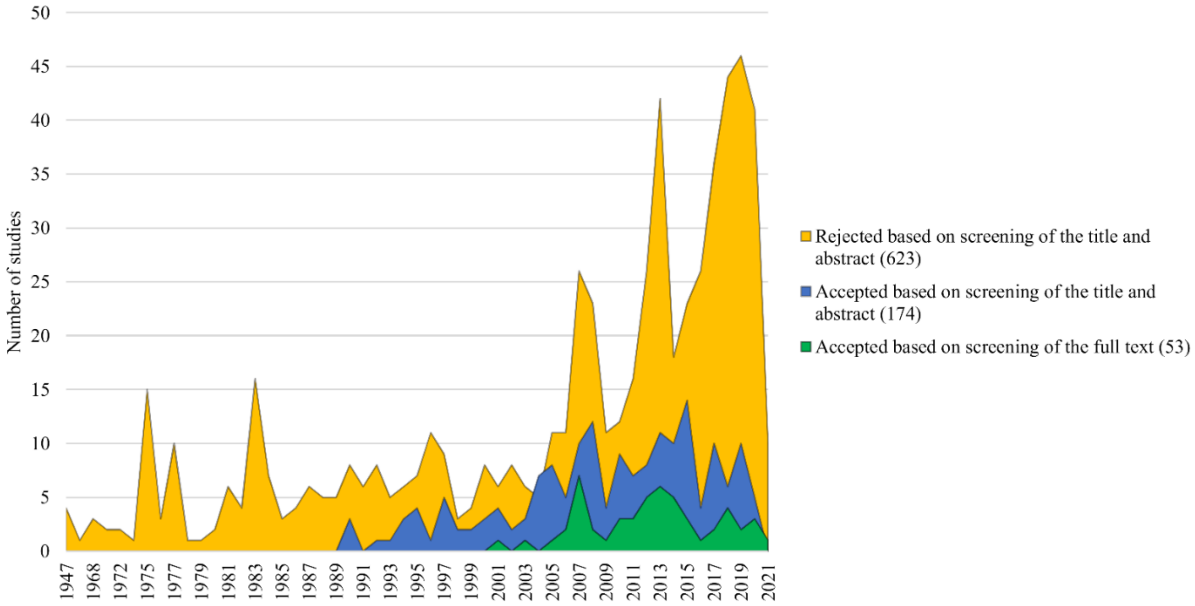


Fig. 4.2 Numbers of publications included and excluded, plotted against year of publication

The study population was highest in India and lowest in Nepal (Table 4.2). The most commonly used test to detect SCM was the California mastitis test (CMT) (n = 21). Data were extracted from the 53 eligible full-text publications, and quantitative data synthesis was performed. The meta-analysis used 38 publications on quarter level SCM prevalence, 20 on quarter level IMI pathogen prevalence by bacterial species, and 15 on quarter level IMI pathogen prevalence by bacterial genus. The global buffalo population was also visualized on a map (Figure 4.3) using data published in a recent review paper on water buffalo (Minervino et al., 2020).

Table 4.2 Quarter level prevalence of subclinical mastitis in water buffalo by country (n = 9) and detection test (n = 5) based on a meta-analysis of 38 studies

Variable name	Categories	No. of studies	No. of animals studied	No. of quarters examined	Pooled proportion	95 % CI	Random effect model heterogeneity I^2
Country	India	14	3,368	13,344	0.19	0.12 to 0.27	99%
	Pakistan	11	3,599	14,169	0.22	0.13 to 0.32	99%
	Bangladesh	1	40	160	0.28	0.21 to 0.35	-
	Nepal	1	50	200	0.09	0.05 to 0.13	-
	Philippines	1	200	751	0.07	0.05 to 0.08	-
	Iran	2	151	601	0.21	0.02 to 0.50	98%
	Egypt	5	640	2,506	0.29	0.18 to 0.41	98%
	Brazil	2	450	1,776	0.18	0.02 to 0.45	99%
	Italy	1	460	618	0.47	0.43 to 0.51	-
Test employed[a]	CMT	21	4,572	17,875	0.21	0.15 to 0.28	99%
	MCMT	2	655	2,573	0.42	0.40 to 0.44	0%
	SFMT	5	1,122	4,488	0.25	0.09 to 0.47	100%
	WST	1	600	2,400	0.24	0.22 to 0.25	-
	SCC	9	2,009	6,789	0.15	0.08 to 0.24	99%
Year of publication	2020	2	510	818	0.46	0.43 to 0.50	0%
	2019	2	144	576	0.22	0.11 to 0.34	89%
	2018	2	255	999	0.26	0.02 to 0.63	99%
	2017	1	217	864	0.17	0.15 to 0.20	-
	2016	1	210	840	0.39	0.36 to 0.43	-
	2015	3	529	2,074	0.24	0.08 to 0.45	99%
	2014	3	719	2,855	0.12	0.04 to 0.22	98%
	2013	4	1,526	5,841	0.14	0.05 to 0.25	99%
	2012	3	1,097	4,380	0.19	0.06 to 0.39	99%
	2011	2	651	2,601	0.29	0.18 to 0.40	91%
	2010	2	550	2,198	0.15	0.02 to 0.38	99%
	2009	1	57	228	0.03	0.01 to 0.06	-
	2008	2	312	1,242	0.10	0.08 to 0.11	0%
	2007	5	1,031	4,080	0.30	0.20 to 0.41	98%
	2006	2	110	439	0.07	0.05 to 0.10	13%
	2005	1	300	1,200	0.59	0.56 to 0.62	-
	2003	1	155	611	0.44	0.40 to 0.47	-
	2001	1	585	2,279	0.07	0.06 to 0.08	-
Overall		**38**	**8,958**	**34,125**	**0.21**	**0.16 to 0.26**	**99%**

[a]CMT=California mastitis test; MCMT=Modified California mastitis test; SFMT= Surf field mastitis test; WST=White slide test; SCC=Somatic cell count.

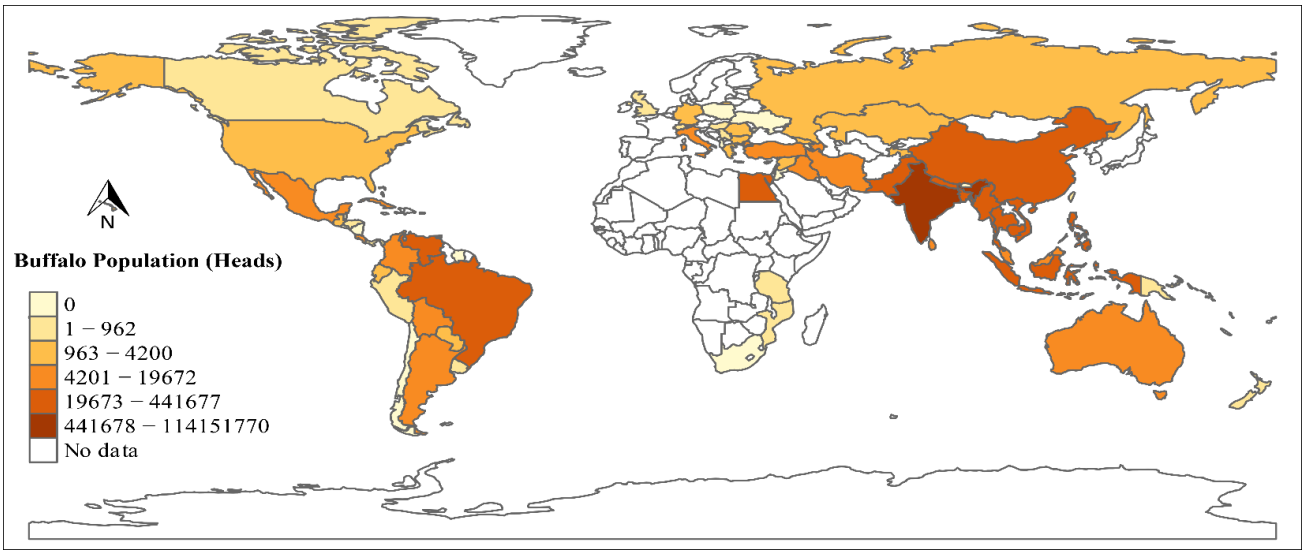


Fig. 4.3 Water buffalo population size in different countries world-wide based on available published data (Minervino et al., 2020)

The different color combinations denote the classified range of prevalence (Map reproduced using published data with the authors' permission)

4.5.3 Prevalence of subclinical mastitis at the quarter level

The CMT test was the most frequently used diagnostic approach to identify SCM. Generally, a test was considered positive if the result was trace or more. Somatic cell count (SCC) was used at various thresholds, ranging from 50,000 to 500,000 cells per mL. The different thresholds used by the various detection tests are listed in Supplementary Table 4.4. The overall pooled prevalence estimate for quarter level SCM was 21 % (95 % CI 16-26 %, I^2 = 99 %) (Figure 4.4). Country-level prevalence of SCM at the quarter level is shown in Table 4.2, although quarter level SCM prevalence differed substantially between publications (3 - 59 %). The pooled prevalence also varied depending on the test employed for SCM detection. The highest pooled prevalence of SCM was 42 % (95 % CI 40 - 44 %) when MCMT was used, based on only two individual publications, and the lowest pooled prevalence was 15 % (95 % CI 8 - 24 %) using SCC based on data from nine publications (Table 4.2).

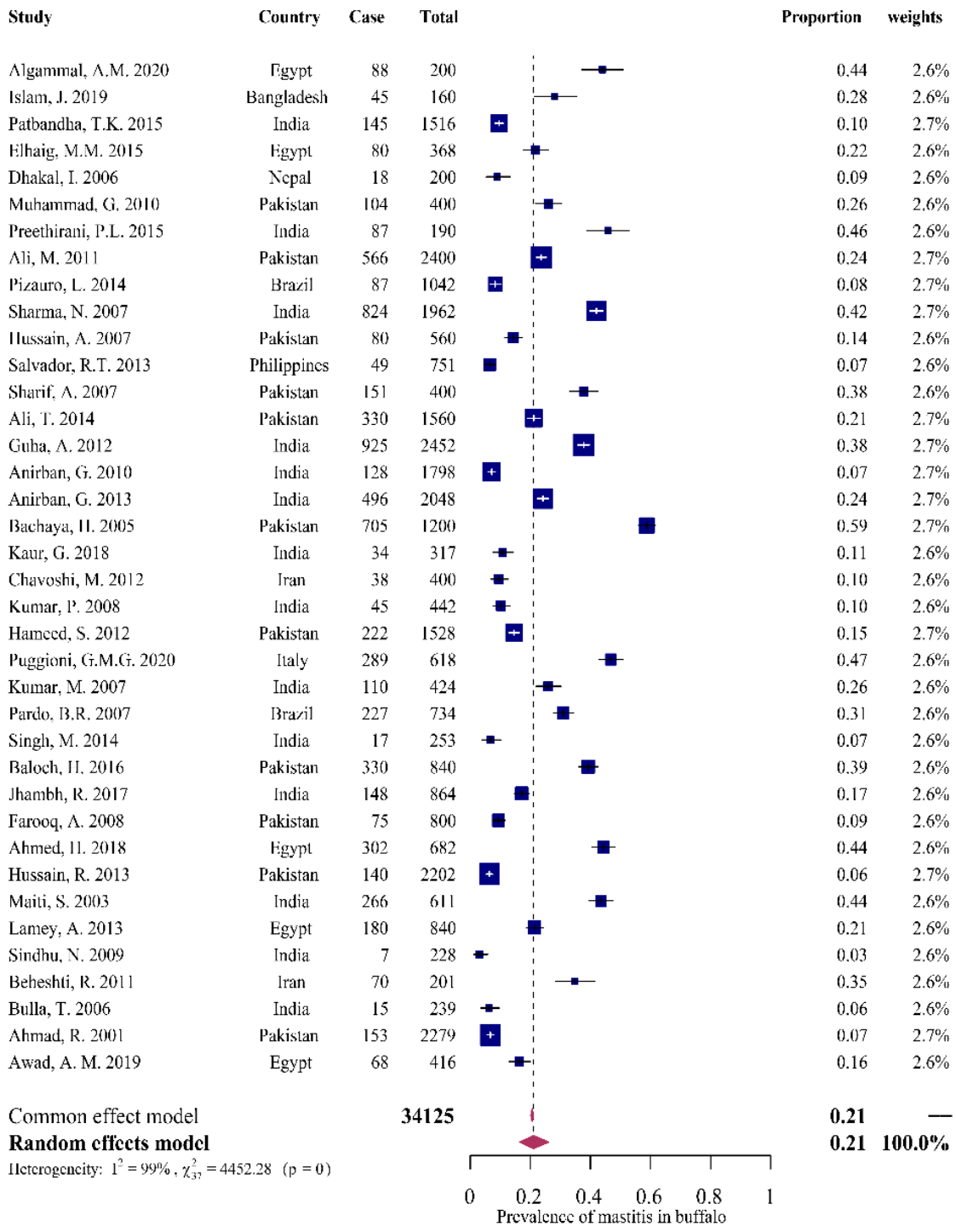


Fig. 4.4 Quarter level prevalence of subclinical mastitis in water buffalo, based on a meta-analysis of 38 studies

Blue squares (■) indicate point estimates, the red diamond (◆) indicates the contribution of individual study weight in the meta-analysis, blue squares with a long horizontal line (—■—) indicate a wide 95 % CI, and blue squares with a plus sign (⊞) indicate a narrow 95 % CI of the point estimate.

4.5.4 Etiology of intra-mammary infection

Genus-level pooled prevalence of IMI was based on 15 publications from four countries (Bangladesh, Brazil, India, and Pakistan). The most prevalent genus was *Staphylococcus* (39 %; 95 % CI 15 - 65 %, I^2 = 99 %), followed by *Streptococcus* (21 %; 95 % CI 13 - 31 %, I^2 = 98 %), *Corynebacterium* (7 %; 95 % CI 2 - 17 %, I^2 = 97 %), *Bacillus* (4 %; 95 % CI 1 - 8 %, I^2 = 94 %), and *Klebsiella* (2 %; 95 % CI 0 - 5 %, I^2 = 94 %) (Figure 4.5). The genus-level prevalence differed substantially between publications for *Staphylococcus* (2-79 %), *Streptococcus* (2-40 %), *Corynebacterium* (2-38 %), *Bacillus* (1-29 %), and *Klebsiella* (1-3 %). The species-level pooled prevalence estimates were based on 20 publications from five countries (Bangladesh, Egypt, India, Iran, and Pakistan). They showed that *S. aureus* was the most prevalent species, with a pooled prevalence of 23 % (95 % CI 14-33 %, I^2 = 97 %), followed by *E. coli* (13 %; 95 % CI 8 - 19 %, I^2 = 95 %). The species-level prevalence also differed substantially between publications for *S. aureus* (5 - 61 %) and *E. coli* (2 - 57 %) (Figure 4.6).

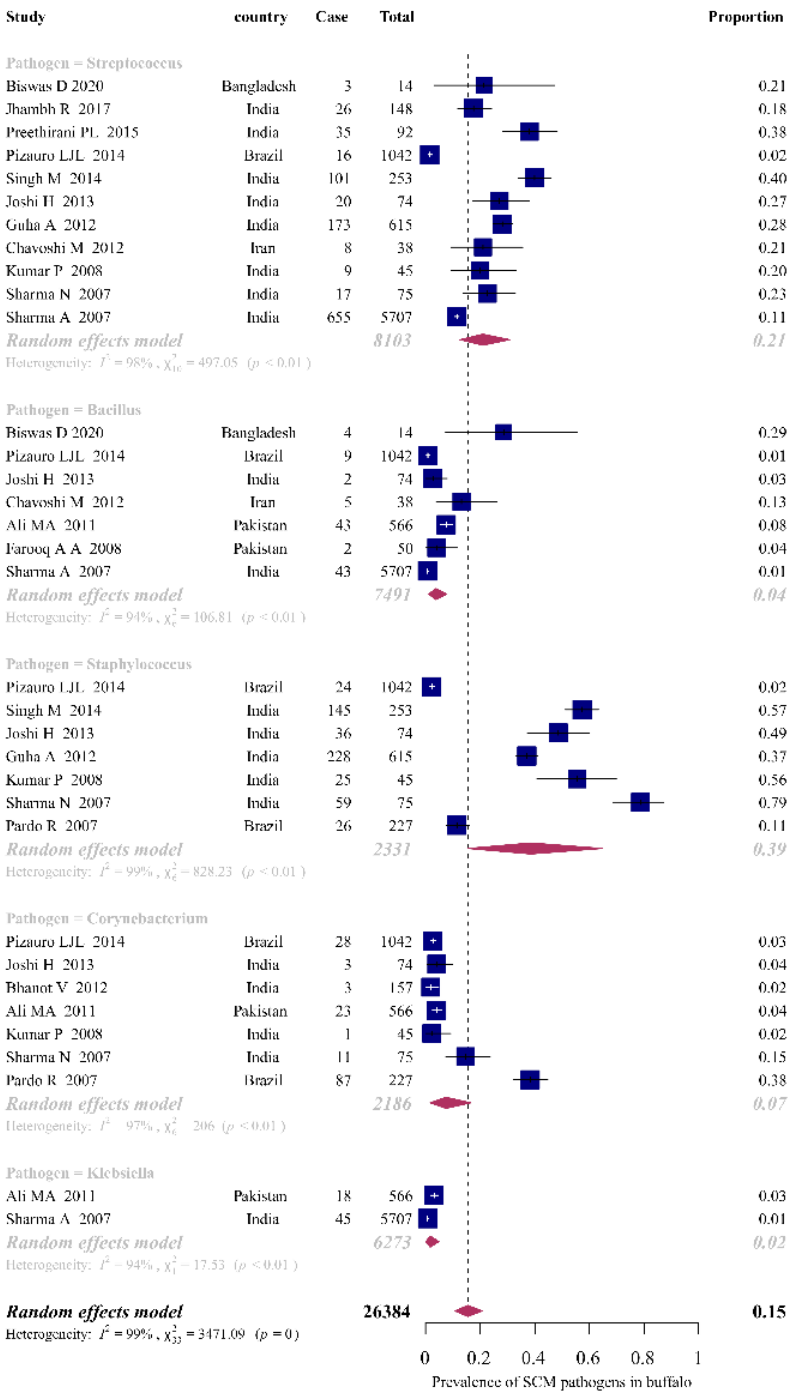


Fig. 4.5 Genus-level pathogen prevalence of subclinical mastitis in water buffalo quarters based on a meta-analysis of 15 studies

Blue squares (■) indicate point estimates, the red diamond (◆) indicates the contribution of individual study weight in the meta-analysis, blue squares with a long horizontal line (—■—) indicate a wide 95 % CI, and blue squares with a plus sign (⊞) indicate a narrow 95 % CI of the point estimate.

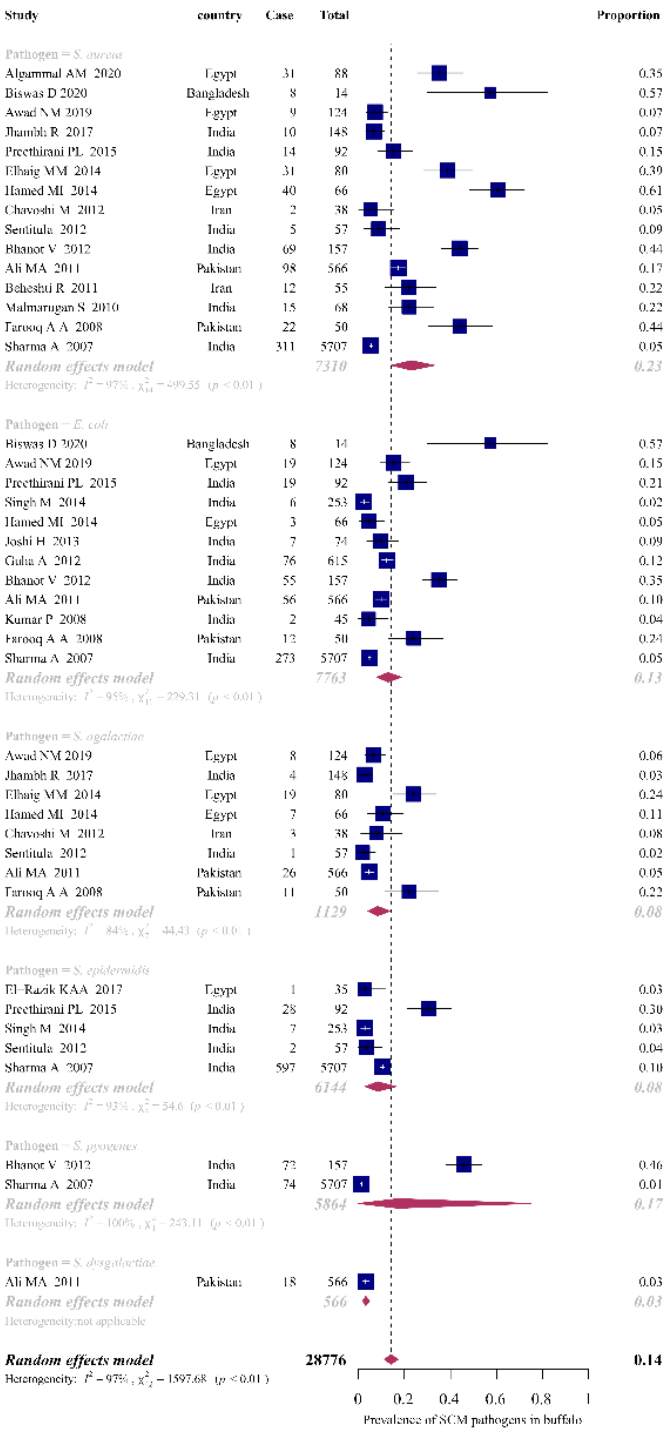


Fig. 4.6 Species-level pathogen prevalence of subclinical mastitis in water buffalo quarters based on a meta-analysis of 20 studies.

Blue squares (■) indicate point estimates, the red diamond (◆) indicates the contribution of individual study weight in the meta-analysis, blue squares with a long horizontal line (—■—) indicate a wide 95 % CI, and blue squares with a plus sign (⊞) indicate a narrow 95 % CI of the point estimate.

4.5.5 Association of herd, animal, and quarter-related risk factors with subclinical mastitis

Among the 53 publications reviewed, five reported management-related risk factors associated with SCM, 10 reported animal-related risk factors, and three reported quarter-related risk factors. An attempt was made to perform a meta-analysis of the risk factors for SCM in water buffalo. However, this was not feasible due to the non-comparability of results between individual publications, e.g., significant variations in risk factor categories. Instead, a narrative description of the significant risk factors identified in publications included in the systematic review is provided below.

For herd-related risk factors, one study reported that water buffalo kept on soil-based floors had a higher prevalence of SCM than those on the sand, which in turn had a higher prevalence than animals kept on cement floors (Baloch et al., 2018). Another study showed that the absence of dry cow therapy was significantly associated with a higher prevalence ($P < 0.001$) of SCM in water buffalo compared with management with dry cow therapy (Medeiros et al., 2011) (Table 4.3). For some herd-related risk factors, statistical significance was not reported. For example, some studies reported a higher prevalence of SCM in water buffalo from larger herds (> 30) than from smaller herds (< 30) and in water buffalo on private farms than in those on government-owned farms (Hussain et al., 2013; Hussain et al., 2018).

Table 4.3 Significant associations reported in the dataset between management-related risk factors and subclinical mastitis in water buffalo

Country	Reference	Numbers of buffalo	Variable	Categories	Prevalence	*P*
Pakistan	Baloch et al. (2018)	422	Flooring type	Cement	19 %	0.05
				Sand	26 %	
				Soil	32 %	
Brazil	Medeiros et al. (2011)	318	Dry cow therapy	Yes	47 %	< 0.001
				No	71 %	

Among the animal-related risk factors identified, young age (< 4 years) in water buffalo was more commonly associated with SCM than older age (> 4 years) (Salvador et al., 2012). Specifically, water buffalo aged 3 years (OR: 4.6; $P < 0.01$), 1-2 years (OR: 3.2; $P < 0.06$), and 4 years (OR: 2.2; $P < 0.05$) were more commonly associated with SCM than water buffalo > 4 years of age (reference group) (Salvador et al., 2012). However, another study reported that older water buffalo (≥ 9 years) was associated with higher

SCM prevalence ($P < 0.05$) than younger animals (3 - 5 years) (Baloch et al., 2016). Water buffalo in higher parities (≥ 4) were reported to have significantly higher SCM prevalence ($P < 0.05$) than those in lower parities (< 4). The odds of SCM in water buffalo in the first month of lactation were 2.1 - 2.5 times higher than for animals at a later lactation stage (Salvador et al., 2012) (Table 4.4). Several non-significant animal-related risk factors for SCM were also reported, with higher prevalence associated with, e.g., the use of high milk-yielding water buffalo breeds such as Murrah, Nilli-Ravi, and Surti compared with non-descriptive native breeds (Srinivasan et al., 2013; Islam et al., 2019), cup-shaped udder compared with bowl-shaped udder (Hussain et al., 2013), and higher daily milk yield (> 3 L/day) compared with lower milk yield (0.5-3 L/day) (Islam et al., 2019).

Table 4.4 Significant associations reported in the dataset between animal-related risk factors and subclinical mastitis in water buffalo

Country	Reference	Numbers of buffalo	Variable	Categories	Odds ratio	Prev-alence	*P*
Pakistan	Baloch et al. (2016)	210	Age	3 to 5 years	-	50 %	<0.05
				6 to 9 years	-	56 %	
				>9 years	-	56 %	
Philippines	(Salvador et al., 2012)	205	Age	1 to 2 years	3.2	46 %	0.06
				3 years	4.6	59 %	0.01
				4 years	2.2	42 %	NS
				> 4 years	Ref	30 %	-
Pakistan	Baloch et al. (2018)	417	Parity	1 to 3	-	23 %	0.02
				4 to 7	-	26 %	
				>7	-	40 %	
Philippines	(Salvador et al., 2012)	205	Lactation stage	Early (0 to 15 days)	2.5	49 %	0.04
				Mid (16 to 31 days)	2.1	52 %	0.09
				Late (> 31 days)	Ref	35 %	-

Among the findings on quarter-specific risk factors, it was reported that bottle-shaped quarters had a higher prevalence of SCM than cylindrical and conical-shaped quarters ($P < 0.05$) (Kaur et al., 2018) (Table 4.5). Flat-end teats, compared with pointed or round-end teats, and hind quarters compared with front quarters, were associated with a higher prevalence of SCM in some studies, although no statistical significance was reported (Hussain et al., 2013; Baloch et al., 2016).

Table 4.5 Significant associations reported in the dataset between quarter-related risk factors and subclinical mastitis in water buffalo

Country	Citation	Numbers of buffalo quarters	Variable	Categories	Prevalence	*P*-value
India	Kaur et al. (2018)	284	Teat shape	Bottle	17 %	< 0.05
				Conical	7 %	
				Cylindrical	10 %	

4.5.6 Publication bias

Publication bias and small-study effects were assessed by visual inspection of the funnel plot (Fig. 4.7). Visual inspection revealed a fairly symmetrical funnel plot, and statistical testing to detect publication bias (using Egger's regression test) was non-significant ($P = 0.5$) for funnel plot asymmetry, suggesting the absence of publication bias due to small study effects.

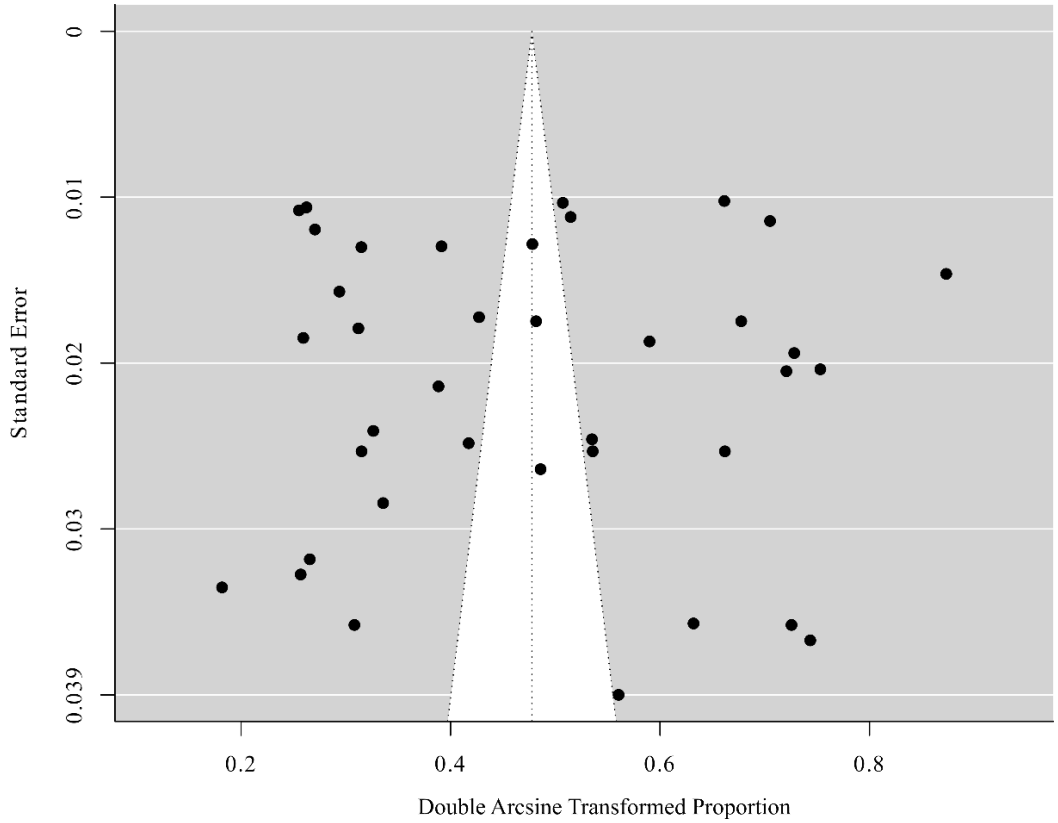


Fig. 4.7 Funnel plot of 38 studies included in the meta-analysis of the quarter level prevalence of subclinical mastitis in water buffalo

The standard error is shown on the x-axis, and the double arsine-transformed proportion of SCM is on the y-axis.

4.6 Discussion

This meta-analysis was conducted to estimate the quarter level prevalence of SCM in water buffalo, to describe the distribution of pathogens causing IMI in SCM, and to summarize animal and quarter level risk factors using currently available global data. It should be noted that the data included in the review primarily represented Asian countries, with only a few studies from other parts of the world. However, this is consistent with the global distribution of the water buffalo population.

The quarter level pooled estimate of SCM prevalence was highest in Italy, Egypt, and Bangladesh. However, there were only one or two reports from some countries, which probably did not truly reflect the prevalence at the country level. Thus, this review indicated that there is scope for more research on SCM in certain regions with substantial buffalo milk production, e.g., Iraq, Italy, Nepal, and the Philippines. The limited number of studies included in the review (n = 53) can be explained by the fact that, in regions where water buffalo are common, research findings are often published in local journals or conference proceedings rather than in peer-reviewed journals. The water buffalo population map developed in this study indicated the presence of water buffalo in 77 countries worldwide. However, scientific evidence on SCM in water buffalo is still lacking in many countries.

There was a significant variation in SCM prevalence between publications. The reported prevalence of SCM in water buffalo at the quarter level ranged from 3 % (95 % CI 1 - 6 %) (Sindhu et al., 2009) to 59 % (95 % CI 56 - 62 %) (Bachaya et al., 2005). The observed heterogeneity ($I^2 > 75$ %) confirmed high between-study variation. Observed differences between publications reviewed in this study related to sample size, population characteristics (e.g., intensive or free-ranging management, use of hand or machine milking system), and animal-specific risk factors (e.g., buffalo breed, age, and parity) (Malmarugan et al., 2010; Bhanot et al., 2012; Abd-Elrahman, 2013). The variation in reported prevalence can also be partly explained by the variety of tests used to detect SCM and the different thresholds used to define SCM. This substantially hampered the interpretation of the overall prevalence values obtained in the meta-analysis.

The pooled prevalence of IMI-causing pathogens was estimated based on microbiological examination of healthy or SCM-affected individual quarter samples. At the genus level, *Staphylococcus* (39 %) was reported to be the most common, which

agrees with findings in studies on IMI in cows and goats (Balemi et al., 2021; Krishnamoorthy et al., 2021b). At the species level, *S. aureus* (23 %) was found to be the most prevalent pathogen in water buffalo in studies included in this review, whereas non-aureus staphylococci (NAS) are reported to be the most common group of bacteria causing IMI in cows or goats (Moroni et al., 2005a; Singha et al., 2021b; Van den Crommenacker-Konings et al., 2021). However, in the studies included in this review, *S. epidermidis* was the only reported NAS species and cannot truly represent the entire NAS group causing IMI in water buffalo. The relatively low proportion of gram-negative bacteria detected in SCM could be explained by the faster removal of these species by a more rapid immune response than gram-positive ones (Günther et al., 2016). Generally, gram-positive species are more frequently associated with chronic infections than gram-negative species.

Interestingly, the pathogen distribution varied widely between the six countries (Bangladesh, Brazil, Egypt, India, Iran, and Pakistan) for which IMI-causing pathogens were reported in the dataset. At the genus level, *Staphylococcus* was frequently reported in India, but reports on *Staphylococcus* were not found in publications from Egypt, Iran, Pakistan, and Bangladesh. At the species level, two publications from Pakistan (Farooq et al., 2008; Ali et al., 2011) reported the prevalence of *S. aureus*. The prevalence of *Streptococcus* spp. was reported in publications from India, Bangladesh, and Brazil. Still, reports on the prevalence of this pathogen were not found in publications from Egypt and Pakistan.

No further meta-regression was performed due to fewer prevalence estimates per subgroup. Therefore, it was not possible to identify the influence of factors such as "test employed", "country", and "breed of the animals" on the prevalence estimates using multivariable subgroup analysis. Our review revealed a significant information gap on risk factors for SCM in water buffalo globally. This needs to be addressed by conducting more research to identify risk factors.

Water buffalo on soil-based flooring had a higher prevalence of SCM than those on sand or cement flooring (Baloch et al., 2018). This makes sense because sand and cement are inorganic matter, which might reduce some level of bacterial contamination. Among the studies in the dataset on animal-specific risk factors, Salvador et al. (2012) reported higher SCM prevalence at an early age, but this was inconsistent with findings by Baloch et al. (2018) of higher SCM prevalence at higher parity. One reason for this discrepancy

could be dissimilar co-variables of the risk factors between publications, such as the pathogen species responsible for SCM. Among the quarter level risk factors, bottle-shaped teats were reported to give a higher prevalence of SCM. Generally, however, risk factor data were minimal, with only a few publications and, in some cases, only a single publication reporting risk factors associated with SCM. Several important risk factors identified for SCM prevalence in water buffalo, e.g., herd size, buffalo breed, milk yield, udder, and teat parameters, were statistically non-significant, or levels of statistical significance were not reported.

Studies showed a higher prevalence of SCM in water buffalo and large variation in the distribution of IMI causing pathogens indicating that there is a need to improve the situation and implement effective management practices. However, most studies did not report the strength of the association resulted a lack of scientific evidences identifying the risk factors of SCM, and further research into the causes behind the associations remained unexplored, which could be an area for future research**.**

4.7 Conclusions

This systematic review and meta-analysis revealed that SCM is a significant disease affecting the water buffalo population worldwide, indicating a need for wide-scale implementation of management practices based on scientific evidence to achieve adequate disease control. However, this field has an extensive global knowledge gap, as the risk factors for SCM in water buffalo are mainly unknown. Therefore, studies are needed to identify risk factors for SCM in water buffalo.

CHAPTER-5

Occurrence and etiology of subclinical mastitis in water buffalo in Bangladesh: A Pilot study

CHAPTER 5

Occurrence and etiology of subclinical mastitis in water buffalo in Bangladesh: A Pilot study

5.1 Abstract

Subclinical mastitis (SCM) in water buffalo are a production disease associated with decreased milk yield and impaired milk quality and safety. Water buffalo is an important livestock species in Bangladesh. Still, information about the occurrence and etiology of SCM in this species is scarce. A cross-sectional study was conducted as part of the Udder Health Bangladesh Programme to i) determine the occurrence of SCM and bulk milk somatic cell count (SCC) in water buffalo in Bangladesh, ii) identify pathogens causing SCM and iii) evaluate penicillin resistance in isolated staphylococci strains. Sixteen buffalo farms in Bangladesh's Bagerhat and Noakhali districts were selected for study, and a bulk milk sample was collected from each farm. In addition, 299 udder quarter milk samples were collected from 76 animals. The bulk milk samples were assessed by direct SCC, and the quarter milk samples by California mastitis test (CMT). The occurrence of SCM calculated at the quarter and animal levels were 42.5 % and 81.6 %, respectively. Milk samples from 108 CMT-positive quarters in 48 animals and 38 randomly selected CMT-negative quarters in 24 animals were investigated using bacteriological culture. The estimated mean bulk milk SCC was 195,000 cells per mL milk (range 47,000-587,000 cells per mL). The estimated quarter level intramammary infection (IMI) on culture was 40.4 %. The identity of isolated bacteria was confirmed by MALDI-TOF mass spectrometry. Non-aureus staphylococci (NAS) were the most common pathogens (24.7 %); among 36 NAS tested, 36.1 % were resistant to penicillin. Thus, SCM prevalence was high on the studied farms, with relatively high penicillin resistance in NAS. Further studies are needed to identify underlying risk factors and develop an udder health control strategy for water buffalo in Bangladesh.

5.2 Introduction

Water buffalo (*Bubalus bubalis*) farming in Asian countries has grown exponentially over the past half-century, contributing around 13 % of global milk production (Siddiky and Faruque, 2018). Water buffalo farming is also becoming popular in Bangladesh due

to the increasing demand for buffalo milk and milk products, greater resistance to many diseases compared with cows, and lower management efforts and feeding costs (Hamid et al., 2016b). A significant impediment to milk production by water buffalo herds is mastitis, which affects the quantity, quality, and safety of milk, causes heavy economic loss, leads to increased use of antibiotics, and impairs animal welfare (Salvador et al., 2012). Clinical mastitis (CM) can be diagnosed by visible changes in the milk, udder, and systemic condition of animals. Still, subclinical mastitis (SCM) remains undetectable in most cases due to a lack of clinical signs (Patel et al., 2019). In water buffalo, SCM is around three-fold more common than CM (Ali et al., 2014) and is responsible for declining milk production, deteriorating milk quality, and reduced milk processability. Subclinical mastitis is also a milk safety concern because of pathogenic microorganisms (Sharma et al., 2011). As the clinical signs remain unnoticed, affected animals may act as reservoirs that shed microorganisms continuously into the environment and affect their herd mates (Ali et al., 2014). Persistent infection also limits the efficacy of antimicrobial treatment by creating a fibrous barrier between the organism and the antibiotic (Putz et al., 2020).

Somatic cells, a combination of leucocytes and epithelial cells released during the regeneration of udder secretory tissue, provide a second line of defense of the mammary gland, so their numbers increase in response to intramammary infection (IMI). These cells are present in high amounts in normal milk, but IMI or stress significantly increases the number of somatic cells in milk (Dang et al., 2007; Alhussien and Dang, 2018). Therefore, milk somatic cell count (SCC) is a reliable parameter for determining milk quality (Sahin et al., 2017). However, it has been suggested that a combination of direct assessment of SCC and indirect assessment by the California mastitis test (CMT) may be the best option to diagnose SCM (Preethirani et al., 2015). As there is no fixed standard for SCC in water buffalo bulk milk, the threshold level of 200,000 cells per mL for cows (Adkins et al., 2017a) has been used as the cut-off value for SCM.

Several studies have assessed the prevalence of SCM in buffalo populations worldwide, ranging from 36 to 48 % at the quarter level (Ali et al., 2011; Salvador et al., 2013; Preethirani et al., 2015). SCM's most reported etiological agents are non-aureus staphylococci (NAS), *Staphylococcus aureus*, *Streptococcus* spp., and *Escherichia coli* (Dhakal, 2006; Patel et al., 2019). Among the staphylococci, NAS are prevalent bacterial species causing IMI (Moroni et al., 2006; Ali et al., 2011; Guha and Guha, 2012;

Locatelli et al., 2013) as well as in SCM and mild forms of clinical mastitis (Frey et al., 2013; Gindonis et al., 2013; Xu et al., 2015). In contrast, *S. aureus* is considered a significant pathogen of buffalo IMI and a concern due to its greater resistance to β-lactam antibiotics (Turutoglu et al., 2009).

A few previous studies have examined the prevalence of SCM in water buffalo in Bangladesh (Islam et al., 2016a; Talukder et al., 2016; Biswas et al., 2020). However, data on buffalo herd-level SCC and studies investigating the susceptibility to β-lactam antibiotics of staphylococci in water buffalo in Bangladesh are scarce. Identifying SCM-causing pathogens and investigating antimicrobial susceptibility in these pathogens are critical prerequisites for implementing effective control of buffalo SCM. In recognition of this, the present study was undertaken within this project to i) determine the occurrence of SCM and bulk milk SCC in water buffalo in Bangladesh, ii) investigate the pathogens causing SCM, and iii) evaluate penicillin resistance in isolated staphylococci strains.

5.3 Material & Methods

5.3.1 Study area and population

Around 40 % of the water buffalo population in Bangladesh is concentrated in coastal areas, including the Noakhali, Bagerhat, and Bhola regions (Faruque *et al.*, 1990). Because of the ease of communication and time constraints, two areas, Noakhali and Bagerhat, were chosen for this investigation. Bagerhat (22.8456°N, 89.5403°E) and Noakhali (22.8246°N, 91.1017°E) are located in the southern coastal part of Bangladesh and have buffalo populations of 21,570 and 13,168 head, respectively (Census, 2010; Huque and Khan, 2017). In Bagerhat, buffalo are reared mainly on household farms, where 1 - 3 milking buffalo are tethered inside the residence boundaries with minimum roughage and concentrate supplements or in a semi-intensive system with ≥ 7 buffalo in enclosed sheds, with or without wallowing facilities in nearby ponds, swamp/marshy land, or rivers. In Noakhali, buffalo are mainly reared in a free-range system (*bathan*), with 50-500 buffalo in each *bathan*. Several closely located *bathans* combine to form a "*killa*" and several *killas* on each island in the Noakhali region. There are also scattered household farms (Habib et al., 2017).

5.3.2 Study design and sampling strategy

A cross-sectional pilot study was performed during September-October 2019 to estimate the prevalence of the SCM status of buffalo in the two selected study districts. A total of 16 buffalo farms were included. The sampling population was 76 lactating buffalo with 299 functional udder quarters (five blocked quarters). In selecting participating farms, a list of buffalo farmers was obtained from Upazila Veterinary Hospital, and 14 household/*bathan* farms (12 in Noakhali, 2 in Bagerhat) with at least one lactating buffalo were selected, based on accessibility and farmer willingness. Two semi-intensive buffalo farms were also selected, one in the Bagerhat region and one in Noakhali (Supplementary Fig. 5.1). A convenient sampling technique (1 - 3 lactating buffalo from each *bathan*/ household and all lactating buffalo from the semi-intensive farms) was applied, due to time limitations and inadequate data on the location of the buffalo farms.

5.3.3 Data collection and processing

A structured questionnaire in English was used to collect data on-farm location (Noakhali / Bagerhat region), education level of the farmer (no formal education /primary /secondary /veterinarian), herd size (total number of buffalo), rearing system (household /*bathan* /semi-intensive), and number of lactating buffalo on each farm. Participation was voluntary, and participants were informed that withdrawal was possible anytime. Written informed consent was obtained from each participant before applying the questionnaire. Data collection was performed in face-to-face interviews with the farmers (farm owner or acting manager) and through on-farm observations following a checklist. Data were collected by administering the questionnaire and made observations on-farm. All interviews were conducted in Bengali, but the questionnaire was completed in English. The completed observation checklists were re-checked for potential inconsistencies at the end of each day.

5.3.4 Analysis and case definition of subclinical mastitis

The SCM status of each buffalo quarter was determined and categorized on a scale from 1 to 5 using the CMT, following the process described by Baloch et al. (2016). A buffalo was considered positive for SCM if at least one of its four quarters had CMT ≥ 2.

5.3.5 Sample collection and preservation

Samples (each 3 - 5 mL) of quarter milk for bacteriological culture were collected aseptically using 70 % ethyl alcohol, following the NMC protocol (Adkins et al., 2017c), from quarters with CMT score ≥ 2. CMT-negative samples were also randomly collected from 24 lactating buffalo. The collected samples were immediately transferred to an insulated icebox and transferred to a freezer (-10 °C to -15 °C) within two hours. The frozen milk samples were transported to the Udder Health Bangladesh laboratory at Chattogram Veterinary and Animal Sciences University within seven days of field visits. The milk samples were stored at -20 ºC at the laboratory, and bacteriological culture was performed within 24 hours.

5.3.6 Bulk milk somatic cell count

After aseptically collecting the composite morning milk sample on each farm, an on-farm bulk milk SCC was performed using a DeLaval cell counter (DeLaval Group, Stockholm, Sweden (Adkins et al., 2017a). This automated device determines SCC optically on a cassette loaded with 60 µL of milk stained with a DNA-specific fluorescent reagent. A digital camera takes pictures of the cell nuclei one-by-one and immediately displays the SCC results, expressed per µL milk.

5.3.7 Bacterial isolation and identification

A total of 146 quarter milk samples, comprising all CMT-positive samples (n = 108) and all randomly selected negative samples (n = 38) from 72 lactating buffalo (no negative samples were taken from the other four buffalo), were cultured in 5 % bovine blood agar following the NMC protocol (Adkins et al., 2017b). After 24 hours of incubation at 37 °C, at least two morphologically similar bacterial colonies were re-cultured on bovine blood agar. The pure cultures were enriched in brain heart infusion broth and incubated at 37 ºC, after which 300 µL 50 % buffered glycerol was added, and the samples were stored at -80 ºC until further use. The pure cultures were also cultured on selective mannitol salt agar to identify *S. aureus* and NAS. Bacterial colonies were identified by colony morphology, color, and hemolytic pattern. All culture media and reagents were manufactured by Oxoid®, Oxoid Ltd, Basingstoke, United Kingdom.

5.3.8 Species confirmation

The isolates from pure cultures were transported to the National Veterinary Institute, Uppsala, Sweden, in Copan Transsystem® (Capon, Brescia, Italy) transport media tubes, for species confirmation using a MALDI-TOF Biotyper 3.0 (Bruker Daltonics GmbH, Bremen, Germany) for validation (score > 1.8) of phenotypic identification accuracy.

5.3.9 Penicillinase test of Staphylococcus spp.

All staphylococci were tested for β-lactamase production using the cloverleaf method described by Mee'aad et al. (2018). The strains *S. aureus* ATCC 29213 and *S. aureus* ATCC 25923 were used for quality control.

5.3.9.1 Statistical analysis

Collected data were imported into Microsoft Excel 2019 (MicrosoftCorp., Redmond, Washington, USA). The data were cleaned, sorted, coded, and checked for inconsistency before export into STATA-IC-17 (StataCorp, 4905, Lakeway Drive, College Station, Texas 77845, USA) for descriptive statistical analysis. Results are presented as frequency and percentage, with 95 % confidence intervals (CI). Demographic characteristics of farmers (location, education, farming system) are presented as frequencies and percentages.

5.4 Results

5.4.1 Descriptive statistics on farms and farmers

Three of the 16 buffalo farms studied were in the Bagerhat region and 13 in the Noakhali region. Farm type was mainly household farms (n = 11), followed by *bathan* (n = 3) and semi-intensive farms (n = 2). Among the farmers, seven had primary-level education, four had no formal education, four had achieved secondary-level education, and a registered veterinarian managed one farm. Twelve farms had 1 - 5 lactating buffalo, two had 6 - 10 lactating buffalo, and the remaining two had ≥ 7 lactating buffalo. The numbers of animals sampled on each farm are listed in Supplementary Table 5.1.

5.4.2 Occurrence of subclinical mastitis and intramammary infection

Estimated SCM occurrence in the buffalo at animal and quarter levels was 81.6 % and 42.5 %, respectively. At the quarter level, the estimated occurrence of IMI was 40.4 %, and the combined occurrence of SCM (CMT ≥ 2) and IMI was 43.5 % (Table 5.1). Among the SCM-affected quarters (CMT score 2-5), a CMT score of 2 was the most frequent (Table 5.2).

Table 5.1 Status of subclinical mastitis at animal and quarter level, based on 299 active udder quarters and 76 lactating water buffalo, on 16 buffalo farms in the Bagerhat and Noakhali regions of Bangladesh

Levels	SCM[1] (CMT ≥2)[2]		IMI[3] (Culture positive)		CMT score 1 (Healthy quarters) +IMI		CMT score ≥2 (SCM quarters) + IMI	
	% (N)	**95 % CI**	**% (N)**	**95 % CI**	**% (N)**	**95 % CI**	**% (N)**	**95 % CI**
Animal	81.6 (76)	71.0 to 89.5	-	-	-	-	-	-
Quarter	42.5 (299)	36.8 to 48.3	40.4 (146)	32.4 to 48.8	31.6 (38)	17.5 to 48.7	43.5 (108)	34.0 to 53.4

[1]SCM: Subclinical mastitis. [2]CMT: California mastitis test (score range 1 to 5). [3]IMI: Intramammary infection. CI: Confidence interval

Table 5.2 Status of subclinical mastitis according to the California mastitis test at quarter level, based on 299 active water buffalo udder quarters and cultures of 146 quarter milk samples from 16 buffalo farms in the Bagerhat and Noakhali regions of Bangladesh

CMT Score[1]	Quarter		IMI[2]	
	% (Numbers)	**95 % CI**	**% (Numbers)**	**95 % CI**
CMT-1	57.5 (172)	51.7 to 63.2	31.6 (38)	17.5 to 48.7
CMT-2	32.4 (97)	27.2 to 38.1	40.0 (80)	29.2 to 51.6
CMT-3	7.7 (23)	4.9 to 11.3	45.5 (22)	24.4 to 67.8
CMT-4	2.0 (6)	0.7 to 4.3	80.0 (4)	28.4 to 99.5
CMT-5	0.3 (1)	0.008 to 1.8	100.0 (1)	2.5 to 100

[1]CMT: California mastitis test (score range 1 to 5). [2]IMI: Intramammary infection. CI:

5.4.3 Bulk milk somatic cell count

The mean bulk milk SCC on the 16 farms was 195,000 cells per mL milk (range 47,000-587,000 cells per mL). In quartile terms, the lowest 25 %, 50 %, and 75 % of farms had a bulk milk SCC value of 129,000, 184,000, and 233,000 cells per mL milk, respectively.

The individual bulk milk SCC count for each of the 16 farms is shown in Supplementary Figure 5.2.

5.4.4 Pathogen distribution and penicillinase production by Staphylococcus spp.

Among the 146 quarter milk samples, 79 (54.1 %) bacterial isolates, of which 60 isolates were confirmed using the MALDI-TOF MS. Ten isolates yielded no bacterial growth on re-culture after shipment, and nine isolates were non-identifiable on a MALDI-TOF spectral scale of > 1.8. The dominant bacteria were NAS, specifically *S. hyicus* (Table 5.3). All 36 NAS isolates were subjected to a penicillinase test, in which 13 (36.1 %) tested positive for β-lactamase production (Table 5.3).

Table 5.3 Pathogen distribution and penicillin resistance identified in quarter milk samples collected from water buffalo in the Bagerhat and Noakhali regions of Bangladesh (confirmed by MALDI-TOF mass spectrometry)

Trait	% (No.)	95 % CI	Penicillin-sensitive NAS	Penicillin-resistant NAS
Non-aureus staphylococci	24.7 (36)	17.9 to 32.5		
Staphylococcus hyicus	(8)		8	0
Staphylococcus hominis	(6)		5	1
Staphylococcus chromogenes	(4)		4	0
Staphylococcus epidermidis	(4)		0	4
Staphylococcus sciuri	(3)		3	0
Staphylococcus arlettae	(3)		0	3
Staphylococcus kloosii	(3)		0	3
Staphylococcus warneri	(1)		1	0
Staphylococcus hemolyticus	(2)		2	0
Staphylococcus capitis	(2)		0	2
Total NAS	**(36)**		**23**	**13**
Streptococcus spp.	0.7 (1)	0.02 to 3.8		
Micrococcus spp.	5.5 (8)	2.4 to 10.5		
Bacillus spp.	2.7 (4)	0.8 to 6.9		
Corynebacterium spp.	2.1 (3)	0.4 to 5.9		
Others (*Rothia*spp., *Deinococcus* spp.)	2.1 (3)	0.4 to 5.9		
*Arcanobacterium*spp.	1.4 (2)	0.2 to 4.9		
Moraxella spp.	1.4 (2)	0.2 to 4.9		
Klebsiella spp.	0.7 (1)	0.02 to 3.8		
Non-specific pathogens	6.2 (9)	2.9 to 11.4		
No growth in sub-culture	6.8 (10)	3.3 to 12.2		

NAS: Non-aureus Staphylococci. 108 subclinical mastitis-positive quarters and 38 negative quarter samples were tested. The frequency of 36 NAS species was below the overall NAS estimates in the Table.

5.5 Discussion

The SCM status in two of Bangladesh's main buffalo farming regions was successfully estimated at animal- and quarter level, and pathogen distribution and penicillin resistance by NAS were determined. SCM's quarter- and animal level occurrence was relatively high, consistent with previous reports from neighboring countries and worldwide (Sharma and Sindhu, 2007; Preethirani et al., 2015; Ahmed et al., 2018). The quarter level occurrence of 42.5% in the present study was in line with the 44 - 46 % reported for other sub-tropical countries such as India and Egypt, where water buffalo production systems resemble those in Bangladesh. Further studies in India have reported higher occurrence (59 - 78 %) (Bhanot et al., 2012; Charaya et al., 2015), while lower prevalence (28 %) has been reported in a small-scale study in Bangladesh (Islam et al., 2019). Compared with the quarter level prevalence, the animal level SCM prevalence was much higher in this study (81.6 %), which aligns with previously reported values ranging from 70 to 78 % (Bachaya et al., 2005; Sentitula et al., 2012). These differences in estimated SCM could be due to differences between study sites, study years, and geographical areas. The criteria applied for SCM detection, climate variations, and underlying animal- or quarter level risk factors (Hussain et al., 2018). Investigating such differences was beyond the scope of this study, but further studies are needed to assess and quantify the farm- and animal level factors that might influence SCM occurrence. Such knowledge could be valuable in controlling mastitis in water buffalo.

The mean bulk milk SCC estimated in the present study was 195,000 cells per mL. This is in line with Mujeeb et al. (2009) and within the acceptable threshold of 200,000 - 500,000 cells per mL of buffalo milk established in India (Panchal et al., 2016; Jhambh et al., 2017). However, it exceeds the cut-off value of 100,000 cells per mL milk recently proposed for Murrah buffalo by NDRI-India and barely meets the cut-off of 200,000 cells per mL milk suggested for neighboring countries such as Nepal and India (Dhakal, 2006; Alhussien and Dang, 2018). The median level of bulk milk SCC was 184,000 cells per mL milk, representing slightly better status. Increased SCC is positively correlated with IMI in water buffalo (Moroni et al., 2006) but the increase in SCC level recorded here was still much lower than that in cows and goats (Moroni et al., 2005b). However, there

was large variation in SCC level between the individual farms, an issue which was not explored further in this study. The level of SCC increases with the entry of IMI-associated pathogens into the udder through the teat canal and with stress conditions due to seasonal, diurnal variations in climate conditions and parity of the lactating buffalo (Alhussien and Dang, 2018; Bombade et al., 2018). To date, relevant data on SCM in water buffalo in Bangladesh have been insufficient. The present study, therefore, makes a timely contribution, especially considering the increasing consumer demand for buffalo milk.

The occurrence of IMI in buffalo was shown to be high in this study (40.4 %). Relative IMI occurrence in healthy and SCM-affected buffalo quarters was similar (40 % and 44 %, respectively). In water buffalo in India, IMI occurrence in SCM quarters of 38 - 45 % has been reported (Singh et al., 2014; Jhambh et al., 2017), consistent with our findings. The most common pathogens in this study were NAS, which is also consistent with findings in other studies on water buffalo (Bansal et al., 2007; Sharma et al., 2007; Kumar, 2009; Singh et al., 2014). *Staphylococcus hyicus* was the most dominant species among the NAS and has been identified previously in IMI in water buffalo in India and Brazil. Non-aureus staphylococci, commonly found in the teat canal, are an important group of SCM pathogens and often resistant to one or more antimicrobials (Condas et al., 2017; Stevens et al., 2018; Traversari et al., 2019). In the present study, 36 % of NAS exhibited penicillin resistance, which is in line with results reported in other studies in India and Brazil (Pizauro et al., 2014; Singh et al., 2014). This level of resistance is high in terms of public health safety. It needs to be confirmed in future studies, which should also explore the underlying cause of the dissemination of antibiotic resistance in buffalo herds in Bangladesh.

The discrepancies between the low bulk milk SCC in the buffalo herds studied (195,000 cells per mL milk) and the higher IMI at the quarter level were not further assessed in the present study. However, several previous studies have found a relatively low SCC level and increased CM incidence in dairy cows (Lam et al., 1997; Peeler et al., 2000; Suriyasathaporn et al., 2000). Environmental conditions and changes in management practices, e.g., such as discontinuation of teat dipping, cleaning of the calving areas, and keeping animals in standing conditions after milking, could result in differences in pathogen exposure, e.g., environmental pathogens. The type of pathogens involved in an IMI may be an important factor associated with the CM (Lam et al., 1997; Peeler et al.,

2000). It is known that the presence of contagious *S. aureus* or *Streptococcus agalactiae* plays a significant role in increases in SCC levels above 500,000 cells per mL milk (Moroni et al., 2018), so the absence of these pathogens in the present study could partly explain the lower SCC level.

Moreover, the increase in SCC in IMI-associated quarters in buffalo may be lower than in dairy cows due to limited phagocytic activity. According to Sahoo et al. (1998), there is a core difference in lysosome-containing hydrolases between cattle and buffalo, with lower concentrations of these enzymes in buffalo, resulting in lower phagocytic activity than in other ruminant species. The requirements of convenient sampling and small sample size-imposed limitations in this pilot-scale study. However, the data obtained reflect the regional SCM situation in water buffalo in Bangladesh and can act as the basis for further national-level research.

5.6 Conclusions

There was a high occurrence of SCM in water buffalo at the quarter and animal levels (42.5 % and 81.6 %, respectively) on buffalo farms in Bangladesh's Noakhali and Bagerhat regions. A moderate rate of SCC was detected in buffalo bulk milk on these farms (7 out of 16 farms exceeded the SCC threshold), which is reasonably good for pre-harvesting buffalo milk in Bangladesh. These findings suggest that appropriate treatment and strategies are needed to control mastitis in water buffalo in Bangladesh. NAS was the most frequently identified pathogen. The NAS strains detected often tested positive for penicillinase production, emphasizing the importance of restricting the use of antibiotics to maintain public health when consuming buffalo milk. A systematic assessment of these pathogens' direct and indirect transmission pathways between water buffalo are urgently needed.

CHAPTER-6

The prevalence and risk factors of subclinical mastitis in water buffalo (*Bubalis bubalis*) in Bangladesh

CHAPTER 6

The prevalence and risk factors of subclinical mastitis in water buffalo (*Bubalis bubalis*) in Bangladesh

6.1 ABSTRACT

Subclinical mastitis (SCM) in water buffalo are responsible for reduced milk yield and quality. This cross-sectional study was carried out to a) estimate the prevalence of SCM, b) identify risk factors associated with SCM, and c) identify farm level risk factors associated with bulk milk somatic cell count (BMSCC). The buffalo farms in this study represented five rearing systems: free-range, semi-free-range, household, semi-intensive, and intensive, providing 3,491 functional quarters of 880 lactating buffalo on 248 farms. The California mastitis test score was used to identify SCM. Bulk milk samples (n = 242) were used for farm level BMSCC. Quarter and buffalo-level risk factors for SCM were measured using questionnaires and observations. The overall SCM prevalence was high at 27.9 % at the quarter level (25^{th} and 75^{th} percentiles: 8.3 % and 41.7 %) and 51.5 % at the buffalo level (25^{th} and 75^{th} percentiles: 33.3 % and 66.7 %). The geometric mean BMSCC was 217,000 cells per mL of milk (ranging from 36,000 - 1,213,000 cells per mL), which is low on average, but some farms could improve substantially. The buffalo-rearing system, udder location (left versus right), teat shape, udder asymmetry, number of milkers, and having a quarantine facility were associated with buffalo udder health. Our findings suggest that mainly using free-range rearing systems may help decrease the prevalence of SCM primarily by employing buffalo breeding and better farm biosecurity, and udder health control strategies can be designed based on our findings.

6.2 Introduction

Subclinical mastitis (SCM) is the inflammation of mammary tissue in the absence of clinical signs. This disease reduces the milk yield in dairy animals, impairs animal welfare, and is associated with milk quality deterioration, making milk less suitable for consumption and processing (Costa et al., 2020). A study by Malik and Verma (2017) reported that the average annual economic loss per buffalo due to mastitis was 70 USD, with mastitis intervention accounting for 55 % of the loss and a drop in milk production accounting for 16 %. Water buffalo (*Bubalus bubalis*) is the second most crucial dairy animal in Asia, and about 74 % of the global buffalo population lives in South Asian

countries (i.e., Bangladesh, Bhutan, India, Pakistan, Nepal, and Sri Lanka) (Minervino et al., 2020).

Diagnosis of SCM is of great importance since this disease remains otherwise unnoticed due to the absence of visible changes in the milk or udder and because SCM contributes to the transmission of intra-mammary infection (IMI) (Kirkeby et al., 2019). Currently, multiple screening tests, such as somatic cell count (SCC) or the California mastitis test (CMT) (Hussain et al., 2018; Aldujaily et al., 2019), are used to detect SCM in water buffalo. Bulk milk somatic cell count (BMSCC) is another helpful indicator representing the udder health status on the farm (Pasquini et al., 2018; Costa et al., 2020). The prevalence of buffalo SCM has been studied in many countries, such as India, Pakistan, and Bangladesh, demonstrating a quarter level prevalence of 10-46 % using CMT (Muhammad et al., 2010; Preethirani et al., 2015; Islam et al., 2019). However, the prevalence is widely variable between the studies and regions.

Water buffalo can be classified into two subspecies: river-type buffalo, which are concentrated in India, Pakistan, Egypt, and Italy, and swamp-type buffalo, which are concentrated in China, Myanmar, and Thailand (Minervino et al., 2020; Zhang et al., 2020). Within these two buffalo subspecies, the river type is widely distributed in Bangladesh, except for some swamp-type buffalo found only in the eastern part of the country (Samad, 2020). About 70 % of the river-type buffalo in Bangladesh are indigenous and non-descriptive, meaning they do not belong to any specific breed, such as Murrah or Nilli-Ravi. A few crossbreeds of indigenous buffalo with Murrah, Nili-Ravi, Surti, or Jaffrabadi can be found on the surrounding Indian border due to migration from India to Bangladesh. These crossbreeds generally produce 5-10 L of milk, about two times more than the indigenous buffalo (Ali et al., 2014; Samad, 2020). High-yield crossbred buffalo generally have an increased risk of SCM (Islam et al., 2019). Buffalo are mainly reared either in a free-range or an intensive system.

Moreover, depending on buffalo-rearing systems in sub-tropical countries, there is limited pasture availability with no or minimal supplementation of concentrates (Tiwari et al., 2007; Habib et al., 2017; Rahman et al., 2019a). Nutritional status is important because specific macro and micronutrients regulate metabolism and immunological function in animals, and it is known that abnormalities in metabolism or immunity can cause health disorders in dairy cows (Sordillo, 2016). For example, insufficient nutrition in dairy cows has been reported to be associated with a negative energy balance and

oxidative stress, predisposing several disease conditions, such as ketosis and mastitis (Esposito et al., 2014; Krishna et al., 2014; Pérez-Báez et al., 2019). Under these circumstances, it is crucial to identify farm, animal, and quarter-related factors that further increase the risk of SCM in water buffalo to improve udder health.

Knowledge of the prevalence and associated risk factors might help identify control strategies for buffalo mastitis. To meet this need, we conducted a nationwide cross-sectional study on water buffalo with the objectives of a) estimating the prevalence of SCM at both an animal and quarter level, b) identifying the risk factors associated with SCM prevalence, and c) identifying the farm level risk factors associated with BMSCC.

6.3 Materials and methods

6.3.1 Study area

Bangladesh is divided into 64 districts and 495 subdistricts named "Upazila". The coastal and semi-coastal districts in Bangladesh, which are surrounded by the Meghna-Ganga and the Jamuna-Brahmaputra River floodplains, are the critical areas of buffalo concentration, where 40 % of the buffalo population in Bangladesh are kept because of the availability of fallow pasture land and green forages (Faruque et al., 1990; Hamid et al., 2016a). Water buffalo farms and individual lactating water buffalo belonging to nine districts were included in the study to effectively represent the buffalo population in Bangladesh (Fig. 6.1).

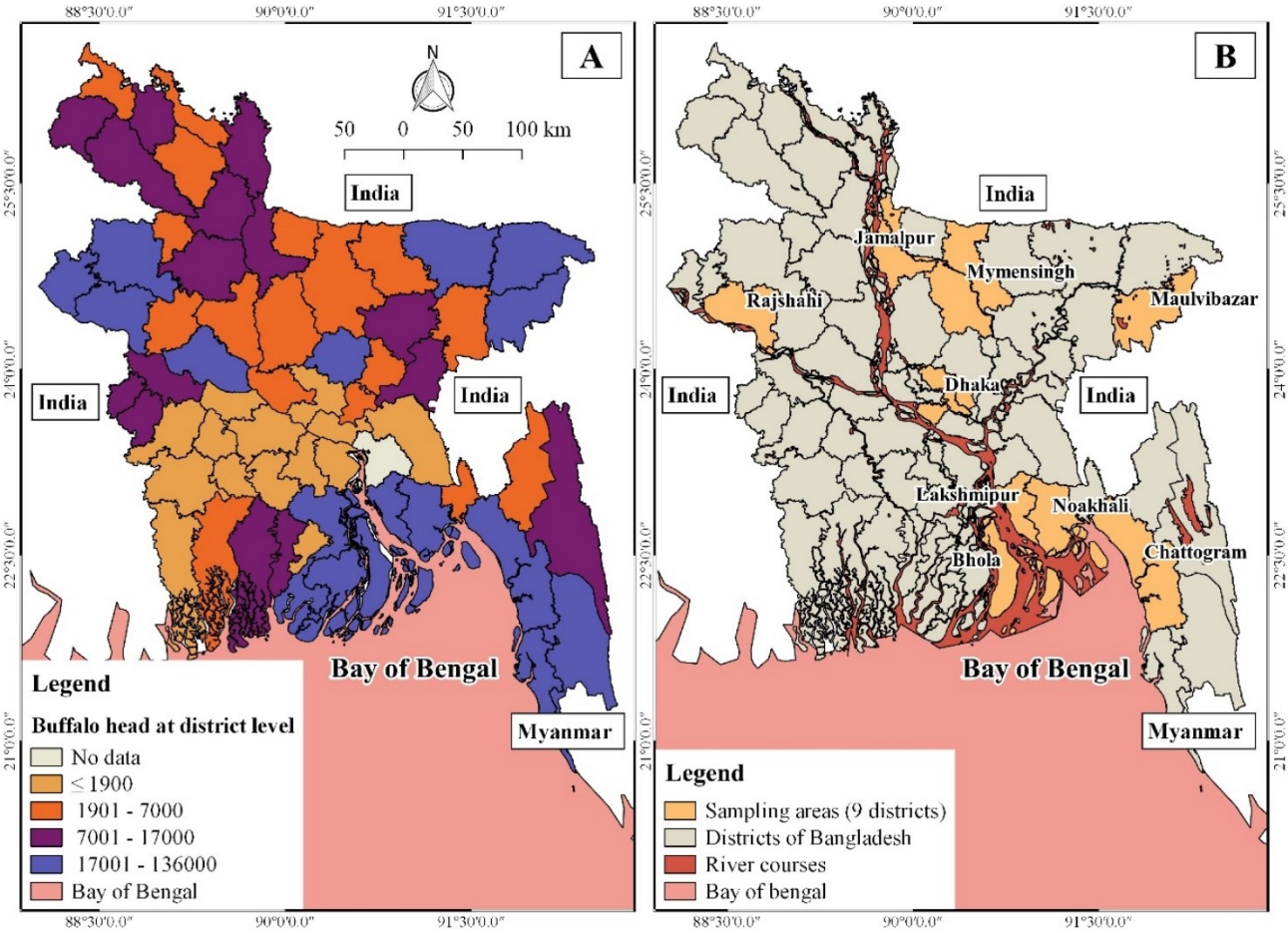


Fig. 6.1 Location of nine buffalo concentrated districts in Bangladesh where buffalo farms were enrolled in the present study.

A. shows the district-level buffalo heads in Bangladesh (Data source: LDDP (2019)). B. The orange indicates the location of the study's nine districts recruited for sampling (Rajshahi, Jamalpur, Mymensingh, Maulvibazar, Dhaka, Lakshmipur, Bhola, Noakhali, and Chattogram).

6.3.2 Production systems

The buffalo farms in this study belonged to five rearing systems: free-range, semi-free-range, household, semi-intensive, and intensive. Free-range buffalo depend on grazing on fallow land in coastal or semi-coastal islands with the supplementation of straw or, at times, small amounts of concentrates. In free-range systems, buffalo are moved from one island to another based on the availability of green forages. In the semi-free-range system, buffalo are transferred to the inlands during the dry season (October to March) due to feed scarcity on the islands and stay there for 3-6 months, depending on roughage

availability. The household rearing system allows 5-7 hours of grazing, combining a supply of a minimal amount of straw, grass, and concentrates. In river-basin areas, the semi-intensive system allows buffalo to stay in sheds at night, graze in nearby pasturelands, and return daily. In an intensive system, buffalo are tied in sheds, stall-fed, and are never allowed to graze.

6.3.3 Sampling design and study period

Presently, the upazila veterinary hospitals (UVH), a part of the government livestock services, and a non-governmental organization (NGO) named "Palli Karma-Sahayak Foundation" (PKSF) collected buffalo population data at the sub-district level in Bangladesh. Data on the number of farms was collected through a census by the UVH every year. About 1.5 million buffalo heads were reported in Bangladesh during 2019-2020 (DLS, 2021). However, this is likely an underestimation of the actual number of buffalo since this census depends on farmers' active reporting of farm data. Since no sampling frame was available, lists of buffalo farms were collected from the buffalo farmers' database of UVH, or PKSF involved with buffalo farmers' support and researchers who had the best knowledge about the buffalo population. Based on their information, one buffalo-concentrated sub-district was selected from each district for sampling. A total of 200 farms to be sampled was estimated according to an expected 50 % prevalence with 5 % absolute precision, assuming an intraclass correlation coefficient of 0.25 for buffalo within farms. A list of buffalo farms was created with the help of UVH and PKSF. The listed buffalo farmers were contacted to request information on the number of lactating buffalo and the farm location. Additionally, four known intensive farms were recruited for sampling because of the limited number of intensive buffalo farms in Bangladesh. Two of the four intensive farms were government-owned, and two more were private buffalo farms. Finally, a total of 248 water buffalo farms from free-range (n = 51), semi-free-range (n = 106), household (n = 33), semi-intensive (n = 54), and intensive (n = 4) buffalo farms were recruited for sampling. The numbers of sampled farms from each study location are given in Table 6.1. About 25 % more farms than the required sample size were included in the study due to uncertainty that all farms could be sampled. For example, the free-range buffalo farms were remotely located and, therefore, sometimes difficult to reach during the morning milking time. Sampling was performed on 1 to 5 lactating buffalo on each farm. All the animals were sampled when

a farm had ≤ 5 lactating buffalo, and five were randomly sampled when a farm had ≥ 5 lactating buffalo. However, on nine farms (five semi-free ranges, three households, and one semi-intensive farm), only one lactating buffalo could be sampled because of non-cooperative animals on those farms. Because of the small number of intensive farms, all the lactating buffalo from these four farms were sampled. Milk sampling and the recording of potential risk factor data (section 6.3.5) were conducted between February 2020 and April 2021 (sampling was interrupted for six months due to the national lockdown in the SARS-CoV2 pandemic from April to September 2020).

Table 6.1 Distribution of 880 buffalo sampled from 248 buffalo farms over five different buffalo-rearing systems in nine buffalo concentrated districts in Bangladesh

District	Total buffalo population	Number of buffalo sampled	Number of farms from different buffalo-rearing systems				
			Free-range	Semi-free-range	Household	Semi-intensive	Intensive
Noakhali	50,680	182	9	113	20	40	-
Jamalpur	11,955	145	17	84	9	35	-
Chattogram	44,494	135	85	38	12	-	-
Rajshahi	18,550	118	-	-	2	116	-
Moulvibazar	55,363	105	-	103	-	2	-
Bhola	52,190	100	48	13	30	7	2
Laxmipur	29,971	52	-	-	-	-	52
Mymensingh	3,087	28	5	15	4	4	-
Dhaka	1,621	15	-	-	-	-	15
Overall	**267,911**	**880**	**164**	**366**	**77**	**204**	**69**

6.3.4 Detection of subclinical mastitis using the California mastitis test and bulk milk somatic cell count

The SCM status of each buffalo quarter was determined and categorized on a scale from 1 to 5 using CMT, following the process described by Baloch et al. (2016). The CMT was performed on each functional quarter of a buffalo before milking was started, after discarding the first 1-2 squirts of milk. A quarter was considered positive when the CMT score was ≥ 2 with no visible signs of CM (any changes in milk or swelling, and/or redness, or painfulness of the mammary gland). A buffalo was considered SCM-positive when one or more of its four functional quarters had SCM. Bulk milk SCC was determined in thoroughly mixed morning bulk milk from all the lactating buffalo on each

farm following Singha et al. (2021a) using a DeLaval cell counter (DeLaval Group, Stockholm, Sweden) (Adkins et al., 2017a).

6.3.5 Questionnaire design and data collection

A questionnaire was developed in English and tested on 16 farms in a pilot study (Singha et al., 2021a) before starting the main study. We prepared two separate datasets: quarter level, buffalo level (dataset A), and farm level (dataset B). Dataset A included the quarter level information on the position of the tested quarters (front left, front right, hind left, or hind right) and teat shape (cylindrical, bottle, or funnel). Buffalo-level information included the age of the buffalo in years (2.5 to 6, > 6 to 8 or > 8 to 20 years), breed (indigenous versus cross breed), stage of lactation (3 months or less, > 3 to 6 months, > 6 to 9 months or > 9 months), average daily milk yield (3 L or less versus 3.1 L to 9 L), udder symmetry (symmetrical or asymmetrical), CM history (yes or no), abortion history (yes or no), and the number of milkers on the farm (single versus multiple). Dataset B included general farm level information such as type of buffalo-rearing system (free-range, semi-free-range, household, semi-intensive or intensive), type of farm area (coastal, semi-coastal, inlands, islands or river basin), season (winter: late December to early February; spring: late February to early April; summer: late April to end of June; rainy: early July to early September or autumn: late September to early December), communication with the employees (good contact with the staff, irregular visits to the farm, rare visits to the farm or managed by farmers themselves), district, buffalo type (river type, swamp type or mixed), wallowing water source (tube-well, deep tube-well, pond, river or rain water), feeding system (stall-feeding, grazing or mixed), supplied feed (no additional food supply, concentrate, roughage and concentrate, locally available straw and roughage, only roughage from grazing), buffalo source (stock, stock or purchased, purchased or contract), history of any buffalo mortality in the last 12 months (yes or no) and restraining during milking (yes or no). Milking was performed by hand, using three traditional techniques: complete hand milking, stripping, or knuckling. Generally, one or more of these techniques is used on a buffalo farm depending on milkers' preference and buffalo teat length or diameter. Complete hand milking involves grasping the teat with five fingers and pressing it against the palm. Stripping involves firmly holding the teat between the thumb and index finger and drawing down the length of the teat while applying pressure. Knuckling consists in bending the thumb and pressing

the teat against the index finger with the nail and end of the thumb. Information related to the milking technique was collected, such as fore stripping (yes or no) and hand milking (full hand, stripping, or knuckling). Other relevant data was also collected, such as udder hygiene (excellent, good, fair, or poor), a score of milkers' hygiene (excellent, good, or fair), feed offered before milking (yes or no), and whether farms use a quarantine facility for the newly purchased buffalo (yes or no). The corresponding author can provide the full version of the questionnaire upon request. Written consent was obtained from the buffalo farmers before administering the questionnaire. Data were collected in face-to-face interviews with the farmers (farm owner or acting manager) and through on-farm observations. All interviews were conducted in Bengali, but the questionnaire was recorded in English. The study was approved and performed in line with the guidelines of the SAU research system (SAU/Ethical Committee/AUP/21/06) of Sylhet Agricultural University, Bangladesh.

6.3.6 Statistical analyses

6.3.6.1 Descriptive statistics

Data were imported into R (version 4.1.2; R Foundation for Statistical Computing, Vienna, Austria) for statistical analysis. The analysis was carried out separately for datasets A and B. The dependent variables considered were, for dataset A, the SCM status of an individual quarter (i.e., healthy or SCM positive) and the SCM status of an individual buffalo, and for dataset B, the BMSCC (cells per mL of milk) as the indicator of SCM status at the farm level. The BMSCC distribution was positively skewed, so a log10 transformation was performed to restore a normal distribution, which was checked using a visual inspection of the histogram. Descriptive statistical analysis was carried out to calculate the prevalence of SCM (at the quarter and buffalo levels) and the summary of BMSCC (at the farm level). To estimate the sampling-corrected prevalence, sampling weights were created based on the total buffalo population in an area, the number of buffalo, and the sampled quarters (Dohoo et al., 2010b). The probability of selecting each buffalo was calculated by dividing the number of buffalo sampled by the total number in an area, as estimated by the UVH/PKSF. Sampling weights were calculated using the inverse of the sampling probability. We then evaluated the buffalo and quarter level prevalence, correcting for weighting and taking clustering by the district into account using STATA SE-17.0 (Stata Corp. College Station, TX, USA).

6.3.6.2 Quarter and animal level risk factor models

Two generalized linear mixed-effects logistic regression models were constructed using the R package "lme4" (Bates et al., 2015) to identify factors associated with SCM from dataset A. For generalized linear mixed-effects logistic regression models, the GLMER function was used. Farm and buffalo ID within farms were used as random effects in these models. Subject-specific effects were used to calculate the odds ratio, meaning that fixed effects in a mixed model represent the effects of that factor within the cluster. The significance of the random effect terms was checked with the likelihood ratio test (LRT) using the latent variable approach (Dohoo et al., 2010a). Then, fixed variables that were significant at $P \leq 0.2$ in the univariable logistic regression were selected for the multivariable logistic regression. The model was manually constructed using a forward selection procedure, applying the maximum likelihood estimation procedure (Dohoo et al., 2010a). The statistical significance of the contribution of individual predictors was determined using Wald's test and the LRT. The presence of confounders was assessed by adding one of the variables in order of significance (the variables with the lowest P-value were added first) and, in each step, determining whether the beta coefficient of other variables in the model changed by more than 30 %, which was deemed confounding. Simultaneously, we assessed the collinearity based on significant changes (> 30 %) in the standard error. If a variable was found to be non-significant ($P > 0.05$), was not a confounder, and no indications of collinearity were seen, the variable was removed from the model in the next step. Finally, significant ($P \leq 0.05$) variables at LRT were presented regarding odds ratio and 95 % C.I.

6.3.6.3 Farm level BMSCC risk factor model

A linear regression model was fitted using dataset B to identify the association of farm level variables with log10-transformed BMSCC (LBMSCC). The linear regression model used the LM function from the R base. Variables significant in the univariable linear regression at $P \leq 0.2$ were used in multivariable linear regression models. Variance inflation factors (VIF) for the factors were also examined to diagnose collinearity (a VIF value ≥ 10 was deemed to indicate collinearity). Variables with > 20 % missing values were removed. We assessed the presence of any confounding variable in the model using a similar procedure as discussed for the logistic regression model. If confounding was present, the confounding variable was kept in the model to correct the coefficient estimates for confounding. If two variables showed collinearity, the most biologically

meaningful variable was kept in the model. To assess the homoscedasticity of the residuals, a Breusch-Pagan test was performed using the library "skedastic" (Farrar, 2020), and we visually inspected the residuals plotted against predicted values of the fitted model. Finally, the significant ($P \leq 0.05$) variables were presented as beta values and their corresponding 95 % CI.

6.4 Results

6.4.1 Descriptive statistics and farm characteristics

A total of 3,520 quarters of 880 lactating buffalo in 248 farms were enrolled in the study, of which 29 quarters could not be sampled because 17 quarters were blocked due to a previous history of CM, seven quarters were congenitally absent, and five quarters of four animals could not be sampled because the animals did not cooperate during the milking. Therefore, 3,491 quarters were examined using CMT. The number of sampled buffalo ranged between 1 to 41 per farm. On average, 67 % of the lactating and 24 % of the total buffalo population per farm were sampled. The average farm size was 22.8 buffalo (ranging from 1 to 278 buffalo per farm) (Supplementary Table 6.1). The studied farms comprised 81.6 % crossbreeds and 18.4 % indigenous non-descriptive breeds. The crossbreeds mainly crossed indigenous non-descriptive breeds with the Murrah, Nilli-Ravi, Jafarabadi, Mediterranean, and swamp-type water buffalo breeds. The majority of the buffalo farms were comprised of river type (79 %), followed by the swamp type (10 %), and then a mixed population of both types (11 %). Buffalo mostly wallowed in water (83 %) but also in muddy places (2 %), and about 14 % wallowed in both areas. Only 1 % of the farms didn't allow the buffalo to wallow. Among the solely grazing-dependent farms, 77 % allowed grazing for 12-18 hours, while the other 23 % allowed for 2-6 hours daily. In the non-grazing-based farms, buffalo were fed once (36 %), twice (53 %), and in some farms, 3-4 times (11 %) a day. All farms performed hand milking, but no farm applied pre- or post-teat dipping. The milker did pre-milking udder stimulation through calf suckling and hand massage. A single person milked all the lactating buffalo in 55 % of the farms, while multiple people performed the milking on 45 %. About 75 % of the farmers got veterinary support from the government or private means. Farmers also got support from UVH through regular deworming and vaccination programs. More of the farmers who participated in the study had a primary education level (grades 1-5) (47 %) than those who had no formal education (32 %), a higher secondary education (18), or a

college degree (3 %). The farmers' ages ranged between 13 to 85 years, and all were male. Only 8 % of the farmers indicated that they knew how to formulate the ration to meet the nutrient requirements of the buffalo.

6.4.2 Quarter and animal level prevalence and farm level BMSCC

The overall prevalence of SCM, corrected for the sampling design, was 27.9 % (95 % CI 26.3-29.7) at the quarter level, with the 25th and 75th percentiles at 8.3 % and 41.7 %, respectively; at the buffalo level, this was 51.5 % (95 % CI 47.8-55.2), with the 25th and 75th percentiles at 33.3 % and 66.7 % respectively (Fig. 6.2). Among the SCM positive quarters, CMT score 2 was most frequently observed irrespective of the type of buffalo-rearing system (Table 6.2). At the farm level, 242 buffalo farms were sampled as we failed to perform BMSCC on six farms because farmers accidentally mixed the buffalo and cow milk in those farms. The geometrical mean BMSCC was 217,000 cells per mL of milk, which varied between the farms and ranged from 36,000 to 1,213,000 cells per mL of milk with the 25 % and 75 % percentiles at 124,000 and 355,000 cells per mL of milk, respectively.

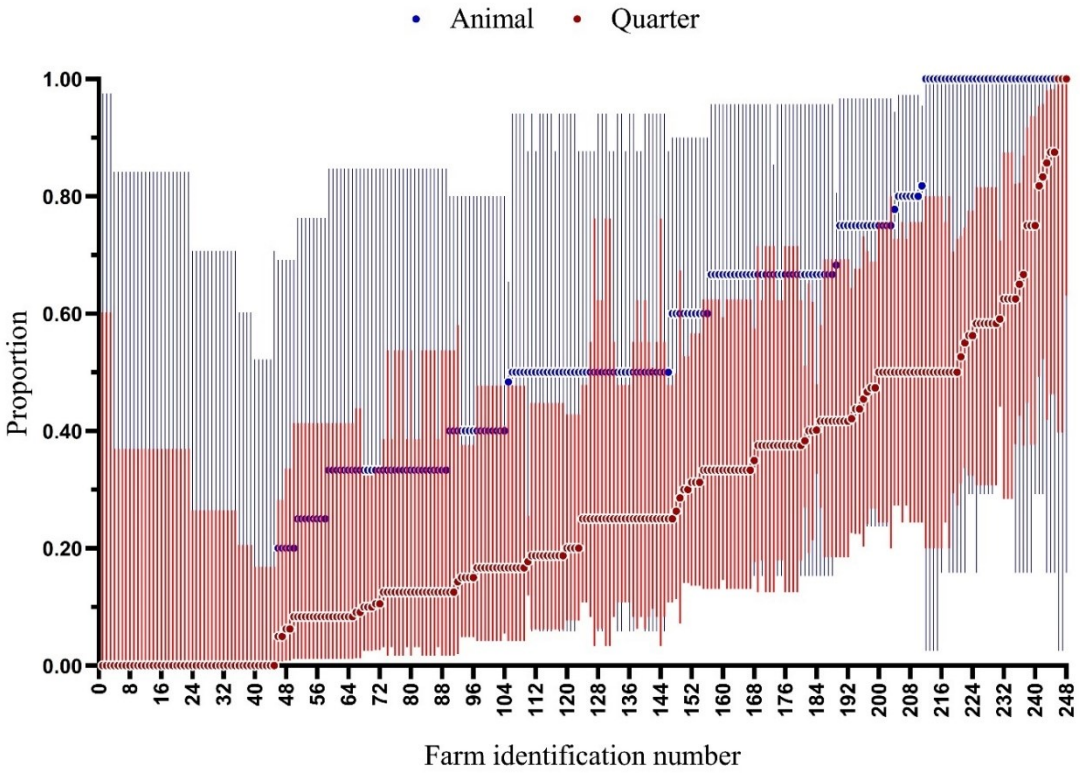


Fig. 6.2 Within farm quarter and animal level prevalence of subclinical mastitis in 248 water buffalo farms in Bangladesh.

Farm identification numbers of each buffalo farm were represented in the X axis (data shorted by the animal level prevalence per farm), and the proportion of quarter and animal levels SCM were presented in the Y axis. The red circles represent the point estimate of the quarter level prevalence of SCM, and the red vertical lines represent the 95 % confidence intervals. The blue circles represent the point estimate of the animal level prevalence of SCM, and the grey vertical lines represent the 95 % confidence intervals in each corresponding buffalo farm.

Table 6.2 Quarter level prevalence of subclinical mastitis based on California mastitis test score (subclinical mastitis at CMT score ≥ 2) on 3,491 buffalo quarters of 880 buffalo from 248 buffalo farms of five different buffalo-rearing systems in Bangladesh

Types of rearing systems	Number of buffalo quarters tested	CMT= 1 n (%)	CMT= 2 n (%)	CMT= 3 n (%)	CMT= 4 n (%)	CMT= 5 n (%)
Free-range	641	484 (75.5)	96 (15.0)	32 (5.0)	21 (3.3)	8 (1.2)
Semi-intensive	814	618 (75.9)	112 (13.8)	57 (7.0)	25 (3.1)	2 (0.2)
Semi-free-range	1,461	1,092 (74.7)	206 (14.1)	101 (6.9)	54 (3.7)	8 (0.5)
Household	308	212 (68.8)	59 (19.2)	19 (6.2)	16 (5.2)	2 (0.6)
Intensive	267	149 (55.8)	47 (17.6)	22 (8.2)	23 (8.6)	26 (9.7)
Overall	**3,491**	**2,555 (73.2)**	**520(14.9)**	**231 (6.6)**	**139 (4.0)**	**46 (1.3)**

6.4.3 Quarter level risk factor model

The univariable analysis of the association between SCM and four quarter level variables is shown in Supplementary Table 6.2. No confounding or collinearity was observed in the multivariable model. In the final model, SCM prevalence was significantly associated with the intensive buffalo-rearing systems, quarters located on the left, and funnel-shaped teat (Table 6.3).

Table 6.3 Multivariable mixed-effects (farm and animal) logistic regression analysis of subclinical mastitis (defined as CMT score ≥ 2) regressed against the quarter level risk factors of 3,455 quarters of 872 buffalo in 248 farms expressed as odds ratio (OR), 95 % confidence interval (95 % CI), and *P* value

Variable name	Categories	Number of observations	OR (95 % CI)	*P*
Quarter position[a]	Right quarters	1,728	Reference	0.006
	Left quarters	1,727	1.3 (1.1 to 1.6)	
Rearing system[a]	Free-range	633	Reference	0.004
	Semi-intensive	810	1.1 (0.5 to 2.1)	
	Semi-free-range	1,441	1.1 (0.6 to 1.9)	
	Household	304	2.0 (0.9 to 4.7)	
	Intensive	267	6.6 (2.2 to 19.5)	
Teat shape[a]	Cylindrical	2,684	Reference	0.003
	Bottle	445	1.3 (0.9 to 1.9)	
	Funnel	326	2.1 (1.4 to 3.3)	

[a] Odds ratio was estimated based on subject-specific beta-estimates

6.4.4 Buffalo-level risk factor model

The univariable analysis of the association between SCM and nine buffalo-level variables is shown in Supplementary Table 6.3. The rearing system confounded the association of SCM with a previous history of CM and contained similar information; therefore, the previous record of CM was removed from the final model. No collinearity was observed in the stepwise forward selection procedure. In the final model (Table 6.4), buffalo with asymmetrical udders, milked by a single milker, and intensive buffalo-rearing systems had higher odds of being SCM positive.

Table 6.4 Multivariable random-effect (farm identification number) logistic regression analysis of the association between subclinical mastitis (defined as at least one of the quarters has a CMT ≥ 2) and various risk factors of 853 buffalo from 246 buffalo farms expressed as odds ratio (OR), 95 % confidence interval (95 % CI), and *P* values

Variable name	Category	Number of observations	OR (95 % CI)	*P*
Udder symmetry[a]	Symmetrical	573	Reference	<0.001
	Asymmetrical	278	1.8 (1.3 to 2.4)	
Number of milkers in the farm[a]	> 1	407	Reference	0.008
	1	444	1.5 (1.2 to 2.1)	
Rearing system[a]	Free-range	155	Reference	0.016
	Semi-intensive	200	1.0 (0.6 to 1.6)	
	Semi-free-range	358	1.1 (0.7 to 1.6)	
	Household	72	1.3 (0.7 to 2.3)	
	Intensive	68	2.7 (1.5 to 5.0)	

[a] Odds ratio based on subject-specific beta-estimates

6.4.5 Bulk milk somatic cell count risk factor model

The univariable association between LBMSCC and 18 farm level variables is shown in Supplementary Table 6.4. Several confounding variables were identified during the model-building process. Adding districts to the model affected the association between farm area type and LBMSCC. The farm area and district contained similar information, but the farm area was biologically more meaningful in association with BMSCC. Therefore, we removed the district from the final model. No collinearity was observed. The type of wallowing area was not included in the final model because of > 20 % of missing values. In the final model (Table 6.5), the winter season was significantly associated with a lower level of LBMSCC than the autumn season. Farms that provide a quarantine facility to the buffalo were also significantly associated with a lower LBMSCC than farms that did not provide any quarantine facility. Farms with a history of buffalo mortality in the previous 12 months were significantly associated with a higher LBMSCC than farms with no history of buffalo mortality.

Table 6.5 Multivariable linear regression analysis of the association between the log10-transformed bulk milk somatic cell count with farm level risk factors in 237 buffalo farms in Bangladesh expressed as co-efficient (β), 95 % confidence interval (95 % CI), and *P* values

Variable	Level	Observations	β	95 % C.I.	*P*
	Intercept		5.59	5.29 to 6.08	<0.001
Season	Winter	75	Reference		<0.001
	Rainy	3	0.06	-0.27 to 0.40	
	Summer	70	0.14	0.04 to 0.23	
	Spring	87	0.26	0.17 to 0.35	
	Autumn	2	0.50	0.10 to 0.91	
History of buffalo mortality	No	119	Reference		0.026
	Yes	118	0.23	0.11 to 0.35	
Having a quarantine facility	Yes	29	Reference		<0.001
	No	208	0.09	0.009 to 0.16	

6.5 Discussion

This cross-sectional study estimated the prevalence and identified the risk factors of buffalo SCM in nine major buffalo-concentrated districts in Bangladesh. Subclinical mastitis is known to be a prevalent disease in water buffalo, with a high buffalo (51-82 %) and quarter level prevalence (28 - 43 %) (Sharif and Ahmad, 2007; Islam et al., 2019; Singha et al., 2021a). Our investigation demonstrates the high prevalence of buffalo SCM at buffalo (52 %) and quarter (28 %) levels. A higher prevalence of SCM at the buffalo level is more probable than at the quarter level because a single SCM-positive quarter results in a buffalo positive for SCM. In our study, the average herd size was medium (22.8 buffalo per herd), and sampled farms did not use teat dipping or antiseptics and generally had poor milking and udder hygiene practices. Therefore, indirect contact between buffalo on the farm may increase the likelihood of transmission of the disease, leading to a higher prevalence of SCM. Alternatively, a large stock density in buffalo farms in Bangladesh (Habib et al., 2017) and the high temperature and humid climate in subtropical countries may cause stress to the buffalo and further increase their susceptibility to SCM (El Nahas et al., 2017; Ahmad et al., 2019). Although a prior small-scale investigation in Bangladesh (Singha et al., 2021a) showed a greater prevalence than our study, the SCM prevalence estimate in our study was corrected for the multistage sampling approach for district-level stratification, thus is more reliable for extrapolation at the population level.

The mean BMSCC in this study was low overall (217,000 cells per mL of milk) and is in line with another study conducted in Bangladesh (Singha et al., 2021a). It also falls within the range of BMSCC values considered acceptable in neighboring countries, such as India (200,000 cells per mL of milk) and Nepal (500,000 cells per mL of milk) (Dhakal, 2006; Alhussien and Dang, 2018). However, the mean BMSCC in this study exceeds the recently proposed BMSCC level of 100,000 cells per mL in Murrah buffalo by the National Dairy Research Institute, India. Bulk milk SCC may fluctuate due to seasonal influences; for example, BMSCC is often expected to be low in winter since the temperature humidity index remains below the critical threshold that poses reduced thermal stress to the buffalo during this season (Choudhary and Sirohi, 2019). The BMSCC in the winter season in our study (172,390 cells per mL) was considerably low and might also be influenced by an overall decrease in mean BMSCC (Supplementary Table 6.1). Our study observed a discrepancy where, although BMSCC was low, a high animal level SCM prevalence (52 %) was observed. A previous study suggested that, in specific NAS-associated IMI, BMSCC may remain low. For example, *Staphylococcus (S.). xylosus*, *S. cohnii*, and *S. equorum* were more frequently isolated from quarters of dairy cows with a low SCC. At the same time, a high SCC was revealed from IMI associated with *S. capitis*, *S. gallinarum*, *S. hyicus*, *S. agnetis,* or *S. simulans* (Condas et al., 2017). This speculation may partly explain the link between BMSCC and SCM prevalence in our study. Our study included five buffalo-rearing systems to represent the nationwide buffalo population in Bangladesh. Therefore, this study's findings can help establish an initial benchmark since BMSCC data in water buffalo are currently limited. If buffalo farmers could access a regular BMSCC testing program, there is scope to improve the farm's udder health by recreating the management practices in farms with low BMSCC.

The left quarter position was associated with a higher prevalence of buffalo SCM in this study. Several other studies have also shown this trend (Moroni et al., 2006; Srinivasan et al., 2013; Ali et al., 2014). The association between quarter position and SCM can be partly explained by the fact that on the studied farms, milking was performed by hand from the left side, increasing the exposure and exerting more pressure on the left quarters (Ali et al., 2014). When milking the right teat, the left teat orifice will be open, allowing IMI pathogens to enter the mammary glands and cause mastitis. Therefore, the left-side milking practice may also allow IMI pathogen contamination from the right teat to the

left teat. However, no previous study has identified whether the left teats are more often infected by different pathogens, such as gram negatives, because of environmental contamination or the contagious transmission between udder glands. The type of hand milking could also play a significant role in teat injury and increase the prevalence of SCM (Hameed et al., 2012), but it was not significant in our study.

A strong association was observed between an intensive buffalo-rearing system and the prevalence of SCM. A previous study (Khan et al., 2019) in Pakistan supported the idea that prevalence was much higher on intensive farms (77 %) than on household farms (23 %). In intensive farms in our study, buffalo lived in stressful conditions due to a lack of access to grazing and wallowing, which can compromise the immunity of the animals. Intensive buffalo farms in our study comprised crossbreed buffalo (e.g., Indigenous × Nili-Ravi and Indigenous × Murrah) that had a higher milk yield compared to the indigenous buffalo, and these crossbreeds are generally known to be susceptible to mastitis (Ali et al., 2014; Charaya et al., 2014). In intensive farms in our study, the herd size was large, ranging from 50-278 animals per herd, and animal density was high in intensive farms, which may also increase the chances of spreading IMI pathogens from infected to healthy buffalo on the farm (Ali et al., 2014).

Funnel-shaped teats were associated with a higher prevalence of SCM than cylindrical teats. A study (Kaur et al., 2018) has suggested that in Murrah buffalo, the cylindrical teat is the most common and has a higher prevalence of SCM, which is contradictory to the results of this study. To our knowledge, no previous studies have discussed the association between teat shape and the prevalence of SCM in water buffalo. In funnel-shaped teats, the skin is loose, generally wrinkled, and rough, making them difficult to clean and may increase exposure to IMI-causing pathogens. Due to the roughness of the teat skin, the funnel-shaped teats also need increased pressure during hand milking, which could increase the chance of teat injury. Moreover, in our study for pre-milking stimulation, calf suckling was the most common, and it was common practice to drag the calf away from the teat. At the same time, any suckling might cause teat injury and make the quarter more susceptible to SCM (Bhandari et al., 2021).

In this study, buffalo with an asymmetrical udder had a higher SCM prevalence. A previous study found that in CM-affected buffalo, udder fibrosis followed by the teat canal and cistern disappearance was observed (Abu-seida et al., 2014). We observed that a significantly higher proportion of buffalo with an asymmetrical udder had a previous

CM history than those with a symmetrical udder. Therefore, it can be speculated that in our study, udder asymmetry may result from a prior infection causing scar tissue, leading to shrinkage of the udder gland. This is attributable to the presence of persistent IMI-causing pathogens from previous clinical cases and the transfer of those pathogens resulting in the development of new cases of IMI.

A single milker was associated with a higher prevalence of SCM in the buffalo than multiple milkers. This is surprising, and it is challenging to hypothesize a causal mechanism behind this. One explanation could be that the milking hygiene was inadequate when a single worker was involved, concerning multiple workers. For example, no teat dipping or hygienic practices were observed between milking one buffalo and the next. Therefore, when a single milker was responsible for milking all the buffalo on the farm, this was more likely to contaminate a contagious pathogen to all the lactating animals in a herd than with multiple milkers. A previous study in Poland demonstrated the transmission of methicillin-resistant *Staphylococcus aureus* among agricultural animals, farm workers, and their household members. Multiple-locus variable number tandem repeat analysis, multilocus sequence typing (ST398), and spa (t034) typing have revealed the same drug-susceptibility profiles in bovine and human isolates (Krukowski et al., 2020). On the buffalo farms in this study, the single milker stayed close to the buffalo while they grazed and did other day-to-day farm management tasks nearby. Usually, during the milking time in the morning, a single person performs the farm environment cleaning (e.g., cleaning the manure) and starts the milking immediately without hand washing or disinfectant. Therefore, IMI pathogens are more likely to spread from the milker to the other buffalo in the herd.

On the other hand, in herds where multiple milkers were involved, the farm environment cleaning was performed by other workers who were sometimes not involved in the milking process. Therefore, it is possible that the milkers had less exposure to the lactating buffalo, reducing the risk of SCM in those animals. However, a higher risk of SCM may also be linked to misclassification bias, resulting from the failure to report the true prevalence, or sampling bias, resulting from underestimating the actual prevalence of SCM. Finally, although it is unclear, it could be speculated that, compared to the multiple milkers, the greater exposure of the single milkers may be the most crucial reason for the higher prevalence of SCM in water buffalo.

Our study evidenced that the winter season was associated with low BMSCC. These findings agreed with earlier studies in India (Bhutia et al., 2019) and Pakistan (Baloch et al., 2018), which showed that there was a lower prevalence of SCM in winter (20-28 %). The association of low BMSCC in buffalo could be speculated to be due to the lower heat stress in winter. Water buffalo generally tolerate cold weather better than hot or humid climatic conditions (Marai and Haeeb, 2010). However, the association may also have to do with the buffalo-rearing systems, and we must be cautious about interpreting the high BMSCC in Autumn. Only intensive farms were studied during the autumn (September to early December), where BMSCC was higher than any other buffalo-rearing system in this study. This shows how the high BMSCC may have been strongly associated with the Autumn season because of the effect of the intensive rearing system over any seasonal influences.

The absence of a quarantine facility was associated with a high BMSCC in this study. The buffalo farmers in this study often expanded the herd by introducing newly purchased buffalo. If quarantine is not followed in these situations, there is a higher likelihood of introducing disease from the new animal to other animals in the herd. Buffalo farmers who are concerned with the provision of quarantine facilities are also expected to be more concerned with the overall farm management than farmers who do not have a quarantine facility.

Buffalo farms with a previous history of buffalo mortality were associated with a high BMSCC. In our study, farmers reported that buffalo mostly died during outbreaks of hemorrhagic septicemia and parasitic diseases, which agrees with a previous study in Bangladesh (Islam et al., 2017). However, neither vaccination nor deworming substantially impacted buffalo mortality. On the other hand, the buffalo-rearing system was strongly linked to farms where animals had previously died. Interestingly, the highest number of animal deaths happened in free-range systems, where 80 % of farmers had no formal education or only a primary educational qualification. They were unaware of buffalo nutrition, disease prevention, or biosecurity science. Free-range farms are also remotely located, making it hard for the farmers to talk to each other and be updated about vital management improvements.

6.6 Conclusion

In conclusion, water buffalo had a high animal and quarter level SCM prevalence. Mean bulk milk somatic cell count was satisfactory but varied significantly between farms. The non-manageable risk factors in our study were the buffalo-rearing system, season, and quarter position. The controllable risk factors were the number of milkers, teat shape, and udder symmetry. Teat shape and udder symmetry are manageable through selective breeding. Our findings indicate that the education of farmers regarding milking technique, hygiene, and rearing system may help further improve udder health on buffalo farms in Bangladesh.

CHAPTER-7

Pathogen group-specific risk factors for intramammary infection in water buffalo

CHAPTER 7

Pathogen group-specific risk factors for intramammary infection in water buffalo

7.1 Abstract

Subclinical mastitis (SCM) is an economically significant disease in water buffalo worldwide. However, information on the intramammary infection (IMI) determinants in water buffalo in low and middle-income (LMIC) countries is limited. A cross-sectional study was conducted to estimate the prevalence of IMI-associated bacteria and identify risk factors for pathogen group-specific IMI in water buffalo in Bangladesh. California mastitis test (CMT) and bacteriological culture were performed on 1,374 quarter milk samples collected from 763 water buffalo in 244 buffalo farms in nine districts in Bangladesh. Quarter, buffalo, and farm-related data were obtained using a questionnaire and visual observations. Mixed effects logistic regression models were used to identify IMI-associated bacterial species risk factors. A total of 618 quarter samples were culture positive. Non-*aureus* staphylococci were the predominant IMI-associated bacterial species, and *Staphylococcus* (*S.*) *chromogenes*, *S. hyicus*, and *S. epidermidis* were the most common ones. The proportion of non-aureus staphylococci or *Mammaliicoccus sciuri* (NASM), *S. aureus*, and other bacterial species identified in the buffalo quarter samples varied between buffalo farms. Risk factors associated with hygiene were milking hygiene and cleanliness of the hind quarters. The odds of IMI by any pathogen [OR: 1.8 (1.3-2.5)] and by NASM [OR: 2.2 (1.5-3.2)] were higher in buffalo herds with poor milking hygiene (opposed to excellent hygiene). Poor cleanliness of the hind quarters (opposed to excellent cleanliness) had a higher odds of IMI caused by any pathogen [OR: 2.0 (1.4-2.7)], NASM [OR: 1.9 (1.3-2.8)], and *S. aureus* [OR: 3.0 (2.9-3.1)]. Farm management-related risk factors were the frequency of milking and buffalo source. Twice daily milking [OR: 3.1 (2.0-4.6)] (opposed to once) and farms with buffalo purchased from another herd [OR: 2.0 (1.2-3.2)] (opposed to own stock) were associated with IMI by any pathogen. Udder symmetry and body condition score (BCS) were risk factors related to buffalo breeding and nutrition. Asymmetrical udder (opposed to symmetrical) was associated with IMI-caused by any bacteria [OR: 1.7 (1.0-2.8)] and with IMI caused by *S. aureus* [OR: 1.3 (1.2-1.4)]. Poor BCS (opposed to good) showed higher odds of IMI by any pathogen [OR: 1.4 (1.0-1.9)] and by NASM [OR: 1.7 (1.1-2.5)]. This study shows that the prevalence of IMI in water buffalo is high. Still, variables

between farms and our data suggest that SCM can be partly controlled by better farm management, primarily by improving hygiene, milking management, breeding, and nutrition.

7.2 Introduction

Subclinical mastitis (SCM) is the inflammation of the mammary gland mainly in response to intra-mammary infection (IMI) in water buffalo (Preethirani et al., 2015; Algammal et al., 2020). Subclinical mastitis has significant effects on both food security and safety by reducing milk yield impairing the quality of milk and milk products (Piccinini et al., 2006; Costa et al., 2020), increasing the culling rate, and increasing the risk of development and spread of antimicrobial resistance (Elahi et al., 2018; El-Ashker et al., 2020). Knowing the risk factors for IMI is essential to determine the most appropriate control measures for IMI in water buffalo.

Previous studies attempted to identify several buffalo and farm level risk factors of buffalo SCM based on high CMT. Animal-related risk factors include age, breed, parity, and lactation stage (Sharif and Ahmad, 2007; Srinivasan et al., 2013; Hussain et al., 2018). Water buffalo may have distinct morphological differences in udder and teat shapes. The udder may be a bowl, globular, or pendulous shaped, whereas the teat may be cylindrical, conical, or funnel-shaped (Kaur et al., 2018). These variations in udder morphology may also influence the entry and establishment of IMI in the mammary gland. Examples of farm-related risk factors of SCM known for water buffalo are flooring materials, rearing system, and type of milking system (Sharif and Ahmad, 2007; Sindhu et al., 2009). Poor hygiene and management may contribute to the exposure of the mammary gland to pathogens, resulting in a high prevalence of IMI. In household buffalo farms, hand milking is a usual milking practice in most South Asian countries. Therefore, trauma caused by incorrect milking procedures may also predispose IMI in these animals (Hameed et al., 2012). Although several risk factors for positive CMT have been identified, few studies have investigated risk factors for specific pathogen groups using bacteriological culture.

Identifying risk factors for IMI is important to identify effective management practices to control buffalo SCM in Bangladesh, which are likely also applicable in buffalo-concentrated neighboring Asian countries with similar management, e.g., India, Pakistan, Iran, Nepal, and Indonesia (Bachaya et al., 2005; Dhakal et al., 2007; Beheshti et al.,

2011; Anirban et al., 2013). Hence the objectives of this study were to estimate the prevalence of IMI-associated bacterial species and to identify risk factors for pathogen group-specific IMI in water buffalo in Bangladesh.

7.3 Materials and methods

7.3.1 Study area description

This cross-sectional study was conducted in nine representative buffalo-concentrated districts in Bangladesh between February 2020 and April 2021. The geographical location of the selected districts are as follows: Noakhali and Chattogram in the south-east, Lakshmipur and Bhola in the south-central, Maulvibazar and Mymensingh in the north-east, Jamalpur in the north-central, Rajshahi district in the north-west, and Dhaka is located in the center of Bangladesh. Buffalo-rearing systems in these regions can be subdivided into five different buffalo-rearing systems: free-ranging, semi-free-ranging, intensive, semi-intensive, and household. Free-range buffalo graze in the fallow land in coastal or semi-coastal islands supplemented with straw or small amounts of concentrates. In a free-ranging system, buffalo are transferred across islands dependent on green roughage availability. Due to feed scarcity on the islands during the dry season (October to March), buffalo are relocated to the inlands for 3-6 months, depending on roughage availability, in the semi-free-range system. The household rearing system offers 5-7 hours of grazing supplemented with a small amount of straw, grass, and concentrates. In river-basin locations, the semi-intensive system lets buffalo stay in sheds, graze nearby pastures, and return daily. Buffalo in intensive systems are tethered in sheds, stall-fed, and never permitted to graze. The selection of buffalo farms in this study has been described in Chapter 6.

7.3.2 On-farm data collection

A questionnaire was developed, including questions about buffalo and farm level information. The questionnaire used was previously described in Chapter 6. The farmers gave written informed consent during interviewing. The data were collected through a face-to-face interview with the farmer and on-farm observations. During the interview, the buffalo farmer gave information on the buffalo source, breed type, and frequency of milking per day. In contrast,animal-related information such as body condition score (BCS), udder symmetry, udder shape, type of hand milking procedure, cleanliness of the

hind quarter, and milking hygiene was assessed by on-farm observations by the interviewer. The study was approved and performed in line with the guidelines of the SAU research system (SAU/Ethical Committee/AUP/21/06) of Sylhet Agricultural University, Bangladesh.

7.3.3 Detection of subclinical mastitis using California mastitis test and collection of quarter milk samples

California mastitis test was performed on the individual buffalo quarters separately without any visible signs of clinical mastitis (CM), and the test results were scored 1 to 5 and classified as either healthy (CMT score 1) or SCM quarter (CMT scores 2 to 5) (Adkins et al., 2017). Milk samples were collected from all the SCM quarters while from healthy quarters only one quarter sample was collected per buffalo. Milk samples from all the healthy and SCM quarters were obtained following aseptic milk sampling procedures (Adkins et al., 2017c) in 15 mL sterile falcon tubes. Milk samples were temporarily stored in a freezer (-10 ºC to -15 ºC) near the field visit area. Frozen samples were transferred within seven days to the Udder Health Bangladesh (UHB) bacteriological laboratory of Chattogram Veterinary and Animal Sciences University (CVASU) and stored at -20 ºC.

7.3.4 Bacteriological culture

Milk samples were subjected to bacteriological culture in the UHB laboratory following the NMC guidelines (Adkins et al., 2017b), with some modifications noted below (Singha et al., 2021a). Quarter milk samples were inoculated (10µL) on 5 % bovine blood agar (BBA). Positive growth was defined when at least three morphologically similar colonies on BBA and a sample was considered contaminated when a sample yielded three or more morphologically different colony types. Gram-positive and gram-negative bacteria were differentiated based on growth characteristics, followed by inoculation of selective agar media: Mannitol Salt agar (MSA), MacConkey agar (MAC), and Eosin Methylene Blue (EMB) agar. Bacterial isolates were preserved at -80 ºC using Brain Heart Infusion Broth and 50 % buffered glycerol for future use. All bacterial culture media and reagents were manufactured by Oxoid (Oxoid Ltd, Basingstoke, United Kingdom).

7.3.5 *Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry*

Bacterial isolates were shipped (Italian Ministry of Health Authorization number: 477015235) to the Mastitis Laboratory, Department of Veterinary and Animal Sciences, Università degli Studi di Milano for further confirmation, using Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS), for identification by MALDI-TOF MS, frozen bacterial isolates were inoculated (10µL) on BBA and incubated for 24 hours. The pure cultures were further sub-cultured to ensure the complete bacterial function of the isolates. One or two colonies were deposited on the target plate using a toothpick, overlaid with 1 µL of α-cyano-4-hydroxycinnamic acid (Bruker Daltonics, GmbH, Bremen, Germany), and were dried. The spectra were acquired with a microFlex™ mass spectrometer (Bruker Daltonics, GmbH). The results were interpreted against the MBT Compass® 4.1 database (Addis et al., 2022). Log scores of ≥ 2.0 were the thresholds used for species confirmation of *S. aureus,* while log scores of ≥ 1.7 were used to confirm the species identification of other species (Cameron et al., 2017).

7.3.6 Bulk milk somatic cell count

Bulk milk SCC was measured in thoroughly mixed morning bulk milk from all the lactating buffalo at each farm using a DeLaval somatic cell counter (DCC) (DeLaval Group, Stockholm, Sweden) (Adkins et al., 2017a). The procedure was followed according to the manufacturer's instructions and described in a previous study of this project (Singha et al., 2021a).

7.3.7 Definition of subclinical mastitis at the quarter, buffalo, and farm level

A buffalo was considered SCM-positive when one or more of its four functional quarters had CMT ≥ 2. A buffalo farm was considered SCM positive when at least one buffalo had a quarter with CMT ≥ 2.

7.3.8 Statistical analysis

Data analysis was done using R statistical software (version 4.1.2; R Foundation for Statistical Computing, Vienna, Austria). Prevalence of IMI in healthy (CMT = 1) or SCM quarter (CMT ≥ 2) samples was calculated by dividing the number of samples positive

for each bacterial species (NAS; *S. aureus*; *Streptococcus*; Gram-negative; or other bacteria) by the total number of tested quarter milk samples. The exact binomial method was used to calculate the proportion's 95 % confidence intervals. The prevalence of IMI-associated bacterial species was calculated at the quarter, animal, and farm levels. Mixed-effects logistic regression models with animal and farm ID as random effects were constructed to test the association between IMI-associated bacterial species and buffalo or farm level variables. The significance of the random effect terms was checked with the likelihood ratio test (LRT) using the latent variable approach (Dohoo et al., 2010a). Variables with a *P* value < 0.20 were included in the multivariable model. The multivariable model was built using a stepwise forward selection procedure, adding each significant variable in the model, starting with the variable having the lowest *P* value. If the beta coefficient in the new model changed by more than 30 % after adding a variable, this was deemed confounding, and the confounder was included in the model. If the standard error in the new model largely changed, it indicated the collinearity with the newly added variable. The most biologically meaningful variable for buffalo IMI was kept in the final model. Finally, significant variables ($P \leq 0.05$) were presented in terms of subject-specific odds ratios (OR) and 95 % C.I., meaning that fixed effects in a mixed model represent the effects of that factor within the cluster (Dohoo et al., 2010a).

7.4 Results

7.4.1 Descriptive statistics

Table 7.1 shows that 1,374 quarters of 763 lactating buffalo from 244 buffalo farms from nine districts were included in the analysis. The prevalence of CMT ≥ 2 quarters was 51 % (95 % CI, 48-54). The quarter level prevalence of CMT ≥ 2 for each district is displayed in Table 7.1.

Table 7.1 Distribution of quarter milk samples collected and analyzed for bacteriological culture in nine water buffalo concentrated districts in Bangladesh.

Districts	N farms	N buffalo	N quarters	CMT ≥ 2	
				N quarters	% (95 % CI)
Noakhali	45	168	292	147	50.3 (44.4 to 56.2)
Jamalpur	35	134	224	107	47.8 (41.1 to 54.5)
Rajshahi	39	104	191	102	53.4 (46.1 to 60.6)
Bhola	37	86	186	97	52.2 (44.7 to 59.5)
Chattogram	38	93	160	68	42.5 (34.7 to 50.6)

Maulvibazar	37	94	146	64	43.8 (35.6 to 52.3)
Laxmipur	2	47	103	69	67.0 (57.0 to 75.9)
Mymensingh	10	23	45	29	64.4 (48.8 to 78.1)
Dhaka	1	14	27	17	63.0 (42.4 to 80.6)
Total	**244**	**763**	**1,374**	**700**	50.9 (**48.3 to 53.6)**

7.4.2 Prevalence of IMI-associated pathogens

In total, 618 quarters were culture positive, 756 were culture negative, whereas 11 samples were found contaminated in bacteriological culture. A total of 46 % (312/674) (95 % CI, 43 -50) CMT = 1 buffalo quarter and 44 % (306/700) (95 % CI, 40 - 48) CMT ≥ 2 buffalo quarters were culture-positive, respectively. Surprisingly, differences in bacteriological culture results between SCM and healthy buffalo quarters based on CMT results were minimal. Bacterial isolates belonging to the *Staphylococcus* genus were common, where *S. chromogenes* was the most prevalent species, followed by *S. hyicus*, *S. epidermidis,* and *S. aureus*. The pathogen distribution is given in Table 7.2.

Table 7.2 Distribution of bacterial species from intramammary infections (N = 618) isolated from 1,374 quarter milk samples of 763 lactating water buffalo from 244 buffalo farms in nine water buffalo concentrated districts in Bangladesh

Percentages were calculated within CMT classes.

Bacterial species	CMT = 1 N quarters (%)	CMT ≥ 2 N quarters (%)	Total N quarters[a] (%)
Staphylococcus			
S. chromogenes	60 (8.9)	66 (9.4)	126 (9.17)
S. hyicus	53 (7.9)	60 (8.6)	113 (8.22)
S. aureus	19 (2.8)	20 (2.9)	39 (2.83)
S. xylosus	18 (2.7)	12 (1.7)	30 (2.18)
S. epidermidis	21 (3.1)	9 (1.3)	30 (2.18)
S. haemolyticus	15 (2.2)	14 (2.0)	29 (2.11)
S. arlettae	6 (0.9)	1 (0.1)	7 (0.50)
S. pasteuri	2 (0.3)	4 (0.6)	6 (0.43)
S. hominis	1 (0.1)	2 (0.3)	3 (0.21)
S. equorum	1 (0.1)	1 (0.1)	2 (0.14)
S. warneri	1 (0.1)	1 (0.1)	2 (0.14)
S. ureilyticus	2 (0.3)	-	2 (0.14)
S. saprophyticus	1 (0.1)	-	1 (0.07)
S. casei	1 (0.1)	-	1 (0.07)
S. capitis	-	1 (0.1)	1 (0.07)
S. kloosii	-	1 (0.1)	1 (0.07)
Mammaliicoccus			

Mammaliicoccus sciuri	39 (5.8)	46 (6.6)	85 (6.18)
Streptococcus			
Strep. uberis	2 (0.3)	1 (0.1)	3 (0.21)
Strep. suis	1 (0.1)	1 (0.1)	2 (0.14)
Strep. canis	1 (0.1)	1 (0.1)	2 (0.14)
Strep. dysgalactiae	1 (0.1)	1 (0.1)	2 (0.14)
Strep. pluranimalium	-	1 (0.1)	1 (0.07)
Gram-negative			
Pseudomonas spp	1 (0.1)	4 (0.6)	5 (0.36)
Acinetobacter spp	1 (0.1)	1 (0.1)	2 (0.14)
Enterobacter spp	1 (0.1)	1 (0.1)	2 (0.14)
Escherichia coli	1 (0.1)	1 (0.1)	2 (0.14)
Stenotrophomonas spp	-	2 (0.3)	2 (0.14)
Klebsiella spp	-	1 (0.1)	1 (0.07)
Ochrobactrum spp	-	1 (0.1)	1 (0.07)
Other bacteria			
Micrococcus spp	13 (1.9)	11 (1.6)	24 (1.74)
Corynebacterium spp	8 (1.2)	7 (1.0)	15 (1.09)
Rothia spp	5 (0.7)	5 (0.7)	10 (0.72)
Lactococcus garvieae	2 (0.3)	3 (0.4)	5 (0.36)
Bacillus spp	4 (0.6)	1 (0.1)	5 (0.36)
Kocuria spp	2 (0.3)	2 (0.3)	4 (0.29)
Actinomyces spp	2 (0.3)	1 (0.1)	3 (0.21)
Arcanobacterium spp	-	2 (0.3)	2 (0.14)
Microbacterium spp	1 (0.1)	-	1 (0.07)
Rhodococcus spp	1 (0.1)	-	1 (0.07)
Brachybacterium spp	-	1 (0.1)	1 (0.07)
Not identifiable[b]	25 (3.7)	19 (2.7)	44 (3.20)
Culture negative	362 (53.7)	394 (56.3)	756 (55.0)
Total quarters examined	**674 (100)**	**700 (100)**	**1374 (100)**

[a] Contaminated samples (n = 11) were excluded from the statistical analysis.

[b] Not identifiable: Bacterial species that could not be confirmed by MALDI-TOF MS log scores of ≥1.7

7.4.3 Prevalence of IMI-associated pathogens

Table 7.3 shows the quarter, buffalo, and farm level prevalence of non-aureus staphylococci, *Mammaliicoccus* spp (NASM), other bacterial species, and *S. aureus*. Non-aureus staphylococci or *Mammaliicoccus* spp were found as the most prevalent bacterial species at the quarter (32 %), animal (45 %), and farm level (72 %).

Table 7.3 Herd, buffalo, and quarter level prevalence of intramammary infection in 1,374 buffalo quarters from 763 buffalo from 244 water buffalo farms in nine buffalo-concentrated districts of Bangladesh

Culture positive regardless of CMT status	N quarters (%)[a]	N buffalo (%)[b]	N farms (%)[c]
Non-aureus staphylococci or *Mammaliicoccus* spp.	439 (32.0)	341 (44.5)	176 (72.1)
Other bacterial species	140 (10.2)	129 (16.9)	91 (37.3)
Staphylococcus aureus	39 (2.8)	37 (4.9)	32 (13.1)
Total	618 (45.0)	462 (60.6)	209 (85.7)

[a] Contaminated samples were excluded from the statistical analysis.
[b] A buffalo was considered positive when at least one of the quarters had an IMI.
[c] A buffalo farm was considered positive when at least one buffalo had an IMI.
[d] Other bacterial species included non-identifiable bacteria species, Gram-negative bacteria species, *Micrococcus* spp, *Corynebacterium* spp, *Rothia* spp, *Lactococcus garvieae*, *Bacillus* spp, *Kocuria* spp, *Actinomyces* spp, *Arcanobacterium* spp, *Microbacterium* spp, *Rhodococcus* spp, *Brachybacterium* spp, and *Streptococcus* spp.

On average, six quarter samples were examined by bacteriological culture per farm (median: 5; range: 1-75). Figure 7.1 shows that the distribution of IMI-associated pathogens per farm and the proportion of culture negatives varied widely across the buffalo farms.

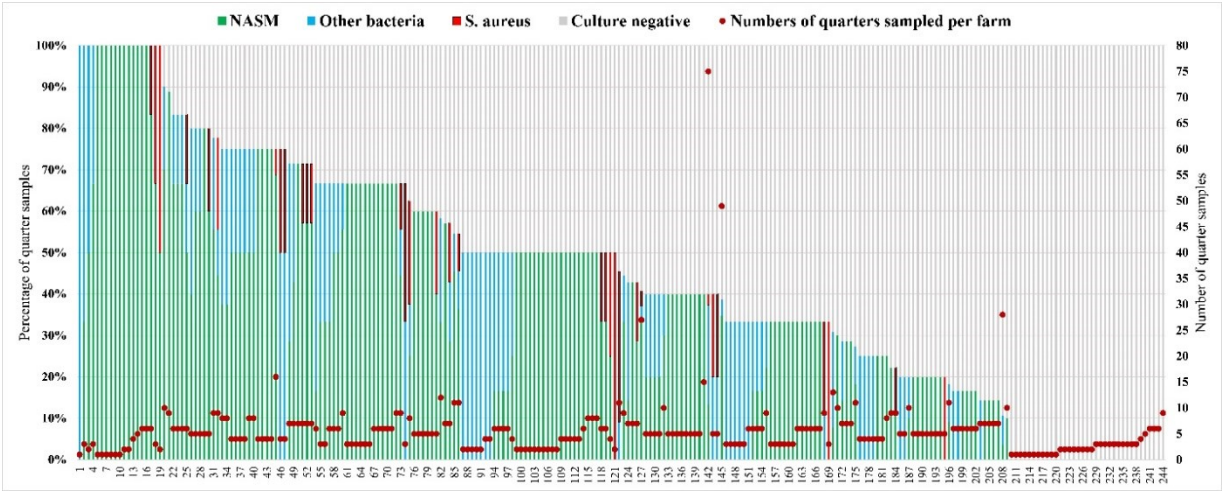


Fig. 7.1 Distribution of culture results of 1,374 buffalo quarter milk samples of 763 buffalo from 244 water buffalo farms in nine buffalo concentrated districts of Bangladesh

Sorted by the percentage of culture-negative samples and the total number of samples per farm. NASM stands for non-aureus staphylococci or *Mammaliicoccus* spp.

7.4.4 Risk factors for intramammary infection

Table 7.4 shows the factors associated with IMI caused by any bacterial species, NASM, and *S. aureus*. Animal-related risk factors of water buffalo IMI were the cleanliness of the hind quarter, BCS, and udder symmetry. Buffalo farm-related risk factors were milking hygiene, frequency of milking per day, and buffalo source. Poor milking hygiene was associated with the risk of IMI by any bacterial species and NASM. Poor cleanliness score of hind quarters was found to be a risk factor for IMI by any bacterial species, NASM, and *S. aureus*. Asymmetrical udders were associated with IMI by any bacterial species and *S. aureus*. Own stock buffalo, on average, had lower BCS than the purchased buffalo, and thereby BCS confounded the association of buffalo source with IMI by any pathogens. The district was associated with the buffalo-rearing system. We kept the rearing system in the model because this is biologically easier to interpret than the effect of districts in Bangladesh. However, the rearing system was statistically non-significant and was excluded from the final model as the model-building process. In the model of IMI caused by NASM, BMSCC and the type of hand milking system were collinear with the milking hygiene, and milking hygiene was chosen to stay in the final model.

Table 7.4 Mixed effects multivariable logistic regression analysis using three separate models for intramammary infection (IMI) by any pathogen, non-aureus staphylococci or *Mammaliicoccus sciuri*, and *Staphylococcus* (*S.*) *aureus* regressed against buffalo and farm level risk factors, using animal and farm as the random effects, based on bacteriological culture data of 1,374 quarters of 763 buffalo in 244 farms in nine buffalo-concentrated districts of Bangladesh.

Subject-specific odds ratios and 95 % C.I. were calculated based on subject-specific beta estimates.

Independent variable	Any bacterial species[a]		NASM[b]		*S. aureus*[c]	
	Odds ratio	95 % C.I.	Odds ratio	95 % C.I.	Odds ratio	95 % C.I.
Milking hygiene						
Excellent	Reference					
Poor	1.8	1.3 to 2.5	2.2	1.5 to 3.2	-	-
Cleanliness of the hind quarter						
Excellent	Reference					
Good	1.5	1.1 to 2.0	1.4	1.0 to 1.9	2.6	2.5 to 2.7
Poor	2.0	1.4 to 2.7	1.9	1.3 to 2.8	3.0	2.9 to 3.1
Frequency of milking per day						
Once	Reference					
Twice	3.1	2.0 to 4.6	-	-	-	-
Buffalo source						
Purchase from others herd	Reference					
Source unknown	1.3	0.4 to 3.9	-	-	-	-
Both own stock or purchased	1.5	0.9 to 2.3	-	-	-	-
Own stock	2.0	1.2 to 3.2	-	-	-	-
Udder symmetry						
Symmetrical	Reference					
Asymmetrical	1.70	1.0 to 2.8	-	-	1.3	1.2 to 1.4
BCS (Score 1-9)						
Good (5)	Reference					
Fatty (6-9)	1.1	0.8 to 1.5	1.0	0.6 to 1.7	-	-
Moderate (4)	1.1	0.8 to 1.5	1.4	1.0 to 2.0	-	-
Poor (1-3)	1.4	1.0 to 1.9	1.7	1.1 to 2.5	-	-

[a] IMI by any bacterial species: The subject-specific odds ratios for the buffalo level variables were calculated from 1,233 quarters of 681 lactating buffalo from 217 farms.

[b] IMI by non-aureus staphylococcus or *Mammaliicoccus sciuri*: The subject-specific odds ratios for the buffalo level variables were calculated from 1,287 quarters of 727 lactating buffalo from 229 farms.

[c] IMI by *Staphylococcus aureus*: The subject-specific odds ratios for the buffalo level variables were calculated from 1,342 quarters of 745 lactating buffalo from 242 buffalo farms.

7.5 Discussion

This study aimed to determine the prevalence of IMI and identify risk factors for IMI-associated bacterial species in water buffalo in Bangladesh. This study demonstrated the predominance of NASM (32 %) in IMI in water buffalo quarters in Bangladesh which is in agreement with our previous work, where we found a prevalence of 25 % (95 % CI 18 - 33 %) in water buffalo in Bangladesh (Singha et al., 2021a). Several previous studies in India also demonstrated NAS as the most prevalent among the culture results (18 - 35 %) in SCM quarters in water buffalo (Joshi et al., 2013; Srinivasan et al., 2013; Charaya et al., 2014). *Staphylococcus chromogenes* were the most common among NAS species in this study, consistent with another German study identifying that *S. simulans* and *S. chromogenes* were responsible for 90 % of the NAS-associated IMI in dairy cows. *Mammaliicoccus sciuri* was another prevalent species identified in this study. This species was formerly known as *S. sciuri* but has recently been reclassified as "*Mammaliicoccus sciuri*" (Madhaiyan et al., 2020; Rosa et al., 2022) and was also reported previously in water buffalo (El-Ashker et al., 2020).

Non-aureus staphylococci may colonize the teat canal of the buffalo mammary gland (Condas et al., 2017; Traversari et al., 2019) but are also known to be associated with SCM or IMI, and occasionally in CM in various dairy species such as cows, buffalo, and goats (Hamel et al., 2020; Santos et al., 2020; Wuytack et al., 2020; Valckenier et al., 2021). Our study surprisingly found a similar prevalence of positive culture results in SCM as in healthy quarters based on CMT, although this was consistent with our previous study (Singha et al., 2021a). If a culture of NASM results from true IMI rather than teat canal colonization in water buffalo, the prevalence of NAS would be expected to be higher in SCM quarters than in healthy quarters, as was previously reported in water buffalo as well as dairy cows (Pisanu et al., 2019; Valckenier et al., 2020; Beuckelaere et al., 2021). This suggests that part of the NASM we cultured may have originated from teat canal colonization rather than true IMI. The absence of a difference in the proportion of culture positives between healthy and CMT-positive quarters may also be explained by the interdependence of microbiota among the quarters of an individual buffalo (Catozzi et al., 2019). It has also been shown in dairy cows that an inflammatory response

to an IMI in one quarter may also evoke an inflammatory response in the other quarters of that animal (Blagitz et al., 2015; Paixão et al., 2017). To what extent the unaffected quarters react may depend on the pathogenicity of the pathogen and the severity of the inflammation (Pyörälä, 2003; Merle et al., 2007; Petzl et al., 2008). This interdependence may further explain the finding that culture results were similar between healthy and CMT-positive quarters.

The prevalence of IMI-causing bacterial species in the buffalo quarters largely varied between farms (Figure 7.1). In a previous study on dairy cows, within farms, the cow-level prevalence of IMI by NAS ranged from 0 to 50 % (Schukken et al., 2009), and the prevalence of *S. aureus* varied from 0 to 73 % (Exel et al., 2023). Still, no such data is available on water buffalo. These differences between farms may result from different management or characteristics of the dominant infecting strains in a herd (Keane, 2019; Leuenberger et al., 2019; Exel et al., 2023). Genotyping of IMI pathogens may help elucidate various strains' transmission potential and routes. A previous study using a bioeconomic simulation model demonstrated that the optimal control measures for an extra-mammary *S. aureus* spillover to milk substantially differed from contagious *S. aureus* strains (Exel et al., 2022). Therefore, genotyping the most critical species may be a valuable next step.

Buffalo with poor cleanliness of the hind quarter had higher odds of IMI by any bacterial species, NAS, and *S. aureus* than buffalo with clean hind quarters. Sub-optimal hygienic conditions of the udder and hind quarter may expose the teat canal to pathogens and result in an IMI (Ndahetuye et al., 2020b). Contamination of hind quarters predisposes mainly to environmental IMI pathogens (Rowe et al., 2019; Fréchette et al., 2021), but interestingly, culture positivity for *S. aureus* was also associated with dirty hind quarters. Since dirt is unlikely the primary source of *S. aureus* IMI, we speculate that dirty hindquarters may be linked to other factors predisposing to *S. aureus* IMI, such as the rearing system or general hygiene. In this study, poor cleanliness of the hind quarter was higher in semi-free-ranging buffalo and in buffalo with poor milking hygiene. This suggests that washing buffalo will not effectively reduce *S. aureus* prevalence. The underlying try risk factors should be identified in future studies.

Poor milking hygiene, such as when farmers did not wash their hands or use antiseptics or teat dipping before or after milking, was associated with higher odds of IMI by any bacterial species than excellent milking hygiene. This finding is consistent with a study

in Brazil which evidenced that poor milking hygiene was associated with IMI and SCC > 400,000 cells per mL of milk in water buffalo quarters (Medeiros et al., 2011). In a review article, poor floor hygiene, wrong hand milking procedures, and dirty milkers' hands were reported to be associated with SCM or IMI in dairy cows in South Asian countries (Bari et al., 2022). Intramammary infection-causing pathogens may be transmitted directly via milkers' hands during hand milking. A study reported that about 16 % of samples taken from milking hands were positive for *S. aureus* (Banu and Zewdu Geberemedhin, 2022), which suggests that dirty hands during hand milking may contribute to the transmission of IMI within animals in a buffalo herd. In our study, farms with poor milking hygiene frequently milked using the stripping method. This hand-milking method involves bending the thumb and pressing the teat against the index finger with the nail and end of the thumb, which may cause injury to the teat skin and the teat canal. Teat skin and sphincter serve as the first line of defense to protect against mastitis pathogens. When the teat is injured, it may lose its protective function and can easily be infected (Rainard and Riollet, 2006; Tolosa et al., 2015). Thus, milking technique and hygiene should be addressed in udder health control efforts.

In this study, buffalo with an asymmetrical udder had higher odds of IMI by any pathogen or *S. aureus* than those with a symmetrical udder. *Staphylococcus aureus* is known to cause chronic subclinical inflammation in the water buffalo mammary gland (Restucci et al., 2019). In our study, a significantly higher proportion of buffalo with an asymmetrical udder had a history of CM than those with a symmetrical udder, suggesting that also CM may result in udder gland atrophy.

Surprisingly, in this study, own-stock buffalo farms had higher odds of IMI by any pathogen than farms with purchased buffalo. Buffalo farms in Bangladesh have limited farm biosecurity as personnel may be shared between different herds to clean the buffalo barn and occasionally for milking. This may be a more critical factor in transmission between farms than purchasing animals. The source of buffalo may be linked with the other factors to predispose an IMI. In this study, farms with their source buffalo frequently had river-type buffalo than swamp or mixed (river and swamp type) type buffalo. River-type buffalo in this study also had higher odds of IMI by NAS than the swamp-type buffalo. River-type buffalo generally produce more milk, and a previous study reported that teat canal diameter and stretchability are correlated with the milk production level (Rathore and Sheldrake, 1977). The teat canal remains open longer in

high-yielding animals (Klaas et al., 2005), which may increase the risk of entering IMI pathogens.

7.6 Conclusions

In conclusion, several udder health problems related to IMI in water buffalo have been identified in this study. NASM was the most common species group responsible for IMI in water buffalo in Bangladesh. We show that the prevalence of IMI-associated bacteria varies between farms. Strain typing of the IMI-associated bacterial species may be helpful in elucidating transmission routes of the major pathogens. Our data suggest that IMI can be partly managed by better farm management, including milking technique, hygiene, and breeding, which can be used to design water buffalo udder health control strategies.

CHAPTER-8

Water buffalo milk chain and associated factors affecting bulk milk somatic cell count, and bacterial counts in Bangladesh

CHAPTER 8

Water buffalo milk chain and associated factors affecting bulk milk somatic cell count, and bacterial counts in Bangladesh

8.1 Abstract

The aims of this study were to a) assess farm udder health by measuring bulk milk somatic cell count (BMSCC); b) compare the hygiene status along the milk chain nodes by measuring total bacteria count (TBC), total non-*aureus staphylococcus* count (TNAS), total *Staphylococcus aureus* count (TSA), and total *Enterobacteriaceae* count (TEC); c) describe milk chain characteristics; and d) identify factors associated with BMSCC at the farm level and bacteria in milk along the buffalo milk chain in Bangladesh. Samples were collected at multiple nodes along the buffalo milk value chain: 122 bulk tank milk samples were collected at the farm level, 109 milk samples at the middlemen level, and 111 milk samples at the milk collection centers. In addition, 35 samples were taken from various milk products at the retail level. The geometric mean BMSCC at the farm level was 254×10^3 cells per mL of milk. The mean value of TBC was 5.2 log10 at the farm level, 6.0 log10 at the middleman level, 6.6 log10 in the milk collection centers, and 7.5 log10 in the product shops. Progressively increasing TBC, TNAS, and TEC levels were observed along the milk chain ($P < 0.05$). A high BMSCC was associated with season (spring versus winter) and buffalo-rearing system (semi-intensive versus intensive). The high TBC was associated with the season (winter versus late autumn), the water sources used when cleaning the milk containers (pond water versus tube-well water), the type of milk (a mix of buffalo and cow milk versus pure buffalo milk), and the cleanliness score of the milk containers (poor versus excellent). The type of farm area (coastal versus river basin) was associated with high levels of TNAS. Our study indicated a moderate BMSCC and identified several factors associated with increased bacterial contamination at the farm and middleman levels. Based on our findings, improving udder health and hygienic practices during milk handling could aid in reducing BMSCC and bacterial contamination and ensure the safety and quality of buffalo milk and milk products consumed in Bangladesh.

8.2 Introduction

Milk provides an excellent nutrient source for many people worldwide, particularly in low- and middle-income countries (LMIC) (Adesogan and Dahl, 2020). However, consuming contaminated milk or milk products may cause serious public health hazards (Cerva et al., 2014; Verraes et al., 2015). As a perishable product, milk must be processed immediately after collection on the farm, especially if there are poor milk cooling facilities. Dairy is primarily consumed in the form of fresh dairy products in LMICs. Demand for these products is projected to increase by 1 % in the next decade, demanding an increase of 1.7 % in global milk production (OECD/FAO, 2019). Cow milk dominates global production (81 %), whereas water buffalo are the principal non-cow dairy production species, contributing 15 % of milk output (Minervino et al., 2020). About 97 % of the total buffalo population resides in Asia; water buffalo are the primary source of milk production in South Asia (Hegde, 2019).

Small-holder farmers in Bangladesh dominate the buffalo farming sector by utilizing fallow land and feed resources, providing income-generation opportunities. Water buffalo are increasingly reared in a free-range system (locally known as "Bathan"), followed by household subsistence systems in the coastal areas, sugarcane belt, and marshland of Bangladesh (Hamid et al., 2016b; Sultana, 2018). However, in Bangladesh, water buffalo only contributed 4 % of the total milk production recorded in 2020-21 (13 million metric tons) (DLS, 2021). Buffalo milk production in Bangladesh is possibly underexploited compared to neighboring high-buffalo milk-producing countries such as India, Pakistan, and Nepal (Samad, 2020). However, similar buffalo-rearing systems and management practices, such as hand milking, inadequate milking hygiene, transportation with insufficient cooling facilities, and traditional milk product processing techniques are also observed in these countries (Tsuji, 2019; Sah et al., 2020; Samad, 2020).

The buffalo milk chain in Bangladesh primarily consists of milk producers at the production level, followed by vendors at the milk collection centers and commercial milk processors or manufacturers (Hamid et al., 2016a). Middlemen and milk product producers primarily collect milk. They collect buffalo milk from farms in remote coastal areas and supply it to large dairy manufacturing companies or sell it to the local market. Traditionally, the buffalo milk trade involves processing the milk into products like yogurt and cheese, using raw or boiled milk without pasteurization. A study in

Bangladesh showed that 78 % of buffalo milk in the Bhola district was used for yogurt production, using the traditional natural fermentation of raw milk (Habib et al., 2021). Milk and milk products can be contaminated directly through lactating buffalo mammary gland intramammary infection (IMI) (Moroni et al., 2006). Bacterial contamination can also occur during milking and milk handling (due to storage, equipment, and hygiene issues), during transportation to milk collection centers, and during the processing and sale of milk products (Sanaa et al., 1993; Tigabu et al., 2015; Islam et al., 2018).

Previous studies have identified the presence of different pathogenic bacteria in buffalo milk and milk products, for example, non-*aureus staphylococcus* (NAS), enterotoxigenic *Staphylococcus*, *Staphylococcus* (*S.*) *aureus*, *Escherichia* (*E.*) *coli*, and other coliform bacteria (Mistry et al., 2015; Bauzad et al., 2019; Giovanni et al., 2020; Al-Rudha et al., 2021). Coliform bacteria, particularly *E. coli,* can contaminate raw milk during storage or transport (Ombui et al., 1994). However, microbial contaminations can vary, depending on the handling and processing steps taken along the buffalo milk chain (Mpatswenumugabo et al., 2019).

Bulk milk somatic cell count (BMSCC) is a crucial indicator of udder health and the prevalence of mastitis, reflecting the milk quality at the herd level. It is monitored at the herd level to ensure the supply of high-quality milk to the consumers. A high BMSCC reflects an elevation of leucocytes in milk in response to IMI. Increased leucocyte activity, caused by high somatic cells and bacteria, can also decrease the quality of milk and milk products by activating proteolytic and lipolytic enzymes such as lipases, oxidases, glycosidases, and proteases (Li et al., 2014; Bauer et al., 2015). In addition to BMSCC, other bacterial indicators are of importance when ranking the hygienic quality of milk, including total bacteria count (TBC), total non-*aureus staphylococcus* count (TNAS), total *S. aureus* count (TSA), and total *Enterobacteriaceae* count (TEC) (Anderson et al., 2011; Berhe et al., 2020). In many countries (for example, Canada, Brazil, and Rwanda), the acceptable limit for TBC in pasteurized milk ranges from 5×10^3 to 5×10^5 CFU per mL of milk (Bauman et al., 2018; Cortinhas et al., 2018; Ndahetuye et al., 2020a). The acceptable limit for TBC in Bangladesh is $< 2 \times 10^4$ CFU per mL of milk (Bangladesh Standards and testing institute (BSTI), 1009:1982).

Several studies have assessed hygienic quality along the dairy cow milk chain in Bangladesh (Khaton et al., 2014; Islam et al., 2016b; Islam et al., 2018) and other LMICs, including India, Sri Lanka, and Ethiopia (De Silva et al., 2016; Rai et al., 2020; Aliyo

and Teklemariam, 2022). However, to the authors' knowledge, limited information is available on the hygienic quality of milk and milk products along the buffalo milk chain. To close this scientific gap, the aims of our study were to a) assess farm udder health by measuring BMSCC; b) compare the hygiene status along the milk chain nodes (in the bulk milk, middleman handling, milk collection centers, and milk products) by measuring TBC, TNAS, TSA, and TEC; c) describe milk chain characteristics; and d) identify factors associated with BMSCC at the farm level and with bacteria in milk along the buffalo milk chain in Bangladesh.

8.3 Material and Methods

8.3.1 Description of the study site and population

Water buffalo is one of the predominant dairy species in Bangladesh. The current buffalo population is 1.5 million (DLS, 2021), of which 40 % is distributed in coastal areas (Faruque et al., 1990). The remaining buffalo population is spread across the Meghna-Ganga and the Jamuna-Brahmaputra flood plains (Sultana, 2018). Together, coastal, semi-coastal, and river basin areas constitute the most significant areas of water buffalo concentration in Bangladesh. The Rajshahi, Jamalpur, Mymensingh, Moulvibazar, Bhola, and Noakhali districts are the country's most important contributors to buffalo milk (Faruque, 2000; Uddin et al., 2016) and, therefore, were selected for this study to represent the buffalo population in Bangladesh effectively. The sub-tropical monsoon climate of Bangladesh is mainly classified into six meteorological seasons: summer (late April to the end of June), rainy (early July to early September), autumn (late September to early October), late autumn (late October to early December), winter (late December to early February), and spring (late February to early April) (Khatun et al., 2016).

Water buffalo in Bangladesh are reared under five major production systems, depending on topography, vegetation pattern, and the seasonal availability of feed resources. Free-range buffalo-rearing systems, such as the bathan (a free-range system with approximately 500 buffalo per farm) and semi-bathan systems, depends on the pasture's seasonal availability. In the bathan systems, buffalo depend on grazing on fallow pasture on islands from spring to autumn. In the semi-bathan systems, buffalo from the islands are shifted to the mainland from late autumn to winter for 3-6 months because of a lack of available pasture on the islands. Up to 20 buffalo are tethered and stall-fed in the household system under available housing facilities. In semi-intensive systems,

approximately 150 buffalo are kept on pasture in river basin areas during the daytime and at housing facilities at night. On intensive farms, up to 170 buffalo are reared for breeding in zero-grazing systems using housing facilities (Uddin et al., 2016; Rahman et al., 2019b; Samad, 2020).

8.3.2 Study design and sampling area

A cross-sectional study was conducted in four buffalo milk chain nodes (farm, middleman, milk collection center, and milk product shop) from seven buffalo-concentrated districts in Bangladesh. The study took place from February 2020 to April 2021 (Fig. 8.1 a and b). A list of the registered buffalo farmers was created with the help of the corresponding *upazila* (the name used for the administrative unit of a sub-district in Bangladesh), veterinary hospital (UVH), and a non-governmental organization named the "Palli Karma-Sahayak Foundation" (PKSF), which worked on buffalo. The chosen buffalo farmers were contacted with a request for information on the farm's location and the middlemen and milk collection centers used. A total of 122 buffalo farms with 1 to 5 lactating buffalo per farm met the selection criteria and were recruited for this project. No organized information, such as lists and contacts, was available for middlemen, milk collection centers, or milk product shops. Therefore, a list was created by applying snowball sampling (Etikan et al., 2016) and collecting data from the selected buffalo farmers and local buffalo milk product shops at each study location. A list of the distribution of the analyzed samples is given in Table 8.1. One of the 122 bulk milk samples was collected from each selected farm. A total of 109 milk samples were analyzed from milk collected at the middleman level and 111 milk samples from the milk collection centers. Another 35 milk products, comprised of yogurt (n = 26), cheese (n = 7), and buttermilk (n = 2), were analyzed for the assessment of bacterial contamination. However, samples from the middlemen node, milk collection centers, and milk product shops were not linked with the source buffalo farm. Data was collected using a pre-structured questionnaire (questionnaires can be found in subsections 12.3, 12.4, 12.5, and 12.6 under Chapter 12 Appendices).

Table 8.1 Distribution of milk and milk product samples (N = 377) collected at four different nodes of the buffalo milk chain in 7 districts of Bangladesh

District	Farm	Middleman	Milk collection center	Milk products shop	Total number of samples
Noakhali	47	44	47	13	151
Bhola	18	18	18	10	64
Maulvibazar	16	16	15	5	52
Rajshahi	15	15	15	5	50
Jamalpur	17	11	11	2	41
Mymensingh	8	4	4	-	16
Dhaka	1	1	1	-	3
Total	**122**	**109**	**111**	**35**	**377**

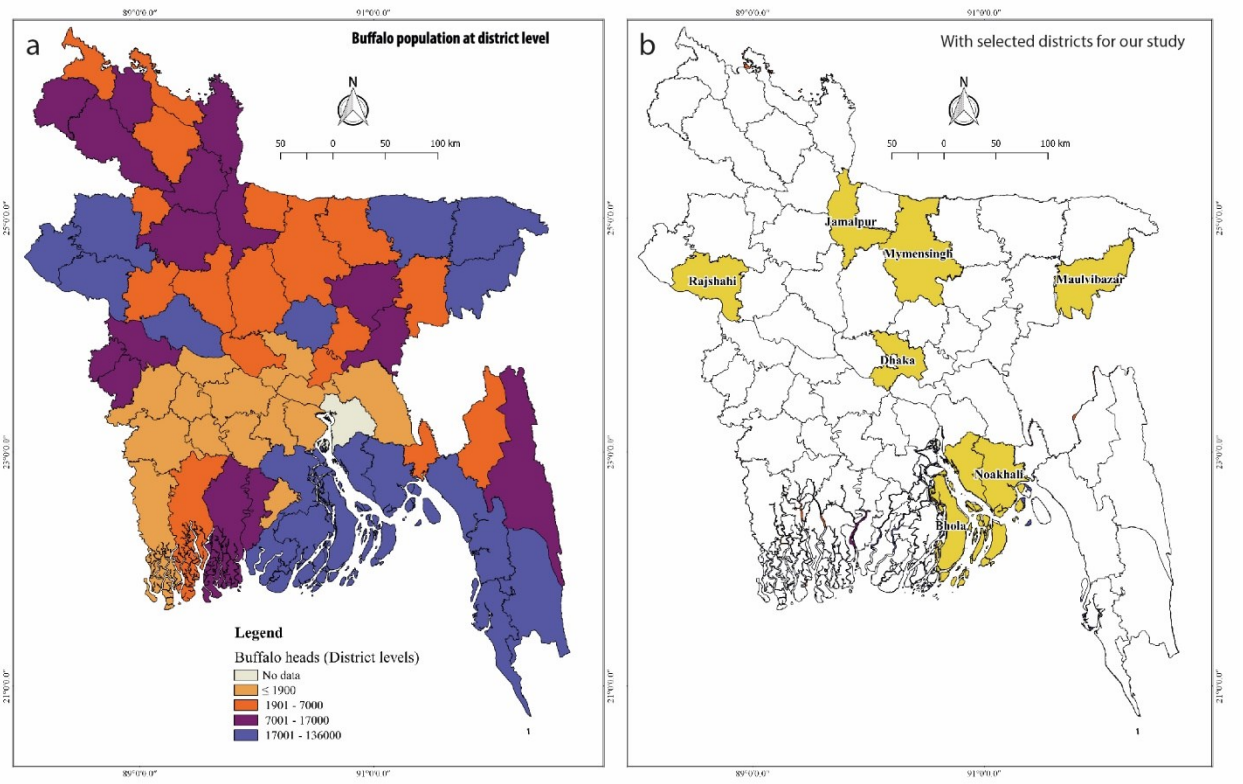


Fig. 8.1 Seven districts that were selected for sampling in the study

(a) District-level buffalo heads in Bangladesh are illustrated in the map of Bangladesh (Data source: LDDP (2019)). (b) The yellow indicates the location of the study's seven selected districts (Rajshahi, Jamalpur, Mymensingh, Maulvibazar, Bhola, Noakhali, and Dhaka).

8.3.3 Collection of samples and handling procedures

After milking all the buffalo at each farm, the bulk milk was thoroughly mixed for 5 minutes, and samples were collected from the top of the bulk tank using a clean, sanitized dipper. 25-30 mL sample of the farm's bulk milk was collected aseptically in a sterile 50 mL screw-capped falcon tube. Two representative aliquots from the same farm's bulk milk were collected. One aliquot was used to perform the BMSCC analysis, and the other was preserved aseptically for bacteriological culture. 10mL bulk milk samples were collected aseptically from the middlemen nodes 1 hour after the collection of bulk milk samples from the farms, and 10mL of mixed bulk milk samples were collected at the milk collection centers one hour after the middlemen delivered the milk. Approximately 30-35 g of milk products (yogurt, cheese, and buttermilk) were collected aseptically from each milk product shop in a 50 mL sterile screw-cap bottle. All collected samples were transferred to an ice box immediately. Upon collecting all samples each day, the samples were frozen and stored at -20 ºC, and bacteriological quantification was performed 24 hours after the samples were stored.

8.3.4 Bulk milk somatic cell counts of farm bulk milk

BMSCC was measured in the thoroughly mixed morning bulk milk, which contained milk from all the lactating buffalo at each farm, using a DeLaval somatic cell counter (DCC) (DeLaval Group, Stockholm, Sweden) (Adkins et al., 2017b). The DCC showed the BMSCC results as cells/ µL of milk multiplied by 1000 to estimate the cells per mL of milk. The measurement was taken immediately at the farm following the collection of the farm bulk milk samples. A BMSCC level of 400×10^3 cells per mL of milk, suggested by Costa et al. (2020), was used as the cut-off to compare the levels in the present study.

8.3.5 Quantification of bacteria

Bulk milk samples from farms, middlemen, milk collection centers, and milk products underwent bacteriological quantification following the National Mastitis Council Protocol 2017 (Adkins et al., 2017b). The total bacteria count, *staphylococcal* count, and *Enterobacteriaceae* count were performed on a Plate Count Agar (PCA), Baird Parker Agar with egg yolk tellurite (BPA), and Violet Red Bile Glucose Agar (VRBG), respectively. All agar media used was manufactured by Oxoid, Basingstoke, UK. To perform bacteria enumerations, 1 mL of the milk samples was mixed with 9 mL of diluent

(sterile 0.9 % NaCl). Samples were serially diluted 10-fold up to 10^{-7}. To estimate the total number of aerobic bacteria in the samples, the pour plate technique was carried out following ISO:4833-1 (2013). From each 10-fold dilution prepared, 1 mL of the sample was mixed with 15-20 mL of molten PCA in a sterile petri dish and incubated aerobically at 30 °C for 72 hours. The *Staphylococci* count was determined using the surface plate technique, following Viçosa et al. (2010). 0.1 mL of the serially diluted sample was taken from each tube after vortexing and plated on the surface onto a solidified sterile BPA plate. The plate was incubated at 37 °C for 48 hours, and then *S. aureus* and NAS were enumerated. To confirm *S. aureus* colonies, five colonies were randomly chosen and tested using a coagulase test. Determination of *Enterobacteriaceae* was performed using the pour plate technique following 5th ed. NMKL-144 (Nordic Committee on food analysis) standards. A 1 mL vortexed sample was mixed with 10-15 mL of VRBG. Then, a second overlay of 5-10 mL of VRBG was added after solidification. Culture plates were incubated at 37 °C for 48 hours. A further oxidase test was conducted on five randomly selected colonies to differentiate *Enterobacteriaceae* from non-*Enterobacteriaceae*. Bacteria enumerations were done considering a countable dilution containing < 300 colonies.

8.3.6 Collection of epidemiological data

A questionnaire was divided into four subsections to describe the buffalo milk chain and investigate factors associated with farm BMSCC and bacteria from the buffalo milk chain nodes. Section A captured data at the farm level and included 45 questions. The data included information on farmers' education level, buffalo-rearing system, geographical area of the farm, the total number of lactating buffalo, number of dry buffalo, average milk yield per day, milker hygiene (excellent: milkers use antiseptic and wash hands; good: milkers only wash hand; poor: milkers don't wash hand), and udder hygiene (excellent: udder is clean and dried; good: udder is clean but not dry; poor: udder is not clean and is wet). Section A also collected data on milk containers, such as the type of milk container, how the containers were cleaned, and the cleanliness score of the milking containers. The cleanliness score was defined using three categories, based on visual observation by the interviewer (excellent: no greasiness or dirt was observed inside or outside the container; good: no greasiness or dirt was observed inside the container; and poor: greasiness or dirt was present both inside and outside the containers). The farmers

in this section also provided generic data on milk storage, transportation, and their ability to obtain a reasonable price when selling the milk.

Sections B and C contained 20 questions and collected information from the middlemen and milk collection centers. Data on milk transportation (such as any cooling materials inside the containers), milk composition (buffalo milk or a mixture of buffalo milk and cow milk), how the containers were cleaned, and the cleanliness score of the milk container was collected.

Section D included eight questions to gather information on milk products: the type of milk used, storage time, and the type of containers used. Qualitative assessments, such as the cleanliness score of the milk containers, were determined during sample collection following subjective visual observation by the interviewer. Information on the type of milk was obtained by cross-questioning the middlemen or personnel at the milk collection centers.

Participation from the farmers, middlemen, and milk product shop owners was voluntary. Each farmer gave written informed consent to participate in the study, and the middleman and milk product shop owners also gave written or oral informed consent. The study was approved and performed in line with the guidelines of the Sylhet Agricultural University Research System (AUP/21/06) in Bangladesh. The questionnaire was pretested and revised based on the comments from the pretest. Each questionnaire was given a unique identification number matching the identification number assigned to the collected samples at each level of the milk chain.

8.3.7 Statistical analyses

Data from the questionnaire-based survey and the bacteriological enumerations were entered into an MS Excel spreadsheet. The data was cleaned, and coding and integrity were checked before importing the dataset into JMP 16.0 for statistical analysis (SAS Institute Inc., North Carolina, USA). BMSCC and bacteria count (TBC, TNAS, TSA, and TEC) data were log10 transformed to achieve normal data distributions. Descriptive statistics were performed using a boxplot for BMSCC at the farm level, and the bacteria count (TBC, TNAS, TSA, and TEC) at each node of the buffalo milk chain (farm, middleman, milk collection center, and milk product). A summary (mean and range) was presented for the quantitative variables, such as the number of lactating animals, the average milk yield at the farm level, and frequency numbers, with percentages calculated

for the generic data, such as problems faced during transportation and storage, and farmers getting the right milk price.

Univariable analysis, a t-test, or a one-way ANOVA was performed to identify the variables ($P \leq 0.20$) to be included in the multivariable regression models to investigate potential associations with BMSCC or bacteria contamination. Three multivariable regression models (Model-1 for BMSCC, Model-2 for TBC, and Model-3 for TSA) were constructed at the farm level and one at the middleman level to identify variables associated with the TBC. During the construction of the multivariable regression models, a maximum likelihood estimation procedure was applied using a manual stepwise forward selection procedure. The presence of any confounding was assessed by removing one variable from the model and evaluating whether the coefficients were changed by 30 % and whether the confounding was biologically meaningful. The interaction was evaluated by constructing two-interaction product terms for any significant main effect, adding them to the model, and examining changes in the *P* values of the main effects. The final model included variables with a $P \leq 0.05$. A variation inflation factor (VIF) and Cook Weisberg test were performed for model fit to identify multi-collinearity and heteroskedasticity, respectively.

8.4 Results

8.4.1 Bulk milk somatic cell counts at the farm level

The geometric mean of BMSCC at the farm level was 254×10^3 cells per mL; the highest value was $1{,}213 \times 10^3$ cells per mL, and the lowest was 36×10^3 cells per mL. Among the farms, 30 % exceeded the BMSCC threshold of 400×10^3 cells per mL.

8.4.2 Bacteria contamination at various nodes of the milk chain

A summary of different bacterial counts (TBC, TSA, TNAS, and TEC) is presented in Table 8.2. The number of samples demonstrating as positive for the included bacteria showed that irrespective of the type of samples, all the tested samples had a countable number of TBC, with 89 - 100 % of the samples being positive for TNAS, 48 - 80 % for TEC, and 13 - 18 % of the samples positive for TSA. A tendency for an increase in TBC was observed along the milk chain (Fig. 8.2 a, b, c, and d).

Table 8.2 Summary statistics of BMSCC and three different bacterial counts presented in mean, range (minimum-maximum), and median for 377 samples collected at four different levels on the buffalo milk chain in 7 districts of Bangladesh

BMSCC/ Bacteria[1]	Sample source (number of samples analyzed)	Number of positive samples (%)	Log10mean[2] (Min-Max)	Median
BMSCC[3]				
	Farm (122)	-	5.4 (4.6 - 6.1)	5.4
TBC				
	Farm (122)	-	5.2 (2.0 - 7.3)	5.2
	Middleman (109)	-	6.0 (3.4 - 8.3)	6.0
	Milk collection center (108)	-	6.6 (3.6 - 9.9)	6.7
	Milk products (35)	-	7.5 (3.6 - 9.9)	7.5
TSA				
	Farm (122)	16 (13.1)	3.3 (3.0 - 3.9)	3.1
	Middleman (109)	15 (13.8)	3.6 (3.0 - 5.3)	3.4
	Milk collection center (111)	20 (18.0)	3.7 (3.0 - 5.7)	3.6
	Milk products (35)	5 (14.3)	3.8 (3.0 - 6.0)	3.8
TNAS				
	Farm (122)	111 (91.0)	4.4 (3.0 - 6.7)	4.2
	Middleman (109)	105 (96.3)	4.9 (3.0 - 7.9)	4.9
	Milk collection center (111)	111 (100.0)	5.4 (3.3 - 8.4)	5.4
	Milk products (35)	31 (88.6)	5.8 (3.6 - 7.7)	5.7
TEC				
	Farm (122)	58 (47.5)	2.9 (2.0 - 5.7)	2.8
	Middleman (109)	66 (60.6)	4.1 (2.0 - 7.4)	3.7
	Milk collection center (111)	85 (76.6)	4.2 (2.0 - 7.0)	4.1
	Milk products (35)	28 (80.0)	4.6 (2.3 - 8.4)	4.8

[1]BMSCC = Bulk milk somatic cell count/ mL of milk; TBC = Total bacterial count/ mL of milk; TSA = Total *Staphylococcus aureus* count/ mL of milk; TNAS = Total *non-aureus Staphylococci*/ mL of milk; TEC = Total *Enterobacteriaceae* count/ mL of milk**.**

[2]To calculate the mean, median, and range, log10 transformed values of the original values were used. Only samples positive for selected bacteria counts were considered when calculating the mean for bacterial counts.

[3]BMSCC records were only available at the farm level on 122 buffalo farms.

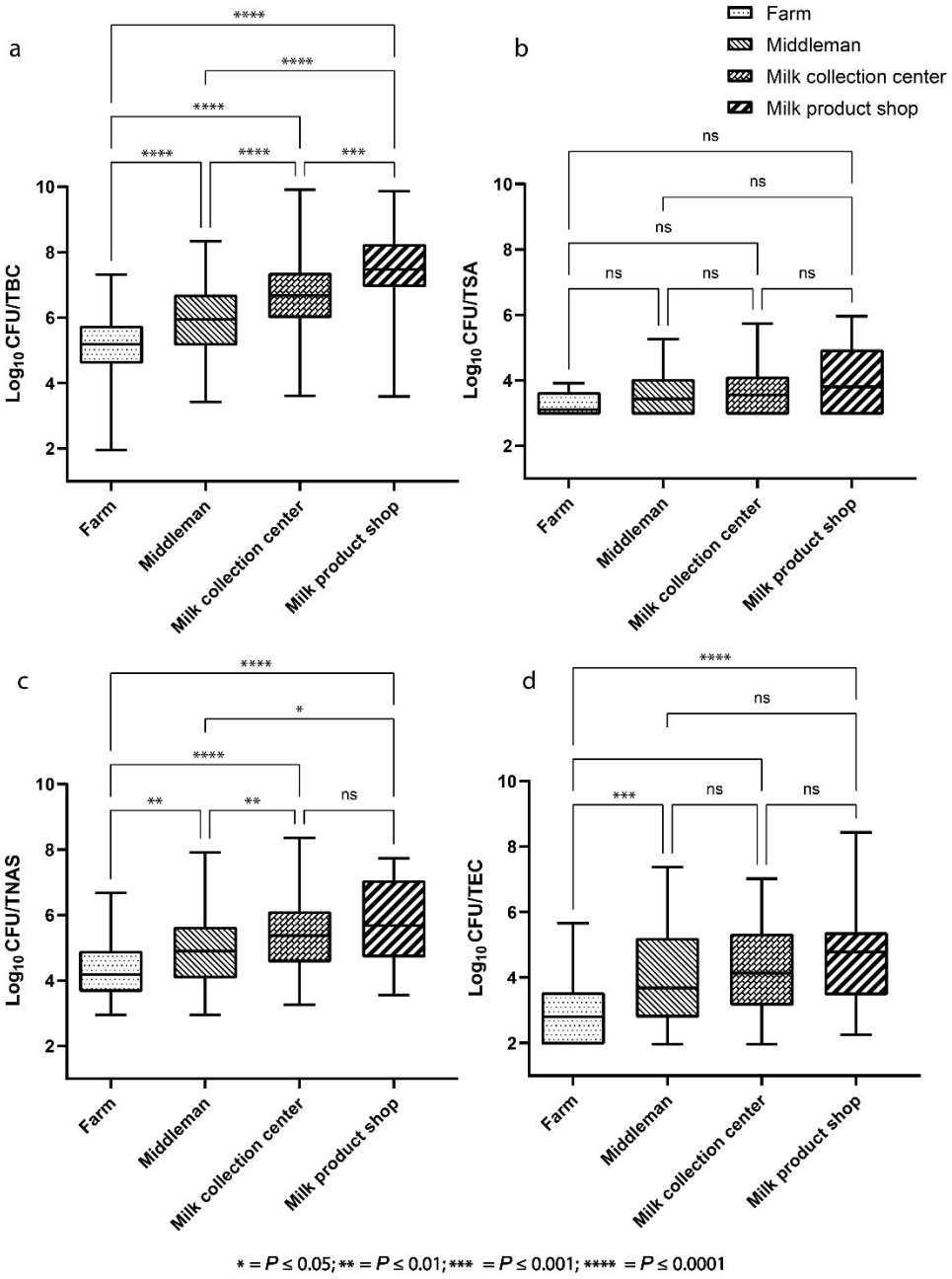


Fig. 8.2 Assessment of bacterial contamination at four different nodes (bulk milk, middleman, milk collection centers, and milk products) of the buffalo milk chain in Bangladesh (using box plots)

(a) represents the total bacteria count; (b) represents the total *Staphylococcus aureus* count; (c) represents the total non-*aureus staphylococcus* count; (d) represents the total *Enterobacteriaceae* count. The horizontal lines show the comparison between the sample type, and the symbol for the significance level is indicated above the line. The level of significance corresponding to the symbol is displayed at the bottom of the Figure.

8.4.3 Buffalo milk chain characteristics

Buffalo farmers in this study were aged between 13 to 85 years old (n =119). A high number of the farmers had a primary education level (Grade I-V) (46 %), followed by farmers with no formal education (32 %) and farmers with a secondary (17 %) or graduate level (5 %). All the farmers involved in the buffalo milk enterprise interviewed were male. The farms used hand milking as their usual milking practice. Poor milking and udder hygiene was observed on most of the farms. About 79 % of the farmers did not use antiseptics or wash their hands before milking, 19 % washed their hands, and 2 % used antiseptics following hand washing. It was observed that, during milking, the udder was dirty and wet on 50 % of the farms; on 37 %, the udder was clean; and on 12 % of the farms, the udders were both clean and dry. None of the farms used pre- or post-milking disinfection. Of the farmers, 17 % sold raw milk from the farm to the local consumers, 70 % sold milk to middlemen, and 14 % sold their milk directly to the milk product shops. 34 % of the farmers responded that hand milking was problematic, as farmers were frequently injured during milking by the non-cooperative buffalo (Table 8.3). The middlemen in this study had a milk-selling experience of between 2 to 40 years. During transportation, 5 % of the middlemen mixed ice with the milk inside the milk containers, while the remaining did not provide any cooling of the milk. Most middlemen (64 %) cleaned the milk containers once daily, while 36 % cleaned them twice daily (Table 8.4). All the milk collection centers mixed buffalo milk with cow milk. The milk remained in the collection center for 0 to 6.5 hours before further processing or sale. During milk storage at the milk collection centers, 96 % of the milk collection centers kept the milk at room temperature (25 -30 ºC), while only 4 % stored the milk in a freezer. About 63 % of the milk products were prepared in retail shops, and 37 % were at the household level. The milk products were mainly (80 %) processed using a mixture of buffalo and cow, followed by pure buffalo milk (20 %). Processed milk products were sold within 1 to 72 hours (Table 8.5).

Table 8.3 Descriptive features of the farmers, hygienic practices, and herd population for 122 buffalo farms located in 7 districts in Bangladesh.

Variable name	Categories	Number (%)	Mean (Min-Max)	Median
Education level	No formal education	37 (32.0)	-	-
	Primary	53 (45.7)	-	-
	Secondary	20 (17.4)	-	-
	Graduation	6 (5.2)	-	-
Who milks the buffalo?	Owner	72 (59.0)	-	-
	The owner, worker, and middleman	53 (40.9)	-	-
Wash the milkers' hands before milking	No	98 (81.0)	-	-
	Yes	23 (19.0)	-	-
Type of milk containers used	Aluminum	69 (58.0)	-	-
	Plastic	32 (26.9)	-	-
	Tin	13 (10.9)	-	-
	Others (Aluminum, Plastic, and glass)	5 (4.2)		
The water source for cleaning the milk containers	River water	25 (21.6)	-	-
	Tube-well water	57 (49.1)	-	-
	Pond water	34 (29.1)	-	-
Containers remain open during storing milk on the farm	No	113 (93.4)	-	-
	Yes	8 (6.6)	-	-
Point of milk sale	Farm	19 (16.5)	-	-
	Middlemen	80 (69.6)	-	-
	Milk product shops	16 (13.9)	-	-
Farmers age	-	-	36.7 (13-85)	35
Farm size (buffalo heads)	-	-	27.3 (3-170)	17
Numbers of lactating buffalo	-	-	7.8 (3-46)	5
Numbers of dry buffalo	-	-	6.6 (0-60)	3
Numbers of heifers	-	-	5.1 (0-95)	2
Numbers of calves	-	-	8.6 (0-51)	5
Average daily milk production per farm	-	-	13.6 (1.5-150)	8

Table 8.4 Descriptive features of milk handling and hygienic practices by the middlemen (N = 109) on the buffalo milk chain in 7 districts of Bangladesh.

Variable name	Categories	Number (%)	Mean (Min-Max)	Median
Trading experience	Non-experienced (≤ 5 years)	2 (2.0)	-	-
	Experienced (> 5 years)	99 (98.0)	-	-
Cooling of milk	No	100 (95.2)	-	-
	Yes (Adding ice to the milk during transportation)	5 (4.8)	-	-
Materials used for covering milk container	Container kept open	63 (57.3)	-	-
	Cloth	1 (0.9)	-	-
	Plastic plate	32 (29.1)	-	-
	Aluminum plate	8 (7.3)	-	-
	Banana leaves	6 (5.5)	-	-
Nature of milk	Buffalo milk	62 (59.6)	-	-
	Cow-buffalo mixed milk	42 (40.4)	-	-
Cleaning frequency of milk containers	Once daily	65 (64.4)	-	-
	Twice daily	36 (35.6)	-	-
The water source used for cleaning the milk container	Pond water	49 (47.6)	-	-
	Tube well water	21 (20.4)	-	-
	Tube well water with detergents	33 (32.0)	-	-
Transport time (in hours) from middleman to milk collection center	-	-	1.5 (0-8.0)	1

Table 8.5 Descriptive features of milk handling and practices associated with milk or milk product processing at the milk collection centers (N = 109) and milk product shops (N = 35) on the buffalo milk chain in Bangladesh.

Nodes	Variable name	Categories	Number (%)	Mean (Min-Max)	Median
Milk collection center	Type of containers used	Aluminum	38 (36.9)	-	-
		Plastic	59 (57.3)	-	-
		Tin	6 (5.8)	-	-
	Stored milk in the freezing facility	Yes	4 (3.6)	-	-
		No	106 (96.4)	-	-
	Storage time (hours) before further processing	-	-	1.4 (0-6.5)	1
Milk product shops	Type of product	Yogurt	26 (74.3)	-	-
		Cheese	7 (20.0)	-	-
		Buttermilk	2 (5.7)	-	-
	Product processing place	Shop	22 (62.9)	-	-
		Household	13 (37.1)	-	-
	Type of containers	Clay	12 (34.3)	-	-
		Plastic	16 (45.7)	-	-
		Glass	7 (20.0)	-	-
	Source of milk purchase	Own shop (own farm and through own contract middlemen)	10 (28.6)	-	-
		Both own farms, contract middlemen, and other farmers or middlemen	25 (71.4)	-	-
	Type of milk	Buffalo milk	7 (20.0)	-	-
		Cow-buffalo mixed milk	28 (80.0)	-	-
	Type of seller	Whole seller	9 (25.7)	-	-
		Retail seller	10 (28.6)	-	-
		Both	14 (40.0)	-	-
		Household seller	2 (5.7)	-	-

8.4.4 Factors associated with bulk milk somatic cell count and total bacteria count at different nodes of the milk chain.

Six variables had a $P \leq 0.2$ in the univariable analysis presented in Table 8.6. Multivariable linear regression analysis at the farm level showed that in model 1, a higher BMSCC was observed in the spring season ($P < 0.001$) compared to the winter season and in the intensive buffalo-rearing system ($P = 0.006$) compared to the semi-intensive system. In model 2, a higher TBC was associated with the winter season ($P = 0.007$) compared to the late autumn season, and milk containers were cleaned using pond water ($P = 0.03$) compared to tube-well water. In model 3, the TNAS count was significantly higher in the coastal or semi-coastal regions ($P = 0.0003$) compared to the farms in the river basin area. (Table 8.7). Neither confounders nor interactions were observed in the models.

A higher TBC in the milk samples from the middleman node was associated with cow and buffalo milk ($P = 0.02$) compared to pure buffalo milk. TBC levels were also significantly higher when middlemen used milk containers with poor cleanliness ($P = 0.004$) compared to excellent cleanliness (Table 8.8). No significant variables existed for the TSA, TNAS, and TEC models. No variables remained significant in the final multivariable models at the milk collection center and milk product shop level. All models fitted well and were free from any collinearity and heteroskedasticity.

Table 8.6 Univariable association between the log10-transformed bulk milk somatic cell count (BMSCC), total bacterial count (TBC), and total non-*aureus staphylococcus* count (TNAS) at the farm and middleman level, with various farm and middleman level variables using t-test or One-way ANOVA

Columns were kept blank where the association had a P value >0.20.

Variables	Categories	Mean (numbers of positive samples)			
		Log10BMSCC	Log10TBC	Log10TNAS	Log10TSA
Farm level					
Season	Winter	5.1 (16)	5.4 (16)	4.2 (12)	
	Late autumn	5.2 (28)	4.6 (28)	3.9 (23)	
	Spring	5.5 (78)	5.3 (78)	4.5 (76)	
	P	***<0.0001***	***0.001***	***0.02***	
Rearing system	Semi-Intensive	5.3 (23)		4.0 (23)	3.0 (1)
	Free-range/bathan	5.5 (15)		4.6 (15)	3.0 (4)
	Household	5.5 (20)		4.4 (19)	3.9 (1)
	Semi-bathan	5.4 (61)		4.4 (52)	3.3 (10)
	Intensive	5.6 (3)		4.3 (2)	-
	P	***0.2***		***0.2***	***0.1***
Geographical area of the farms	River basin	5.3 (62)	4.9 (62)	4.0 (52)	3.2 (10)
	Inland	5.5 (20)	5.3 (20)	4.4 (19)	3.9 (1)
	Coastal or semi-costal area	5.6 (40)	5.5 (40)	4.9 (40)	3.3 (5)
	P	***<0.0001***	***0.01***	***<0.0001***	***0.1***
The water source used for cleaning the milk containers[1]	River water	-	5.3 (24)	4.4 (22)	3.1 (9)
	Tube-well water	-	5.0 (57)	4.1 (51)	3.4 (5)
	Pond water	-	5.6 (34)	4.8 (32)	3.7 (2)
	P	-	***0.009***	***0.004***	***0.04***
Middleman level[2]					
Nature of milk	Buffalo milk	-	5.8 (62)	4.7 (61)	
	Cow-buffalo mixed milk	-	6.1 (41)	5.3 (38)	
	P	-	***0.04***	***0.01***	
Cleanliness of the containers	Excellent (Clean inside and outside)	-	5.4 (13)		
	Poor	-	6.3 (30)		
	Good (Clean inside)	-	5.8 (59)		
	P	-	***0.04***		

[1] BMSCC is not biologically associated with the water source cleaning milk containers. Thus, is kept blank.

[2] BMSCC data was unavailable at the middleman level. Thus, the LBMSCC column was kept blank.

Table 8.7 Results from three multivariable linear regression models for the association between the log10-transformed bulk milk somatic cell count (BMSCC), total bacterial count (TBC), and total non-aureus staphylococcus count (TNAS), with farm level factors in Bangladesh expressed as co-efficient (β), 95 % confidence interval (95 % CI), and *P* values

Models	Variable name	Categories	β ± SE	95 % CI	*P*
Model 1: Bulk milk somatic cell count[1]					
		Intercept	4.86 ± 0.09	4.67 to 5.04	< 0.001
	Season	Winter	Reference		< 0.001
		Late autumn	0.14 ± 0.09	-0.04 to 0.31	
		Spring	0.51 ± 0.08	0.36 to 0.67	
	Rearing system	Semi-Intensive	Reference		0.002
		Household	0.18 ± 0.08	0.01 to 0.34	
		Free-range/bathan	0.21 ± 0.09	0.03 to 0.39	
		Semi-bathan	0.24 ± 0.07	0.10 to 0.37	
		Intensive	0.62 ± 0.17	0.27 to 0.97	
Model 2: Total bacteria count[2]					
		Intercept	4.59 ± 0.18	4.23 to 4.95	< 0.001
	Season	Late autumn	Reference		0.018
		Spring	0.54 ± 0.20	0.14 to 0.93	
		Winter	0.63 ± 0.30	0.05 to 1.22	
	The water source used for cleaning the milk containers	Tube-well water	Reference		0.026
		River water	0.22 ± 0.24	-0.25 to 0.68	
		Pond water	0.50 ± 0.18	0.14 to 0.86	
Model 3: Total *non-aureus Staphylococcus* count[3]					
		Intercept	3.98 ± 0.12	3.75 to 4.22	
	Geographical area of the farms	River basin	Reference		<0.001
		Inland	0.38 ± 0.23	-0.08 to 0.84	
		Coastal or semi-costal area	0.87 ± 0.18	0.51 to 1.22	

[1] Based on records from 116 buffalo farms

[2] Based on records from 111 buffalo farms

[3] Based on records from 109 buffalo farms

Table 8.8 Multivariable linear regression analysis of the association between the log10-transformed total bacterial count (TBC), with middleman level factors in 102 middleman buffalo milk chain samples in Bangladesh expressed as co-efficient (β), 95 % confidence interval (95 % CI), and *P* values

Variable name	Categories	β ± SE	95 % CI	*P*
	Intercept	5.87 ± 0.12	5.64 to 6.11	
Type of milk	Buffalo milk	Reference		0.02
	Cow-buffalo mixed milk	0.24 ± 0.10	0.04 - 0.44	
	Excellent	Reference		0.01
Cleanliness of the containers	Good	0.006 ± 0.14	-0.27 - 0.28	
	Poor	0.47 ± 0.16	0.16 - 0.79	

8.5 Discussion

This study showed moderate levels of BMSCC in milk from buffalo farms and high levels of TBC, TNAS, and TEC at various nodes in the milk value chain. The overall mean BMSCC at the farm level was 254 × 10^3 cells per mL, which falls below the acceptable threshold of 400 × 10^3 cells per mL used for buffalo milk in Italy (Costa et al., 2020) and the neighboring countries, Nepal and India, (Dhakal, 2006; Alhussien and Dang, 2018). However, about 30 % of the herds in the present study exceeded the abovementioned threshold. A high SCC is considered a reliable parameter for indicating inflammation in the mammary gland; therefore, farms with a high BMSCC may be associated with possible IMI in buffalo. A previous study reported that SCC in buffalo quarters substantially increases in the presence of an IMI, particularly when IMI is mainly caused by *Streptococcus* spp and *S. aureus* (Moroni et al., 2006). This indicates that IMI may be the most critical factor driving BMSCC, suggesting that improving buffalo udder health might reduce BMSCC.

Our study found that the spring season and intensive buffalo-rearing were associated with a high BMSCC level on buffalo farms. Seasonal variations in BMSCC have been reported in earlier studies on dairy cows and goats in the Netherlands and Canada (Sargeant et al., 1998; Olde Riekerink et al., 2007; Koop et al., 2009). However, the influence of the seasons on BMSCC is not well understood in buffalo. Nevertheless, the seasonal effect on BMSCC is a descriptive rather than a risk factor, and the possible explanations for these findings are mainly speculative. In the present study areas, buffalo were moved to the islands during spring, which might put the animals in a stressful

condition due to the long transportation (both walking and traveling by boat), with lactational stress as a response to the surge in milk yield. A high BMSCC may reflect this management stress in spring.

In our study, larger farm sizes were observed in the intensive buffalo-rearing system, i.e., farm size was between 50-170 animals and generally had a high stock density, which may also increase the chances of spreading IMI pathogens in the herd (Bari et al., 2022). Nevertheless, the animals lacked access to grazing and wallowing facilities, putting them in stressful conditions and possibly compromising their immunity. Animals in intensive farms in this study were primarily high-yield, crossbred buffalo (e.g., Indigenous × Nili-Ravi and Indigenous × Murrah). It is known that there is a correlation between high milk yield and increased BMSCC (Costa et al., 2020). Altogether, the increased chances of IMI and stressful conditions may have led to a high BMSCC on the intensive farms in this study.

Buffalo farmers in the present study did not use BMSCC or any qualitative mastitis screening test, such as the California mastitis test. Therefore, milk quality in terms of udder health status from buffalo farms in this region of Bangladesh is mainly unknown. Our study presents BMSCC on buffalo farms for the first time in Bangladesh. Currently, there is no regulatory enforcement for setting a threshold for BMSCC, and there are no dairy herd improvement programs like those found in the USA and Canada (Hand et al., 2012; Troendle et al., 2017). Therefore, the buffalo farmers in this study with high BMSCC are likely not motivated to upgrade their farm management based on BMSCC results. Enforcing regular testing of BMSCC and implementing a penalty or premium system may help regulate the farms, thereby improving udder health and milk quality on the buffalo farms.

The buffalo milk chain in Bangladesh is informal and has remained underregulated in terms of hygiene practices. The highest level of TBC was identified in the terminal milk chain nodes, e.g., milk products, compared to the lowest TBC levels observed in the farm bulk milk. The levels of TBC at the included nodes (5.2 log10 per mL at the farm level, 6.0 log10 at the middleman level, 6.6 log10 at milk collection centers, and 7.5 log10 per mL in milk products) were much higher than the acceptable threshold of 4.3 log10 (2×10^4) per mL set by the BSTI (1009:1982), the regulatory body for food safety in Bangladesh. This increased level of TBC in the terminal milk chain is consistent with a previous study from Bangladesh (Islam et al., 2018) and in studies from other LMICs,

such as Rwanda and Zimbabwe (Mhone et al., 2011; Ndahetuye et al., 2020a). There may be several reasons for increasing levels of TBC, for example mixing milk from different farms, bacterial growth due to long transport times in ambient temperatures, and unhygienic processing steps (Artursson et al., 2018; De Vries et al., 2020; Ndahetuye et al., 2020a). Therefore, it is advised that microbiological evaluation of milk quality be enforced, and farmers should be encouraged by offering a premium price for higher milk quality.

Our study demonstrated that the winter season compared to late autumn and the use of pond water compared to deep tube-well water for cleaning the milk containers were significantly associated with a higher level of TBC at the farm level. These findings can be partly explained by the fact that, during winter, there is usually a lowering of the water level in the studied areas, which makes river transport difficult by boat, causing the need for additional walking time to reach the mainland. On the other hand, in late autumn, the buffalo are transported to the mainland due to the greater availability of feed resources there, meaning they are closer to the location of milk collection centers on the mainland. This dramatically reduces the post-milking transportation time from the farm to the milk collection center, which may act as a protective factor in reducing bacteria contamination levels.

Cleaning the milk container using tube-well water makes sense as a means of reducing the level of contamination compared to river or pond water. This also agrees with an Ethiopian study demonstrating that tap water use correlates with lower TBC than other water sources, such as river and pond water (Aliyo et al., 2022). Using pond and river water may affect the milk containers' cleanliness and compromise the milk's microbiological quality. Ponds or rivers are generally located close to the manure reservoir. They could act as a source of cross-contamination of the water used to clean the containers (Lopes et al., 2021). Therefore, it is recommended to use tube-well water to clean the milk containers, which may likely reduce the TBC. At the middleman level, mixed cow and buffalo milk and a poor cleanliness score for the milk containers were significantly associated with high TBC, consistent with the findings of Aliyo et al. (2022). A mix of milk from different sources resulted in an overall high TBC level. Hence, from the findings of this study, cleaning the milk containers with clean tube-well water and avoiding mixing buffalo and cow milk could improve the hygienic quality of the milk.

Non-*aureus staphylococcus* increased between the farm node (4.2 log10) and the milk products node (5.7 log10). Previous studies show that non-*aureus staphylococc*us species can colonize the teat canal of water buffalo during episodes of IMI (Pisanu et al., 2019; Singha et al., 2021a), this can thereby easily contaminate milk during milking. We identified higher levels of NAS associated with farms located in coastal or semi-coastal areas. In coastal or semi-coastal areas, buffalo farms are distantly located. Transportation depends on boats and walking, with the transport time to the milk collecting center reaching up to 6 hours, promoting bacterial growth. To reduce bacterial contamination during transportation, it is advised to use an insulated milk container with a cooling facility.

An increased level of *Enterobacteriaceae* was recorded in the milk products (4.6 log10) compared to the farm bulk milk samples (2.9 log10). Other studies from Bangladesh and elsewhere show similar results (Mhone et al., 2011; Knight-Jones et al., 2016; Islam et al., 2018). Islam et al. (2018) reported that all milk samples from a chilling center and final processed milk were contaminated with coliforms, and two-thirds were contaminated with *E. coli*. Another study in Kenya identified that milk containers were frequently contaminated with coliform bacteria (Ombui et al., 1994). The presence of *Enterobacteriaceae* indicates the fecal contamination of milk. There was inadequate udder and milker hygiene on the farms in the present study, which might lead to milk contamination. This is supported by a previous study in Bangladesh (Islam et al., 2018). Regardless of the type of milk producers, there is a need to create motivation to follow the hygienic cleaning of milk containers to ensure safe milk for the consumers. Since the milk products is produced using a traditional natural fermentation of raw milk, producing raw milk products, even with cooling of milk during transport, is quite a challenge. Therefore, introducing pasteurization at the milk collection center level probably could be a feasible and useful step to improve the microbiological quality of buffalo milk products for example, in Noakhali and Bhola district where people primarily consume natural fermented buffalo milk products such as, yogurt.

8.6 Conclusions

The mean BMSCC was moderately high on the buffalo farms. Regular testing of BMSCC on the farm and microbiological evaluation of milk before milk processing is required to ensure safety and milk quality. Microbial contamination increased from farm to milk

product shops, indicating bacterial proliferation during milk transportation. Therefore, cooling milk during transportation could improve milk quality. Contamination by NAS and *Enterobacteriaceae* highlights insufficient hygienic practices during milking and subsequent milk handling. Cleaning milk containers with tube-well water and avoiding mixing cow milk with buffalo milk were identified as beneficial practices. Our findings suggest that better hygienic practices during milk handling could help reduce bacterial contamination and increase public safety when consuming buffalo milk and milk products in Bangladesh.

CHAPTER-9

Prevalence of foodborne zoonotic pathogens in milk and milk products along the buffalo milk value chain in Bangladesh

CHAPTER 9

Prevalence of foodborne zoonotic pathogens in milk and milk products along the buffalo milk value chain in Bangladesh

9.1 Abstract

Buffalo milk and milk products are highly valued due to their superior nutritional quality and health benefits. Therefore, assessing the prevalence of zoonotic pathogens in buffalo milk is crucial for ensuring the safety of the consumers. The present study aimed to estimate the prevalence of important foodborne zoonotic pathogens in milk and milk products and identify the associated factors in Bangladesh's buffalo milk chain nodes. One hundred and forty-three samples were collected from farm bulk milk (n = 34), middlemen (37), milk collection centers (n = 37), and milk product shops (n = 35). They were analyzed using RT-PCR to estimate the prevalence of seven important zoonotic pathogens: *Staphylococcus aureus*, *Escherichia* (*E.*) *coli*, Shiga toxin-producing *Escherichia* (*E.*) *coli O157:H7*, *Campylobacter* (*C.*) *jejuni*, *Listeria* (*L.*) *monocytogenes*, *Salmonella* (*S.*) *enterica*, and *Yersinia* (*Y.*) *enterocolitica*. *Escherichia coli* was the most prevalent pathogen along the milk chain nodes. The prevalence of the pathogens was high over the buffalo milk value chain. Three classical enterotoxin-encoded genes for *E. coli O157:H7* were tested, e.g., *eae*, *stx1*, and *stx2,* of which the *stx2* genotype was most prevalent and was most common in milk products (74 %). *L. monocytogenes* and *Y. enterocolitica* were more prevalent in the farms (65-79 %) than in the later milk value chain nodes. The prevalence of *S. enterica* was low (0-2.9 %) in all the milk chain nodes, and all the samples were negative for *C. jejuni.* These results suggest that buffalo milk consumers in Bangladesh are at risk for *L. monocytogenes* and *Y. enterocolitica* in the farms and *E. coli O157:H7* with *stx2* genotype at the milk product shops. *S. enterica* and *C. jejuni* are not frequent contaminants of the buffalo milk chain in Bangladesh. Avoiding plastic made containers and dirty cloths for cleaning milk containers may help reduce the contamination in the buffalo milk value chain.

9.2 Introduction

Foodborne diseases affect about 600 million people, resulting in 0.4 million deaths yearly, including 1.3 million children under five years (WHO, 2022). Microorganisms causing foodborne illnesses are mainly associated with consuming harmful toxins or chemicals contaminated with food or water (CDC, 2020). Food-producing animals act as the reservoir of many foodborne zoonotic diseases, and food products serve as vehicles for transmitting the causal agents (Heredia and García, 2018). The illness primarily results from ingesting contaminated foodstuffs either by the infection through vegetative pathogenic microorganisms or the intoxication by bacterial toxins (Addis and Sisay, 2015). The microorganism's toxins or virulence factors are responsible for the pathogenesis and detrimental effects on human health (Abebe et al., 2020). Bacteria are associated with two-thirds of human foodborne diseases, with a relatively heavy burden affecting low and middle-income countries (Abebe et al., 2020). Foodborne diseases also have economic consequences for the healthcare system, food producers, and distributors and require specific attention from regulatory authorities (Abebe et al., 2020; CDC, 2021). Despite increased global awareness of foodborne infections as a threat to public health safety and socio-economic development, food safety remains ignored, especially in low- and middle-income nations.

Milk and milk products are nutritionally rich and considered important components for many healthy diets but, if contaminated, can be a source of human pathogenic microorganisms (Sonnier et al., 2018). Milk is a suitable growth medium for many microorganisms due to its high nutritional value with protein, sugars, and lipid contents (Monnet et al., 2015; Abebe et al., 2020). Previous studies primarily evidenced the presence of zoonotic bacteria in cows, sheep, and goats (Rahimi et al., 2010; Artursson et al., 2018; Aragão et al., 2021). Studies have found zoonotic microorganisms like *Staphylococcus* (*S.*) *aureus*, *Escherichia* (*E.*) coli, and enteropathogenic *E. coli O157:H7* in bulk milk and cheese samples from dairy cows (Boss et al., 2016; Guzman-Hernandez et al., 2016; Jaakkonen et al., 2019). Shiga toxin-producing (STEC) ability of *E. coli O157:H7* was previously reported from dairy cows' milk mainly for three virulent genes such as *stx1*, *stx2*, and *eae* (Caine et al., 2014; Desmarchelier and Fegan, 2016; Disassa et al., 2017). *Campylobacter* (*C.*) *jejuni*, *Listeria* (*L.*) *monocytogenes*, *Salmonella* spp, and *Yersinia* (*Y.*) *enterocolitica* were also previously identified in cow bulk milk and milk products (Raeisi et al., 2017; Jaakkonen et al., 2019; Diriba et al., 2021; Gebeyehu

et al., 2022). Although 35 % of total milk production in Asian countries accounted for buffalo milk (Minervino et al., 2020), still there only a few published studies (Godinho et al., 2020; Khan et al., 2022) reported the prevalence of these zoonotic pathogens from buffalo milk in low- and middle-income countries.

The water buffalo-rearing system in Bangladesh mainly includes free-ranging systems on islands in the coastal or semi-coastal areas and a semi-intensive or intensive buffalo-rearing system in the inland. The buffalo milk trading also has different levels of handlers, for example, middlemen, milk collection, and milk products shops (Hamid et al., 2016a; Singha et al., 2021c). Bangladesh's buffalo milk value chain consists of activities and processes, including production, processing, trading, and consuming milk and milk products (Hamid et al., 2016a; Singha et al., 2021c). The farms are generally located remotely. Thus, transportation to the milk collection center takes a long time, and the absence of a milk cooling facility during transport affects milk safety (Habib et al., 2017). Thus milk and milk products from buffalo milk could be a source for transmitting zoonotic pathogens between humans and animals. Guaranteeing adequate hygienic status at the farms and during milk handling along the stages of production is required to achieve quality and safety during the consumption of milk and milk-derived products. A considerable obstacle to adequately addressing food safety concerns is the lack of data, e.g., prevalence and factors associated with foodborne pathogen contamination in buffalo milk and milk products, which would enable policymakers to set public health priorities and allocate resources.

The present study aimed to apply real-time polymerase chain reaction (RT-PCR) to estimate the prevalence of seven important zoonotic pathogens: *S. aureus*, *E. coli*, Shiga toxin-producing *E. coli O157:H7*, *C. jejuni*, *L. monocytogenes*, *Salmonella* spp, and *Y. enterocolitica* and to identify the risk factors associated with these pathogens in milk and milk products in the buffalo milk chain nodes in Bangladesh.

9.3 Materials and Methods

9.3.1 Ethical statement

The study was approved and performed in line with the guidelines of the Sylhet Agricultural University Research System (AUP/21/06). Written informed consent was

given by the buffalo farmers, middlemen, milk collection center, and milk product shop owners before participation in this study.

9.3.2 Study design

This cross-sectional study was conducted from February 2020 to April 2021 in four buffalo milk chain nodes (farm, middleman, milk collection center, and milk product shop) in 6 districts in Bangladesh (Noakhali, Bhola, Maulvibazar, Mymensingh, Jamalpur, and Rajshahi). The farmers' list was created with the help of the Upazila Veterinary Hospital (UVH) and a non-governmental organization named the "Palli Karma-Sahayak Foundation" working on buffalo farmers in Bangladesh. A list was created by collecting data from the buffalo farmers and local milk product shops at each study location. From there, the farms, middlemen, milk collection centers, and milk product shops were randomly recruited for this study based on the sample size estimation. The sample size was calculated to estimate a 50 % prevalence with 95 % confidence and a final margin of error of 0.17 using https://statulator.com/SampleSize/ss1P.html. We required 34 samples for each type of milk chain sample.

9.3.3 Collection of samples

One bulk milk sample was collected from each buffalo farm. Two milk samples were collected aseptically before mixing (middleman) and another after mixing in the milk collection center. Milk product samples (yogurt, cheese, and butter) were also collected from the study areas. Samples taken from the middlemen, milk collection centers, and milk product shops were not linked with the source buffalo farm. From each mixed milk sample (bulk milk from a farm, middleman, and milk collection center), an aliquot of 10 mL and 30-35g of milk product (i.e., yogurt, cheese, and milk cream) were aseptically collected in a 15 mL and 50 mL falcon tube, respectively. Upon arrival at the laboratory, the samples were stored immediately on ice and at -20 ºC.

9.3.4 Questionnaire data collection

A questionnaire was developed and divided into four subsections to determine risk factors associated with the zoonotic bacteria related to the buffalo milk chain nodes. Section A captured data at the farm level and included 45 questions. Data included information such as farmers' education level, buffalo-rearing system, geographical area

of the farm, the total number of lactating buffalo, dry buffalo, and average milk yield per day. Section A also collected data on milk containers, such as the type of milk container, how the containers were cleaned, and the cleanliness score of the milking containers. The cleanliness score was defined using three categories, based on visual observation by the interviewer: excellent (if no greasiness or dirt was observed inside or outside the container), good (if no greasiness or dirt was observed inside the container), and poor (if greasiness or dirt was present both inside and outside of the containers). Sections B and C contained 20 questions and collected information from the middlemen and collection centers. Data on milk transportation, such as any cooling materials inside the containers, the nature of milk composition (buffalo milk or a mixture of buffalo milk and cow milk), how the containers were cleaned, and the cleanliness score of the milk container. Section D included eight questions to gather information on milk products, such as the nature of milk, storage time, and the type of containers used. Qualitative assessments, such as the cleanliness score of the milk containers, were determined during sample collection following subjective visual observation by the interviewer. Information on mixing buffalo milk with cow milk was obtained by cross-questioning the middlemen or personnel at the milk collection centers.

9.3.5 Genomic DNA extraction and purification

The samples were subjected to DNA extraction in the Microbial genetics and bioinformatics laboratory under the Department of Microbiology, University of Dhaka, Bangladesh. DNA extraction was performed using Maxwell® 16 Cell DNA Purification Kit (Promega, UK) with the Maxwell® 16 Instrument platform (Promega, UK). One mL of the sample was centrifuged at 16,000 rcf for 10 minutes (Oikonomou et al., 2014). Then the supernatant was discarded, and the remaining pellet was used for DNA extraction according to the manufacturer's instructions. The DNA samples were eluted in 200 µL elution buffer (Promega, UK) and were stored at -20 °C until further processing. DNA concentration and purity were analyzed using NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific, Waltham, Massachusetts, USA) at a wavelength of 260nm and A260/A280, respectively. DNA samples were considered appropriate for downstream if the DNA concentration was ≥ 9 ng/ µL and 260/280 absorbance ratios (> 1.6 to ≤ 2). Then the DNA samples were delivered to the Molecular

Pathology Laboratory, Department of Veterinary and Animal Sciences (DIVAS), Università degli Studi di Milano (UNIMI).

9.3.6 Real-time polymerase chain reaction

Quantitative PCR was performed following the MIQE guidelines (Bustin et al., 2009) in a final 15 µL reaction volume using the CFX 96 System (Bio-Rad Laboratories, USA). Each reaction volume contained 7.5µL of 2x Mix EVA Green (SsoFast EvaGreen® Supermix, Bio-Rad Laboratories, USA) and primers specific for target genes (Table 9.1). The PCR reaction was carried out using the same thermal profile for all the targets (2 min at 50 ºC, 3 min at 95 ºC, and 39 cycles of 10s at 95 ºC and 30s at 60º C). To assess melting curves, PCR products were incubated at 55 ºC for 60s then increased to 95 ºC at 0.5 ºC increments for 10s. The PCR efficiencies were determined using four-fold serial dilutions of DNA, prepared from ATCC strains of the bacteria of interest, such as *S. aureus* ATCC 19048, *E. coli* ATCC 11229, *E. coli* O157:H7 ATCC 35150, *Listeria monocytogenes* ATCC 13932, *Yersinia enterocollitica* DSM 4780, *Salmonella enterica* ATCC 13076, and *Campylobacter jejuni* ATCC 33291. ATCC and DSM bacterial strains were obtained from the American type culture type collection (MD, USA) and German collection of microorganisms and cell cultures (Braunschweig, Germany), respectively. No template controls were included in the assays. The results were analyzed using the software Bio-Rad CFX Maestro 1.0 (Bio-Rad Laboratories, USA). The samples with a threshold of CT < 35 cycles were considered positive for the presence of the targeted gene.

Table 9.1 Primer sequences used for RT-PCR to identify the presence of the 9 target genes of seven zoonotic bacteria from the extracted DNA samples

Primers were selected from previously published studies (Cremonesi et al., 2014; Cremonesi et al., 2016)

Bacteria	Gene	Sequences (5'-3')	Accession number	Amplicon size	Primer concentration[a]
Staphylococcus aureus	*htrA*	GAAGTAATATCAGACAAATCAAATCAGTACC	NC_009782	92bp	500nM
		TCTTCCGGTAAAGTTAATGGCTTCTG			
Escherichia coli	*yccT*	GCAGCGTGGTGGCAAAA	CP10315	56bp	400nM
		CGTGACCACCTTGATTGCAT			

Escherichia coli O157:H7	*eae*	GTAACAATGTCAGAGG CGAGTTG	AE005174	72bp	600nM
		CCACCGCTTGCTTTCAG TTTAA			
	stx1	GGATTTCGTACAACAC TGGATGATC	M16625	67bp	400nM
		GATCAACATCTTCAGCA GTCATTACA			
	stx2	ACCCCACCGGGCAGTT	X07865	59bp	500nM
		CGCGCCTGATAGACAT CAAG			
Listeria monocytogenes	*inlA*	TAACAGACACGGTCTC G CAAA	CP013288	66bp	400nM
		TCCCTAATCTATCCGCC TGAAG			
Yersinia enterocollitica	*yst*	TGGAGCATTCGGCCAA GAA	X65999	60bp	400nM
		ATTGGTGTCGATAATG CATCACTGA			
Salmonella enterica	*invA*	TGGAAAGGGAAAGCC AGCTT	M90846	68bp	500nM
		AATAGCGTCACCTTTG ATAAACTTCA			
Campylobacter jejuni	*cadF*	TGAACCAAGAGAAGG TGCTTTGT	FJ946061	76bp	400nM
		AAAACCAAAATGACCT TCCAAAGAAATAGTT			

[a] Appropriate primer concentration was selected based on the reproducibility ($R^2 > 0.99$) and Efficiency (> 85 %) of the selected primers (forward and reverse) to detect the targeted genes specific to each of the ATCC positive controls (*S. aureus* ATCC 19048, *E. coli* ATCC 11229, *E. coli* O157:H7 ATCC 35150, *Listeria monocytogenes* ATCC 13932, *Yersinia enterocollitica* DSM 4780, *Salmonella enterica* ATCC 13076, and *Campylobacter jejuni* ATCC 33291).

9.3.7 Statistical analysis

Data analysis was performed using R (version 4.2.2; R Foundation for Statistical Computing, Vienna, Austria). The presence of the zoonotic bacteria (*S. aureus*/ *E. coli*/ *E. coli O157:H7/ L. monocytogenes*/ *Y. enterocollitica*/ *S.* enterica/ *C. jejuni*) was a binary outcome variable (yes or no). The prevalence of each pathogen was calculated for each milk chain node, namely, bulk milk, middleman, milk collection center, and milk products, by dividing the total number of positives by the total number of samples tested. A chi-squared test was performed to analyze the difference in the prevalence of each pathogen between the four different milk chain nodes. For categorical variables, a Fisher's exact test was performed to compare the difference between categories of a variable for each RT-PCR positive zoonotic pathogen. Variables with a $P \leq 0.2$ were

presented with the prevalence of RT-PCR-positive zoonotic pathogens and corresponding *P*-values.

9.4 Results

9.4.1 Descriptive statistics

Table 9.2 shows that 143 samples were collected, comprising 108 bulk milk samples from three different milk chain nodes (farm, middleman, and milk collection center) and 35 milk products (yogurt, cheese, and buttermilk) from the milk product shops.

Table 9.2 Distribution of milk and milk product samples (N = 143) collected at four different buffalo milk chain nodes in 6 districts of Bangladesh

District	Farm bulk milk	Middleman	Milk collection center	Milk product shop			Total number of samples
				Yogurt	Cheese	Buttermilk	
Rajshahi	4	1	8	2	3	-	18
Jamalpur	6	4	3	2	-	-	15
Mymensingh	4	3	2	-	-	-	9
Maulvibazar	4	4	8	-	3	2	21
Bhola	5	5	5	10	-	-	25
Noakhali	11	20	11	12	1	-	55
Total	**34**	**37**	**37**	**26**	**7**	**2**	**143**

9.4.2 Prevalence of the zoonotic bacteria along the buffalo milk chain

The overall prevalence of most pathogens in milk and milk products was very high. Table 9.3 shows that *E. coli* was the most prevalent (81-97 %) pathogen along the buffalo milk chain nodes and had an exceptionally high prevalence in farm bulk milk. The presence of *E. coli O157:H7* virulence-specific genes such as *eae* and *stx2* genes significantly differed along the milk chain and was higher in milk products than at the farm level. The prevalence of *S. aureus* increased over the milk value chain, but *L. monocytogenes* and *Y. enterocolitica* decreased over the milk chain and started extremely high in farm bulk milk. The prevalence of *Salmonella* spp. was relatively low (0-2.9 %), and *C. jejuni* was absent along the milk value chain.

Table 9.3 Prevalence of RT-PCR identified zoonotic pathogens from the milk and milk product samples (N = 143) collected from 6 buffalo-concentrated districts of Bangladesh.

Pathogen name	Samples collected at the milk value chain nodes (N = 143)				*P* [a]
	Farm bulk milk (34)	Middleman (37)	Milk collection center (37)	Milk products (35)	
	Number of positives (%)				
S. aureus	3 (8.8)	5 (13.5)	8 (21.6)	11 (31.4)	0.081
E. coli	33 (97.1)	30 (81.1)	33 (89.2)	31 (88.6)	0.207
E. coli O157:H7 *eae*	1 (2.9)	3 (8.1)	11 (29.7)	12 (34.3)	0.001
E. coli O157:H7 stx1	3 (8.8)	0 (0.0)	0 (0.0)	3 (8.6)	0.081
E. coli O157:H7 stx2	17 (50.0)	10 (27.0)	13 (35.1)	26 (74.3)	<0.001
Listeria monocytogenes	22 (64.7)	16 (43.2)	2 (5.4)	5 (14.3)	<0.001
Yersinia enterocolitica	27 (79.4)	0 (0.0)	7 (18.9)	1 (2.9)	<0.001
Salmonella enterica	1 (2.9)	0 (0.0)	5 (13.5)	1 (2.9)	0.04
Campylobacter jejuni	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	-

[a] *P* value was obtained from a chi-squared test by comparing each zoonotic pathogen prevalence among the samples from four buffalo milk chain nodes (farm bulk milk, middleman, milk collection center, and milk product).

9.4.3 Factors associated with the prevalence of the zoonotic pathogens in the buffalo milk chain

Several variables were associated with the prevalence of zoonotic pathogens in the buffalo milk chain nodes (Table 9.4). Importantly, bulk milk from the buffalo farms in the Bhola district had a higher prevalence of *L. monocytogenes and Y. enterocolitica* than in the other districts. The prevalence of *E. coli O157:H7* was higher in free-ranging (62 %) than in the intensive (25 %) buffalo-rearing system. At the middleman level, milk containers that were dried using cloth had a higher prevalence of *E. coli O157:H7*. Plastic milk containers had a higher prevalence of *S. aureus* and *E. coli O157:H7* at the middleman or milk collection centers. At the milk collection center level, *S. enterica* and *Y. enterocolitica* had a higher prevalence in buffalo-cow mixed milk than pure buffalo

milk. In the milk products, the prevalence of *E. coli O157:H7 eae* was higher in cream and yogurt than the cheese.

Table 9.4 Univariable association between variables associated with the prevalence of zoonotic pathogens (*P* < 0.20) on 143 samples collected from four milk chain nodes (farm, middleman, milk collection center, and milk product shop) in six buffalo concentrated districts of Bangladesh

Milk value chain	Variables	Categories	*N*	*S. aureus*		*E. coli*		*E. coli O157:H7*		*Y. enterocolitica*	
				Positive (%) [a]	*P* [b]	Positive (%)	*P*	Positive (%)	*P*	Positive (%)	*P*
Bulk milk (n = 34)	Season	Late autumn	11			11 (100.0)	***0.12***	3 (27.3)	***0.14***	11 (100.0)	***0.01***
		Spring	19			19 (100.0)		12 (63.2)		15 (78.9)	
		Winter	4			3 (75.0)		3 (75.0)		1 (25.0)	
	District	Noakhali	11					6 (54.5)	***< 0.01***	9 (81.8)	***0.02***
		Bhola	5					5 (100.0)		5 (100.0)	
		Maulvibazar	4					3 (75.0)		1 (25.0)	
		Mymensingh	4					3 (75.0)		4 (100.0)	
		Jamalpur	6					0 (0.0)		6 (100.0)	
		Rajshahi	4					1 (0.25)		2 (50.0)	
	Rearing system	Free-range	26					16 (61.5)	***0.11***		
		Intensive	8					2 (25.0)			
	Milk container cleaning frequency per day	Once	29								
		Twice	4								
	Do they dry the milk containers upside down?	Yes	30							25 (83.3)	***0.18***
		No	4							2 (50.0)	
	Keep bulk milk containers open.	Yes	30					18 (60.0)	***0.08***		
		No	3					0 (0.0)			
	Sieving the bulk milk	Yes	2								
		No	32								
	Milkers' hygiene	Good	9							9 (100.0)	***0.15***
		Poor	25							18 (72.0)	
	Previous history of clinical mastitis in the last 12 months	Yes	31			31 (100.0)	***0.09***				
		No	3			2 (66.7)					
	Mixing CM milk in the bulk milk	Yes	27							23 (85.2)	***0.13***
		No	7							4 (57.1)	

Middleman (n = 37)	District	Noakhali	20			19 (0.0)	***0.01***			
		Bhola	5			4 (80.0)				
		Maulvibazar	4			3 (75.0)				
		Mymensingh	3			3 (100.0)				
		Jamalpur	4			1 (25.0)				
		Rajshahi	1			0 (0.0)				
	Season	Late autumn	7			4 (57.1)	***0.06***			
		Spring	6			4 (66.7)				
		Summer	20			19 (95.0)				
		Winter	4			3 (75.0)				
	Type of container	Aluminum	13	4 (30.8)	***0.05***					
		Plastic	23	1 (4.3)						
	Type of trader	Farmer or middleman	18			12 (66.7)	***0.09***			
		Wholesalers	16			15 (93.8)				
	How the container is dried	Sundry	15			14 (93.3)	***0.17***	1 (6.7)	***0.01***	
		Cloth or tissue	16			11 (68.8)		**9 (56.3)**		
Milk collection center (n = 37)	Type of trader	Farmer or middleman	20							
		Wholesalers	17							
	Nature of milk	Buffalo milk	25						1 (4.0)	***< 0.01***
		Buffalo-cow mixed milk	11						6 (54.5)	
	Type of container	Aluminum	14					4 (28.6)	***0.17***	
		Plastic	18					10 (55.6)		
		Tin	4					3 (75.0)		
Milk product shop (n = 35)	Type of products	Yogurt	26							
		Cheese	7							
		Cream	2							
	Type of container	Earthen	10	2 (20.0)	***0.04***					
		Plastic	18	9 (50.0)						
		Glass	7	0 (0.0)						

9.5 Discussion

Our study aimed to estimate the prevalence and identify the risk factors associated with the presence of zoonotic pathogens such as., *S. aureus*, *E. coli*, pathogenic *E. coli O157:H7* strains encoded by three selected genes such as., *eae*, *stx1*, and *stx2*, *L. monocytogenes*, *Y. enterocollitica*, *S. enterica*, and *C. jejuni* in buffalo milk chain in Bangladesh. The prevalence of *E. coli* was high (81-97 %) along the buffalo milk chains in our study, but it was significantly high (97 %) in farm bulk milk. A prior study in Vietnam showed a high prevalence of *E. coli* (71 % in the rectal swabs) in water buffalo (Vu-Khac and Cornick, 2008). Another study in Bangladesh found a higher prevalence

of *E. coli.* (84 %) in slaughtered water buffalo fecal samples (Islam et al., 2008). Several investigations in dairy cows demonstrated that the prevalence of *E. coli* was comparatively lower in bulk milk (22-34 %) than in the milk collection centers (39 %) and traditional cheese prepared from raw milk (80 %) (Awadallah et al., 2016; Disassa et al., 2017; Ranjbar et al., 2018; Liu et al., 2021). However, no previous study has been conducted to explore the prevalence of *E. coli* in milk along the buffalo milk value chain.

Escherichia coli contamination in buffalo farms may result from fecal contamination in bulk milk (Coroian et al., 2010; Desmarchelier and Fegan, 2016). *E. coli* can also contaminate the milk through infected food handlers who practice poor personal hygiene or by water-containing human discharges (Chye et al., 2004). Earlier studies in dairy cows showed that a high prevalence of *E. coli* in milk was also associated with poor cleaning of milk containers, storing milk for a prolonged period (> 4 hours) without any refrigeration, prevalence of mastitis, and no disinfection of water or feed equipment (Awadallah et al., 2016; Disassa et al., 2017). These factors can also be linked to our study situation, where most of the buffalo farms were located on the islands, where it took a long time (4-6) hours to reach the milk collection centers. Finally, 91 % of the farmers kept open during hand milking, and milk hygiene was poor, e.g., 76 % of milkers did not use any antiseptics or wash their hands before hand-milking, and about 82 % of the farmers mixed the CM milk samples in the bulk tank. The cold chain was not maintained, and hygiene was poor during handling, possibly related to *E. coli* contamination in bulk milk in buffalo farms in Bangladesh. It is suggested that overall hygiene should be improved to reduce the *E. coli* contamination in Bangladesh's buffalo milk value chain.

In this study, STEC *E. coli* virulent genes such as *eaeA*, *stx1*, and *stx2* increased over the buffalo milk chain, especially since the prevalence of *stx2* was high in farms (50 %) and milk products (74 %). Prevalence of *stx-2* exceeded the studies on water buffalo in Italy (1 %) and Vietnam (30 %) (Borriello et al., 2012; Liu et al., 2021). The prevalence of *stx1* and *eaeA* (5-11 %) at buffalo farms in these studies is consistent with our findings. However, a Bangladeshi study reported a higher prevalence (82 %) of *stx1* or *stx2* in water buffalo (Islam et al., 2008). These studies used feces or rectal swabs, not buffalo milk. Previous studies in dairy cows demonstrated that these virulent genes varied from bulk milk to milk filters. In bulk milk, *eaeA* (5-18 %), *stx-1* (6-9 %), and *stx2* (5-8 %) were detected with a lower prevalence, whereas was increased in milk filter, *eaeA* (64

%), *stx1* (46 %), and *stx2* (27 %) (Sonnier et al., 2018). A recent Bangladeshi study demonstrated a higher prevalence of *stx1*, *stx2,* and *eae* (40-57 %) in cattle, poultry, and human diarrheal patients (Parvej et al., 2020). However, it is not known if transmission occurred between livestock and human. In a free-range system, buffalo, cattle, sheep, and sometimes chicken and duck are reared in the same farm area where buffalo farm workers mainly live closely within the farm boundary. Therefore, in the buffalo-rearing system, there could be a risk of the spread of these virulence genes in close interaction between human and livestock species. Previous studies on dairy cows show that using plastic-made milk containers and milk handling at the middleman were associated with a higher prevalence of *E. coli O157:H7* (Disassa et al., 2017). Poor hygiene during the transportation and processing of raw buffalo milk by the middleman, milk collection center, and milk product shops may contaminate the milk with *E. coli* virulence genes in the buffalo milk chain. However, further studies are needed to identify the transmission routes to establish effective control measures for pathogenic *E. coli O157:H7* contamination in the buffalo milk value chain.

The prevalence of *S. aureus* in our study ranged between 9-31 % increasing over the buffalo milk value chain in our study. A previous study in Ethiopia showed that the prevalence of *S. aureus* was 16 % in buffalo bulk milk which is higher than this study (Mansour et al., 2017). The increasing trend of *S. aureus* is not comparable since no previous study reported the prevalence of *S. aureus* in the buffalo milk chain other than bulk milk. In dairy cows, previous studies show that the prevalence of *S. aureus* was higher in cheese (33-40 %) than in bulk milk (13-25 %) (Alghizzi and Shami, 2021; Lemma et al., 2021). The presence of *S. aureus* in raw milk might be due to clinical or subclinical mastitis or during unhygienic handling and milk processing. It may threaten potential public health hazards for humans (Pyz-Łukasik et al., 2015). Previous studies in dairy cows showed that rearing systems, farm size, poor hygiene during milk handling, and water source for cleaning the milk containers could be associated with the higher prevalence of *S. aureus* in bulk milk (Borena et al., 2023). A high stocking density was observed in the buffalo farms due to inadequate land, and the farms were very closely located to each other, which could increase the chances for transmission of *S. aureus* within herds.

The prevalence of *L. monocytogenes* and *Y. enterocolitica* was high in the buffalo farms but was lower in later milk value chains. Previous studies in dairy cows demonstrated

that the prevalence of *Listeria* monocytogenes was higher in raw bulk milk (19-40 %), which was higher than in traditional milk products such as Yogurt (5 %), butter (1 %), cheese (8 %) (Seyoum et al., 2015; Akrami-Mohajeri et al., 2018). However, no previous study reported the prevalence of *Listeria* spp. in water buffalo. Similarly, *Y. enterocolitica* was also found as prevalent in bulk milk from dairy cows (32-46 %) and water buffalo (25 %) but was low in milk products, e.g., commercial or traditional cheese (0-12 %) and yogurt (0 %) (Rahimi et al., 2014; Darwish et al., 2015). *Listeria* is known as widely distributed in natural environments such as soil and may survive in broader temperatures ranging from 0-45 ºC (Liu et al., 2005). A previous study reported a high prevalence of *L. monocytogenes* (20 %) in dairy silos (Waak et al., 2002). Another study corroborated that poor silage quality and poor animal cleanliness were mainly associated with the exogenous contamination of *L. monocytogenes* in bulk milk (Sanaa et al., 1993). Water buffalo in Bangladesh generally depends on grazing and green roughages with contaminated soil which could be associated with an increased prevalence of *L. monocytogenes* at the farm bulk milk. *Yersinia enterocolitica* was associated with a higher prevalence in the Autumn season in Iran. During autumn, the temperature is neither low nor high, which could provide a better growth requirement for *Y. enterocolitica* (Darwish et al., 2015). However, a previous study reported that *L. monocytogenes* and *Y. enterocolitica* did not grow at a low pH (< 4.5) and temperature ranging from 5-19 ºC (Bhaduri et al., 1995). Processing of traditional buffalo milk products probably increases the acidity of the milk products which alternatively may reduce the contamination by *L. monocytogenes* and *Y. enterocolitica* in buffalo milk (Rahimi et al., 2014; Darwish et al., 2015). To reduce the prevalence at the bulk milk pasteurization of the milk may help reduce the contamination by *L. monocytogenes* and *Y. enterocolitica* in Bangladesh.

Salmonella enterica was hardly found (0-2.9 %), and *C. jejuni* was absent in all the buffalo milk value chain samples. Previous studies in Ethiopia demonstrated the prevalence of *Salmonella* spp varied between 10-20 % in bulk milk and milk collection centers (21 %) in dairy cows (Geletu et al., 2022), which was higher than the prevalence (0-4 %) in buffalo milk in Brazil and India (Godinho et al., 2020; Khan et al., 2022). For optimum growth in milk, *Salmonella* requires 35-37 ºC (El-Gazzar and Marth, 1992). Therefore, the higher prevalence of *Salmonella* is probably related to a high ambient temperature in Ethiopia (ranging from 17 to 37 ºC) which is much lower in Bangladesh

(18-28 ºC) (CCKP, 2021a, b). Prevalence of *C. jejuni* was mainly reported from bulk milk in dairy cows ranging from 3.1 to 20 % in different countries such as., Italy, Tanzania, and Egypt (Serraino et al., 2013; El-Zamkan and Hameed, 2016; Kashoma et al., 2016). However, studies on water buffalo are limited except for Serraino et al. (2013), who reported the absence of *C. jejuni* in water buffalo farms in Italy. Our findings may suggest that raw milk and milk production from the water buffalo milk chain were safe from contamination by *Salmonella enterica* and *C. jejuni* in Bangladesh.

In our study, we found the district was a risk factor, and the bulk milk samples collected from the Bhola district had a high prevalence of *E. coli O157:H7*, *Y. enterocolitica*, and *Listeria monocytogenes* (80-100 %). The district was linked with the buffalo-rearing system. For example, the Bhola district had only a free-ranging system, with a higher prevalence of *E. coli O157:H7* than the intensive system. This makes sense because grazing is the mainstay of free-range farms where milk can be contaminated with soil and dirt during handling. Because of inadequate hygiene procedures during milk handling and insufficient transport facilities in remote areas, longer transit durations were necessary, which may have contributed to increased bacterial contamination in the Bhola district.

Milk container material and drying method was found to be a risk factor for the contamination by *E. coli O157:H7* and *S. aureus* at the middleman and milk product shops. Plastic-made containers had a higher prevalence of *E. coli O157:H7 eae* and *S. aureus*. Plastic milk containers are more complicated to clean than glass or stainless-steel ones, and spoilt milk from the day before can readily contaminate with pathogenic microorganisms (Disassa et al., 2017). The use of cloth was more often related to a higher prevalence of *E. coli O157:H7* than sun drying the container. The cloths used to clean and wipe the containers were unclean and occasionally used by the staff for their own usage, so they could contaminate milk during shipment if overused.

9.6 Conclusions

The prevalence of STEC *E. coli O157:H7* and *S. aureus*, potential human pathogenic organisms, increased along the buffalo milk value chain in Bangladesh. On-farms, however, *Y. enterocolitica* and *L. monocytogenes* were the most common. Pathogenic bacteria in milk can be reduced by replacing plastic milk containers with glass or stainless steel and not using a dirty cloth to clean them. Our findings indicate numerous pathogenic

zoonotic bacteria circulating throughout the buffalo milk value chain, posing an emerging risk to public health safety and necessitating identifying appropriate interventions in future studies.

CHAPTER-10

GENERAL DISCUSSION

CHAPTER 10

GENERAL DISCUSSION

The thesis aimed to assess the udder health and milk quality in water buffalo by studying 1) the prevalence and risk factors of SCM and IMI-causing pathogens and 2) bacterial contamination and the prevalence of zoonotic pathogens in the buffalo milk value chain in Bangladesh. The practical importance of the study findings of this thesis and their use as a means to improve udder health and milk quality will be discussed in this chapter. The chapter will start with a summary of the main results of this thesis.

10.1 Main findings of the thesis

10.1.1 Water buffalo udder health

In previous studies, the risk factors for SCM in water buffalo were largely unknown, with little available information on water buffalo SCM and IMI in Bangladesh (Chapter 4). An important finding of this thesis was the high prevalence of animal and quarter level SCM, with the overall mean BMSCC being satisfactory. A higher prevalence of SCM and BMSCC was observed in intensive farms than in free-range or semi-intensive farms. Funnel-shaped teats and asymmetrical udders were associated with a high prevalence of SCM (Chapter 6). Non-aureus staphylococci were the most prevalent IMI-causing pathogens and showed marked resistance to penicillin (Chapter 5). Poor milking hygiene and cleanliness of the hind quarters were associated with a higher prevalence of NASM. A significant difference between farm IMI and BMSCC indicated room for improvement (Chapter 7). In conclusion, udder health can partly be improved by employing better milking hygiene, animal cleanliness, and controlled breeding in buffalo farms in Bangladesh.

10.1.2 Milk quality

Milk handling operations are diverse in different buffalo-rearing systems. Therefore, similar control measures might not be equally effective. A progressive TBC, TNAS, and TEC increase was observed over the milk value chain. At the farm level, higher bacterial contamination occurred in coastal areas than in river basin areas and when the milk container was cleaned using pond or river water rather than tube-well water. Mixing cow milk with buffalo at the middleman level increased bacterial contamination (Chapter 8).

The overall prevalence of most zoonotic pathogens in milk and milk products was very high. The prevalence of STEC *E. coli O157:H7* and *S. aureus* increased over the milk value chain. On-farms, *Y. enterocolitica,* and *L. monocytogens* were the most prevalent pathogens. Using plastic milk containers compared to glass containers and wiping the milk containers using the same cloth were associated with zoonotic bacterial contamination at the middleman and milk collection center levels (Chapter 9).

10.2 Importance of the findings

10.2.1 Water buffalo udder health

It is known that udder health is challenging in dairy farms as it reduces milk production and affects milk products' overall technological traits (Bobbo et al., 2017; Pegolo et al., 2022). Even after technological improvements and veterinary support, mastitis remains one of the significant critical threats to the dairy industry, affecting animal welfare, increasing the costs associated with treatment and replacement of productive animals, and creating a risk for public health through the dissemination of antimicrobial resistance (Neculai-Valeanu and Ariton, 2022; Ferchiou et al., 2023; Prack McCormick et al., 2023). To make an accurate decision, it is crucial to ensure that the mammary gland of a high-yielding animal should be protected against the possible intrinsic and extrinsic factors predisposing an animal to IMI. Mastitis is a multifactorial disease, and therapeutic management is mainly ineffective (Zigo et al., 2021; Zapata-Salas et al., 2022). Therefore, there is an utmost necessity to identify the risks and initiate control, prevention, and promotion of mastitis by employing udder health monitoring programs. To fulfill this aim, we demonstrated several risk factors for SCM and IMI in water buffalo (Chapters 5-7), which could provide a sound basis for interventions to improve udder health in water buffalo in Bangladesh.

10.2.2 Milk quality

Milk quality can be assessed through nutritional composition, somatic cell count (SCC), and microbial contamination. Buffalo milk is superior to cows' milk because of its higher nutritional value. Particularly buffalo milk possesses higher levels of fat, lactose, proteins, and vitamins, including vitamins A and C. Buffalo milk fat is less stable and has larger fat globules and more saturated milk fatty acids. The protein in Buffalo milk has larger casein micelles and is enriched with minerals (Abd El-Salam and El-Shibiny,

2011). Previous studies have demonstrated that high-SCC milk affects the organoleptic grade of yogurt, coagulation, eye production, and ripening in cheese-making (Li et al., 2014; Moradi et al., 2021). Consumption of raw buffalo milk is becoming more popular among consumers because raw milk is typically thought to be superior in organoleptic properties of milk such as greater taste and flavor, which many feel may be reduced in pasteurized or boiling milk. Milk consumption may contribute to public health risks when milk is consumed in the form of raw milk, mainly if the milk contains potential pathogenic microorganisms for humans (Abebe et al., 2020). Several outbreaks have been reported caused by consuming raw milk (Verraes et al., 2015). Therefore, identification of the risk factors and effective control and prevention measures are necessary to prevent zoonotic pathogens from contaminating the buffalo milk chain in Bangladesh. To meet this need, several possible interventions within the buffalo milk chain may be considered based on our study findings (Chapter 6, 8-9) to improve the BMSCC and reduce the bacterial contamination in milk, which may influence the quality of milk products and ensure the safety of milk consumers.

10.3 Application of the findings

10.3.1 Design of tools to improve milk quality

There is a need to design tools to advise buffalo farmers on improving productivity and milk quality through better feeding, milking and hygiene, animal health, and breeding (Hayashi et al., 2014; Vyas et al., 2020). These tools should support farmers' implementation of evidence-based practices (Derks et al., 2014). Based on our suggested interventions, the buffalo milk value chain could be designed with diagnostic tests, quality control, and incentive programs to enhance product quality and consumer satisfaction and increase the market for buffalo milk in Bangladesh.

10.3.2 Designing the interventions

We identified several problems at the primary production level on farms, mainly related to the lack of knowledge farmers had of buffalo husbandry and health. The issues at the level of the milk chain organization were related primarily to long and poorly managed transport chains and poor quality control at milk collection centers and retail. Altogether, these problems lead to low milk yield per animal and a lack of enthusiasm from farmers to shift from cow to buffalo production. To solve these problems, there is a need for (1)

education of the farmers, (2) better organization of the milk chain, and (3) better buffalo genetics. If addressed together, we expect buffalo production to improve substantially regarding milk yield per animal, the number of animals and farms, and better product quality and food safety.

10.3.3 Implementation of the interventions

A systemic redesign towards improved production and the safety of buffalo milk is also required to implement interventions successfully. One possible way to achieve these transformational changes is "Reflexive interactive design". This approach aims to achieve design goals for sustainable development by addressing complex problems, focusing on the main actors of the system, such as animals, farmers, middlemen, and finally, the consumers (Romera et al., 2020). It also needs to assess the farmers' perceptions towards the interventions to attain the well-being of humans and animals and to reconnect the new system with the rest of society. The socio-cultural and politico-economical structure which leads to the farmers' decisions can be studied using a perception studies approach, such as ethnographic fieldwork and frame analysis (Jackson et al., 2022; Ida et al., 2023). Finally, the interventions may be applied and assessed for successful improvements, which can then be introduced into policy-making to support farmers' uptake of the practices on farms.

10.4 Limitations of the study

10.4.1 Generalizability of the findings

Some results for the prevalence of SCM, IMI, and BMSCC could be less useful shortly, whereas others will be useful. For example, a free-range system may not be sustainable due to the limited availability of fallow pastureland, while semi-intensive systems can extend further. However, these effects on the generalizability of this study's conclusion are probably limited because the maximum number of buffalo farms was included from different buffalo-rearing systems in Bangladesh, representing the actual buffalo population contributing to milk production in the country.

10.4.1.1 Small sample size and reliability of diagnostic tests of zoonotic pathogens

The sample size for the zoonotic pathogens study for the buffalo milk value chain was reasonably low for identifying the risk factors. However, this was the first study to

determine the prevalence of several zoonotic pathogens from Bangladesh's water buffalo milk chain. Some hypotheses on the most critical factors associated with zoonotic pathogen contaminations were generated. Also, the use of PCR in this study may not be able to differentiate between live and dead organisms. However, this is also probably a minor limitation of this study because, in practice, there is a lack of biosafety-ensured laboratory facilities to culture zoonotic pathogens in buffalo-concentrated areas. Therefore, PCR would probably be a more convenient and rapid process to use as a diagnostic test for identifying zoonotic pathogens from buffalo milk and milk products.

10.5 Significance of the study from the broader perspectives

10.5.1 Historical perspectives

Milk is an energy and nutrient-dense food that can help prevent starvation, malnutrition, and stunting. Milk is a biological fluid that is more accessible than any other source of nutrients in developing countries (Prasad and Kothari, 2021; Kh'ng et al., 2022; Cimmino et al., 2023). In Bangladesh, national milk production is insufficient to serve the entire population (DLS, 2022). In this situation, ruminant production is encouraged in response to their capability of transforming low-quality feed into milk. However, cattle have already been exploited in the country through crossbreeding and intensifying the intensive system; therefore, further improvement would create a greater demand for feed resources. On the other hand, buffalo have been an important livestock species in Asia for a long time (Samad, 2020), and buffalo milk production can be exploited, contributing sufficiently to meeting the current milk deficit to ensure food security.

10.5.2 Climate change

Continuous climate change strongly affects low-lying Bangladesh, making the country less fit for dairy cattle production and directly threatening people’s income and food security. Mitigating actions are therefore urgently needed. Due to the resilience of water buffalo to high salt concentrations, the water buffalo is one of the best livestock species for coping with the new situation resulting from climate change (Samad, 2020). However, we should also rethink increasing the livestock population, taking a lesson from many developed countries that are now trying to reduce the detrimental effect of livestock on climate change (Bennetzen et al., 2016). Other alternative food sources are also rising in global interest, including plant-based milk, vegan meals, and laboratory-

grown food replacing animal-derived food (Fresán and Sabaté, 2019; Goma and Phillips, 2021; Tong et al., 2022). However, optimal land use can be met by utilizing livestock and is necessary to protect the ecosystem. We should consider increasing dairy production in a sustainable livestock production system while emphasizing reducing the effect on the climate, e.g., enteric methane emission (Kebede et al., 2014; Carlson et al., 2023).

10.5.3 Problems other than udder health

Buffalo udder health is an important issue to address when attempting to meet milk production demand and ensure the quality of buffalo milk. However, the present study did not cover other research areas needing further research to ensure animal health. Parasitic and other infections, reproductive problems, and nutritional deficiencies remained significant problems for water buffalo health (Kumar, 2009; Harizt et al., 2021). Alongside these, heat and oxidative stress affect immunity and predispose the production and progression of many diseases in water buffalo; they also need future research (Kapila et al., 2018; Yadav et al., 2021).

CHAPTER-11

SUMMARY

CHAPTER 11

SUMMARY

The study aimed to assess the udder health and quality of milk and milk products along Bangladesh's water buffalo milk value chain.

Chapter 1: Includes the general introduction illustrating the scientific gaps and presents the dissertation's aims.

Chapter 2: Review of literature, highlighting the potential for water buffalo population and milk production and current knowledge by scrutinizing the scientific publications.

Chapter 3: Overview of the methods, selection, and surveying of the study area, animal selection, sampling, and laboratory examination, followed by the statistical analysis.

Chapter 4: Prevalence, etiology, and risk factors for subclinical mastitis in water buffalo (*Bubalus bubalis*): A systematic review and meta-analysis.

A systematic review and meta-analysis were performed to determine the quarter level pooled prevalence of SCM in water buffalo, the distribution of pathogens causing IMI, and associations between herd, animal, and quarter-related risk factors and the prevalence of SCM. Publications on SCM in water buffalo were retrieved from four electronic databases (PubMed, Scopus, Embase, and Web of Science). A total of 53 publications were deemed eligible for the study, providing data on 8,958 buffalo and 34,125 quarters. The pooled prevalence of SCM was 21 % (95 % CI 16-26 %) at the quarter level. *S. aureus* was the most frequently reported bacterial species causing IMI (23 %; 95 % CI 14-33 %), followed by *E. coli* (13 %; 95 % CI 8-19 %). SCM risk factors in water buffalo were assessed by aggregating data from publications in the systematic review. This showed that soil-based flooring, the absence of dry cow therapy, a young age, early lactation stage, and bottle-shaped teats are associated with increased SCM prevalence. However, risk factors for SCM in water buffalo are primarily unknown, indicating a need for further studies. This systematic review and meta-analysis revealed that SCM is a significant disease affecting the water buffalo population worldwide. This suggests a need for wide-scale implementation of management practices based on scientific evidence to achieve adequate disease control. However, this field has a sizeable global knowledge gap, as the risk factors for SCM in water buffalo are mainly unknown. Therefore, studies are needed to identify risk factors for SCM in water buffalo.

Chapter 5: Occurrence and etiology of subclinical mastitis in water buffalo in Bangladesh: A Pilot study.

A pilot study was conducted on sixteen buffalo farms in Bangladesh's Bagerhat and Noakhali regions, and a bulk milk sample was collected from each farm. In addition, 299 udder quarter milk samples were collected from 76 animals. The farm bulk milk samples were assessed using direct SCC, and the quarter milk samples using CMT. The occurrence of SCM calculated at the quarter and animal levels was 42.5 % and 81.6 %, respectively. Milk samples from 108 CMT-positive quarters in 48 animals and 38 randomly selected CMT-negative quarters in 24 animals were investigated using bacteriological culture. Estimated mean bulk milk SCC was 195,000 cells per mL of milk. The estimated quarter level intramammary infection (IMI) on culture was 40.4 %. The identity of isolated bacteria was confirmed using MALDI-TOF mass spectrometry. *Non-aureus staphylococci* were the most common pathogens (24.7 %); among 36 NAS tested, 36.1 % were resistant to penicillin. Thus, SCM was high on the study farms, with relatively high penicillin resistance in NAS.

Chapter 6: Prevalence and risk factors for subclinical mastitis in water buffalo (*Bubalis bubalis*) in Bangladesh.

A nationwide cross-sectional study was carried out to estimate the prevalence and the associated risk factors of SCM in water buffalo. The study represented five rearing systems: free-range, semi-free-range, household, semi-intensive, and intensive, providing 3,491 functional quarters from 880 lactating buffalo on 248 farms. The CMT score was used to identify SCM. Bulk milk samples (n = 242) were used for farm level BMSCC. Quarter and buffalo-level risk factors for SCM were measured using questionnaires and observations. The overall SCM prevalence was high, at 27.9 % at the quarter level and 51.5 % at the buffalo level. The geometric mean BMSCC was 217,000 cells per mL of milk, which is low on average, but some farms could improve substantially. The buffalo-rearing system, udder location (left versus right), teat shape, udder asymmetry, number of milkers, and presence of a quarantine facility were all associated with buffalo udder health. Our findings suggest that mainly using free-range rearing systems may help decrease the prevalence of SCM. This would also be supported by improving buffalo breeding and better farm biosecurity. Udder health control strategies can be designed based on our findings. Our findings indicate that the education

of farmers regarding milking technique, hygiene, and rearing system may help further improve udder health on buffalo farms in Bangladesh.

Chapter 7: Pathogen group-specific risk factors for intramammary infection in water buffalo.

A cross-sectional study was conducted to estimate the prevalence of IMI-associated bacteria and identify risk factors for pathogen group-specific IMI in water buffalo in Bangladesh. Bacteriological cultures were performed on 1,374 quarter milk samples collected from 763 water buffalo in 244 buffalo farms in nine districts in Bangladesh. A total of 618 quarters of samples were culture positive. Non-*aureus* staphylococci were the predominant IMI-associated bacteria, and *S. chromogenes*, *S. hyicus*, and *S. epidermidis* were the most common species. The proportion of Non-aureus staphylococci or *Mammalliococcus* spp. (NASM), *S. aureus* and other bacterial species identified in the buffalo quarter samples varied between farms. Risk factors associated with IMI were milking hygiene and cleanliness of the hind quarters. The odds of IMI from any pathogen and NASM were higher in buffalo herds with poor milking hygiene. Poor cleanliness of the hind quarters had a higher likelihood of IMI caused by any pathogen, NASM, and *S. aureus*. Farm management-related risk factors were the frequency of milking and buffalo source. Twice daily milking and farms with buffalo purchased from another herd were associated with IMI from any pathogen. Udder symmetry and BCS were risk factors related to buffalo breeding and nutrition. The asymmetrical udder was associated with IMI caused by any bacteria and with IMI caused by *S. aureus*. Poor BCS showed higher odds of IMI from any pathogen and NASM. This study shows that the prevalence of IMI in water buffalo is high. Still, variables between farms and our data suggest that SCM can be partly controlled through better farm management, primarily by improving hygiene, milking management, breeding, and nutrition. Several udder health problems related to IMI in water buffalo have been identified in this study. Our data suggest that IMI can be partly managed through better farm management, including milking technique, hygiene, and breeding, which can be used to design water buffalo udder health control strategies.

Chapter 8: Water buffalo milk chain and associated factors affecting bulk milk somatic cell count and bacterial counts in Bangladesh.

This study aims to describe the milk hygiene parameters and chain characteristics of unpasteurized raw milk sold to consumers to improve milk hygiene. A quantitative study design evaluated somatic cell counts, total bacterial counts, and specific gram-negative (Enterobacteria) and gram-positive (Staphylococcus) pathogens in 377 aseptically collected milk samples. Samples were collected at multiple nodes along the buffalo milk value chain: 122 bulk tank milk samples were collected at the farm level, 109 milk samples at the middlemen level, and 111 milk samples at the milk collection centers. In addition, 35 samples were taken from various milk products at the retail level. It was found that progressively increasing somatic cell counts and bacterial counts, including potential pathogens, occurred along the milk chain. A seasonal increase in spring was found, varying based on the farming system (semi-intensive versus intensive). Other factors included water purity and cleanliness of containers, mixing buffalo and cow's milk, and the location of the water buffalo milk producer (coastal or river basin). This study demonstrated how improving udder health and milk hygiene along the water buffalo milk value chain would increase the safety and quality of water buffalo milk in the study area.

Chapter 9: The prevalence of foodborne zoonotic pathogens in milk and milk products along Bangladesh's buffalo milk value chain.

This study aimed to estimate the prevalence of zoonotic pathogens in milk and milk products and identify the associated factors in the buffalo milk chain nodes in Bangladesh. One hundred and forty-three samples were collected from farm bulk milk (n = 34), middlemen (n = 37), milk collection centers (n = 37), and milk product shops (n = 35). They were analyzed using RT-PCR to estimate the prevalence of seven important zoonotic pathogens: *S. aureus*, *E. coli*, Shiga toxin-producing *E. coli O157:H7*, *C. jejuni*, *L. monocytogenes*, *S. enterica*, and *Y. enterocolitica*. *Escherichia coli* was the most prevalent pathogen along the milk chain nodes. The prevalence of the pathogens was high along the buffalo milk value chain. *E. coli O157:H7* classical enterotoxin encoded genes (e.g., the *stx2* genotype) were most prevalent in milk products, compared to earlier in the milk value chain (74 %). *L. monocytogenes* and *Y. enterocolitica* were more prevalent in the farms (65-79 %) than in the later milk value chain nodes. The prevalence of *S. enterica* was relatively low (0-2.9 %) in all the milk chain nodes, and all the samples were negative for *C. jejuni.* These results suggest that buffalo milk consumers in Bangladesh are at risk from *L. monocytogenes* and *Y. enterocolitica* in the

farms and *E. coli O157:H7* with a *stx2* genotype at the milk product shops. *S. enterica* and *C. jejuni* are not frequent contaminants in the buffalo milk chain in Bangladesh. Avoiding plastic-made containers and dirty clothes used for cleaning milk containers may help reduce contamination in the buffalo milk value chain.

CHAPTER-12

CONCLUSIONS

CHAPTER 12

CONCLUSIONS

This study aimed to improve Bangladesh's buffalo milk value chain by studying udder health and quality. The main conclusions of the study are as follows:

- The prevalence of SCM was high (animal-52 % and quarter-28 %), with free-range or semi-intensive buffalo-rearing systems associated with less SCM compared to intensive systems. The prevalence of SCM can partly be controlled through a breeding program focusing on improving teat shape and udder symmetry (Chapters 5 and 6).
- Overall, BMSCC was satisfactory, but there was a significant variation between farms; therefore, there is room for improvement (Chapter 6).
- The prevalence of IMI was high, with NASM as the most common bacteria. Improved milking hygiene and cleanliness of the hind quarter could help reduce IMI (Chapter 7).
- Milk handling operations are diverse in different rearing systems, and one control measure may not fit all systems (Chapter 8).
- A progressive increase in TBC and TEC was seen in the milk value chain. Better management on farms, such as using tube-well water to clean milk containers, and better milk handling at the middleman stage, such as milk transportation with a cooling facility and cleaning of milk containers, could reduce bacterial contamination (Chapter 8).
- The overall prevalence of most pathogens in milk and milk products was very high. Improved milk handling at milk collection centers and shops should be promoted, and using the same cloth to clean the container and using plastic milk containers for carrying milk should be avoided (Chapter 9).

FURTHER RESEARCH OPPORTUNITIES

This thesis work aims to explore the opportunities for an increase in water buffalo milk production by improving udder health and improvement of milk quality. This could help develop a safe, sustainable, resilient, and profitable water buffalo milk chain in Bangladesh. However, now the evidence-based advice must be implemented by the farmer's education and qualitative and quantitative estimation of effectiveness in practice in the next step. Scopes for future studies remain open to initiate a participatory approach where together with farmers and other key actors along the milk chain, (1) identify key factors that currently limit improvements to animal health and milk quality in the buffalo-rearing and milk value chain and (2) jointly agree on ways to address these factors. Subsequently, this will together evaluate the tested strategies for addressing the challenges. Figure 12.1 shows that interventions directed at the farmers should lead to changes in farmers' behavior and better animal health, leading to better milk quality. Intervention at the milk chain level should lead to better milk chain quality, product quality, and consumer satisfaction.

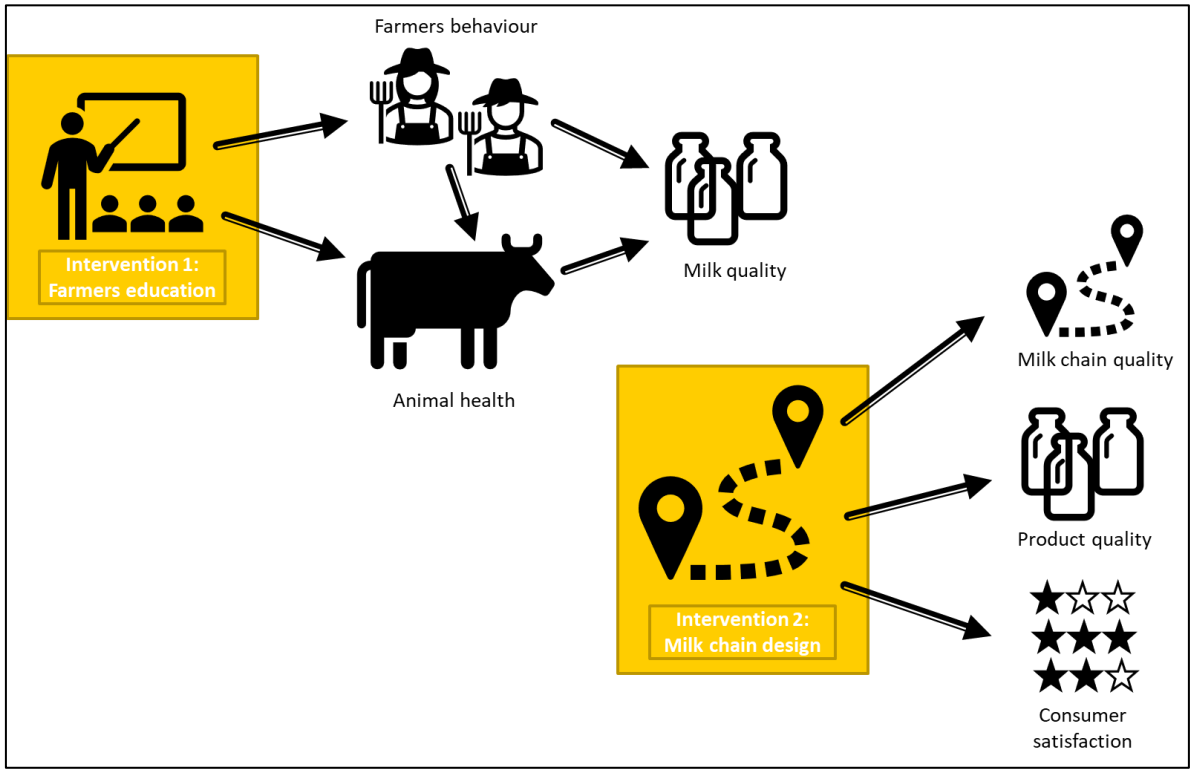


Fig. 12.1 Outline of the two interventions, intervention 1 being farmers' education and intervention 2 being the redesign of the milk chain

REFERENCES

Abd-Elrahman AH 2013: Mastitis in housed dairy buffalo: incidence, etiology, clinical finding, antimicrobial sensitivity and different medical treatment against *E. coli* mastitis. *Life Science Journal* **10** 532-538.

Abd Al-Galil A, Khalil A 2016: Ultrasonographic diagnosis of subclinical mastitis in buffalo (*Bubalus bubalis*). *Research Opinions in Animal and Veterinary Sciences* **6** 261-267. http://dx.doi.org/10.20490/ROAVS/16-044.

Abd El-Salam MH, El-Shibiny S 2011: A comprehensive review on the composition and properties of buffalo milk. *Dairy Science and Technology* **91** 663-699. http://dx.doi.org/10.1007/s13594-011-0029-2.

Abebe E, Gugsa G, Ahmed M 2020: Review on major food-borne zoonotic bacterial pathogens. *Journal of Tropical Medicine* **2020** 4674235. http://dx.doi.org/10.1155/2020/4674235.

Abu-seida A, Kotb E, Fadel M 2014: The correlation between ultrasonographic and laboratory findings of mastitis in buffalo (*Bubalus Bubalis*). *Global Veterinaria* **13** 68-74. http://dx.doi.org/10.5829/idosi.gv.2014.13.01.84171.

Addis M, Sisay D 2015: A review on major food borne bacterial illnesses. *Journal of Tropical Disease and Public Health* **3** 1-7.

Addis MF, Maffioli EM, Penati M, Albertini M, Bronzo V, Piccinini R, Tangorra F, Tedeschi G, Cappelli G, Di Vuolo G, Vecchio D, De Carlo E, Ceciliani F 2022: Peptidomic changes in the milk of water buffalo (*Bubalus bubalis*) with intramammary infection by non-aureus staphylococci. *Scientific Reports* **12** 8371. http://dx.doi.org/10.1038/s41598-022-12297-z.

Addis MF, Tanca A, Uzzau S, Oikonomou G, Bicalho RC, Moroni P 2016: The bovine milk microbiota: insights and perspectives from omics studies. *Molecular Biosystems* **12** 2359-2372. http://dx.doi.org/10.1039/c6mb00217j.

Adesogan AT, Dahl GE 2020: MILK Symposium Introduction: Dairy production in developing countries. *Journal of Dairy Science* **103** 9677-9680. http://dx.doi.org/10.3168/jds.2020-18313.

Adkins PRF, Middleton JR, Fox LK, Pighetti G, Petersson-Wolfe C 2017a: Somatic cell count: *Laboratory handbook on bovine mastitis*. 2nd Edition. National Mastitis Council, New Prague, MN 56071 USA. 88-93.

Adkins PRF, Middleton JR, Fox LK, Pighetti G, Petersson-Wolfe C 2017b: Diagnostic equipment and materials: *Laboratory Handbook on Bovine Mastitis*. 2nd Edition. National Mastitis Council, New Prague, MN 56071 USA. 13-23.

Adkins PRF, Middleton JR, Fox LK, Pighetti G, Petersson-Wolfe C 2017c: Sample collection and handling: *Laboratory handbook on bovine mastitis*. 2nd Edition. National Mastitis Council, New Prague, MN 56071 USA. 7-12.

Ahmad M, Bhatti JA, Abdullah M, Ullah R, Ul Ain Q, Hasni MS, Mahboob A, Rashid A, Qaisar I, Rashid G, Uddin R 2019: Different ambient management intervention techniques and their effect on milk production and physiological parameters of lactating NiliRavi buffalo during hot dry summer of subtropical region. *Tropical Animal Health and Production* **51** 911-918. http://dx.doi.org/10.1007/s11250-018-1774-5.

Ahmad R 2001: Studies on mastitis among dairy buffalo. *Pakistan Veterinary Journal* **21** 220-221.

Ahmed H, Straubinger R, Hegazy Y, Ibrahim S 2018: Subclinical mastitis in dairy cattle and buffalo among small holders in Egypt: Prevalence and evidence of virulence of *Escherichia coli* causative agent. *Tropical Biomedicine* **35** 321-329.

Akrami-Mohajeri F, Derakhshan Z, Ferrante M, Hamidiyan N, Soleymani M, Conti GO, Tafti RD 2018: The prevalence and antimicrobial resistance of *Listeria* spp in raw milk and traditional dairy products delivered in Yazd, central Iran (2016). *Food and Chemical Toxicology* **114** 141-144. http://dx.doi.org/10.1016/j.fct.2018.02.006.

Al-Rudha AM, Khalil NK, Altaai NA 2021: Evaluation of bacterial contaminants and heavy metals in cow and buffalo raw milk sold in Baghdad governorate. *Iraqi Journal of Veterinary Sciences* **35** 101-105. http://dx.doi.org/10.33899/ijvs.2021.131744.1999.

AL-Saadi BQH, AL-Shakh MAA 2015: Some physiological factors influence somatic cell counts and milk contents associated with mastitis in Iraqi buffalo. *World Journal of Pharmaceutical Research* **4** 214-224.

Aldujaily A, Al-Alo K, Mohmoud M 2019: Hematology, bacteriology and antibiotic resistance in milk of water buffalo with subclinical mastitis. *Veterinary Research* **23** 1-8.

Alexander KA, Carlson CJ, Lewis BL, Getz WM, Marathe MV, Eubank SG, Sanderson CE, Blackburn JK 2018: The ecology of pathogen spillover and disease emergence at the human-wildlife-environment interface: *The Connections Between Ecology and Infectious Disease*. Springer International Publishing, Gewerbestrasse 11, 6330 Cham, Switzerland.

Algammal AM, Enany ME, El-Tarabili RM, Ghobashy MO, Helmy YA 2020: Prevalence, antimicrobial resistance profiles, virulence and enterotoxin-determinant genes of MRSA isolated from subclinical bovine mastitis samples in Egypt. *Pathogens* **9** 362.

Alghizzi M, Shami A 2021: The prevalence of *Staphylococcus aureus* and methicillin resistant *Staphylococcus aureus* in milk and dairy products in Riyadh, Saudi Arabia. *Saudi Journal of Biological Sciences* **28** 7098-7104. http://dx.doi.org/10.1016/j.sjbs.2021.08.004.

Alhussien MN, Dang AK 2018: Milk somatic cells, factors influencing their release, future prospects, and practical utility in dairy animals: An overview. *Veterinary World* **11** 562-577. http://dx.doi.org/10.14202/vetworld.2018.562-577.

Ali M, Ahmad M, Muhammad K, Anjum A 2011: Prevalence of sub clinical mastitis in dairy buffalo of Punjab, Pakistan. *Journal of Animal and Plant Sciences* **21** 477-480.

Ali T, Rahman A, Qureshi MS, Hussain MT, Khan MS, Uddin S, Iqbal M, Han B 2014: Effect of management practices and animal age on incidence of mastitis in Nili Ravi buffalo. *Tropical Animal Health and Production* **46** 1279-1285. http://dx.doi.org/10.1007/s11250-014-0641-2.

Aliul H, Paul A, Mahmood R, Mizanur R, Ahmed S 2021: Investigation of prevalence and risk factors of subclinical mastitis of dairy buffalo at Bhola district of Bangladesh. *Asian Journal of Medical and Biological Research* **6** 697-704.

Aliyo A, Seyoum A, Teklemariam Z 2022: Bacteriological quality and antimicrobial susceptibility patterns among raw milk producers and vendors in Gomole district, Borena zone, Southern Ethiopia. *Infection and Drug Resistance* **15** 2589-2602. http://dx.doi.org/10.2147/idr.S364578.

Aliyo A, Teklemariam Z 2022: Assessment of milk contamination, associated risk factors, and drug sensitivity patterns among isolated bacteria from raw milk of Borena zone, Ethiopia. *Journal of Tropical Medicine* **2022** 1-9. http://dx.doi.org/10.1155/2022/3577715.

Anderson M, Hinds P, Hurditt S, Miller P, McGrowder D, Alexander-Lindo R 2011: The microbial content of unexpired pasteurized milk from selected supermarkets in a developing country. *Asian Pacific Journal of Tropical Biomedicine* **1** 205-211. http://dx.doi.org/10.1016/s2221-1691(11)60028-2.

Anirban G, Sandeep G, Anshu S 2010: Assessment of chemical and electrolyte profile as an indicator of subclinical mastitis in riverine buffalo (*Bubalus bubalis*). *Haryana Veterinarian* **49** 19-21.

Anirban G, Sandeep G, Anshu S 2013: Evaluation of milk biochemical constituents as indicators of subclinical mastitis in Murrah buffalo (*Bubalus bubalis*). *Buffalo Journal* **29** 51-58.

Aragão BB, Trajano SC, de Oliveira RP, Sobral da Silva DM, de Carvalho RG, Juliano MA, Pinheiro Junior JW, Mota RA 2021: Multiresistant zoonotic pathogens isolated from goat milk in Northeastern Brazil. *Comparative Immunology, Microbiology and Infectious Diseases* **79** 101701. http://dx.doi.org/https://doi.org/10.1016/j.cimid.2021.101701.

Artursson K, Schelin J, Thisted Lambertz S, Hansson I, Olsson Engvall E 2018: Foodborne pathogens in unpasteurized milk in Sweden. *International Journal of Food Microbiology* **284** 120-127. http://dx.doi.org/10.1016/j.ijfoodmicro.2018.05.015.

Assefa A, Bihon A 2018: A systematic review and meta-analysis of prevalence of *Escherichia coli* in foods of animal origin in Ethiopia. *Heliyon* **4** e00716. http://dx.doi.org/10.1016/j.heliyon.2018.e00716.

Awad N 2019: Prevalence, etiology and antibiogram of mastitis in buffalo in Sohag governorate. *Assiut Veterinary Medical Journal* **65** 124-134. http://dx.doi.org/10.21608/avmj.2019.169201.

Awadallah MA, Ahmed HA, Merwad AM, Selim MA 2016: Occurrence, genotyping, shiga toxin genes and associated risk factors of *E. coli* isolated from dairy farms, handlers and milk consumers. *The Veterinary Journal* **217** 83-88. http://dx.doi.org/10.1016/j.tvjl.2016.09.014.

Bachaya H, Iqbal Z, Muhammad G, Yousaf A, Ali H 2005: Subclinical mastitis in buffalo in Attock district of Punjab (Pakistan). *Pakistan Veterinary Journal* **25** 134.

Badua AT, Boonyayatra S, Awaiwanont N, Gaban PBV, Mingala CN 2020: Methicillin-resistant *Staphylococcus aureus* (MRSA) associated with mastitis among water

buffalo in the Philippines. *Heliyon* **6** e05663. http://dx.doi.org/10.1016/j.heliyon.2020.e05663.

Balemi A, Gumi B, Amenu K, Girma S, Gebru M, Tekle M, Ríus AA, D'Souza DH, Agga GE, Kerro Dego O 2021: Prevalence of mastitis and antibiotic resistance of bacterial isolates from CMT positive milk samples obtained from dairy cows, camels, and goats in two pastoral districts in southern Ethiopia. *Animals* **11** 1530. http://dx.doi.org/10.3390/ani11061530.

Baloch H, Rind R, Rind MR, Kumar V, Baloch N, Oad RK 2018: Effect of diverse factors on the frequency of clinical and subclinical mastitis in Kundhi buffalo of Sindh, Pakistan. *Pakistan Journal of Zoology* **50** 1913-1920.

Baloch H, Rind R, Umerani AP, Bhutto AL, Abro SH, Rind MR, Abro R, Rizwana H, Kamboh AA, Baloch AK 2016: Prevalence and risk factors associated with sub-clinical mastitis in Kundhi buffalo. *Journal of Basic and Applied Sciences* **12** 301-305.

Bansal BK, Hamann J, Lind O, Singh ST, Dhaliwal PS 2007: Somatic cell count and biochemical components of milk related to udder health in buffalo. *Italian Journal of Animal Science* **6** 1035-1038. http://dx.doi.org/10.4081/ijas.2007.s2.1035.

Banu MG, Zewdu Geberemedhin E 2022: Occurrence and antimicrobial susceptibility of *Staphylococcus aureus* in dairy farms and personnel in selected towns of West Shewa Zone, Oromia, Ethiopia. *PLoS One* **17** e0277805. http://dx.doi.org/10.1371/journal.pone.0277805.

Bari MS, Rahman MM, Persson Y, Derks M, Sayeed MA, Hossain D, Singha S, Hoque MA, Sivaraman S, Fernando P, Ahmad I, Samad A, Koop G 2022: Subclinical mastitis in dairy cows in south-Asian countries: a review of risk factors and etiology to prioritize control measures. *Veterinary Research Communications* **46** 621-640. http://dx.doi.org/10.1007/s11259-022-09948-x.

Barkema HW, De Vliegher S, Piepers S, Zadoks RN 2013: Herd level approach to high bulk milk somatic cell count problems in dairy cattle. *Veterinary Quarterly* **33** 82-93. http://dx.doi.org/10.1080/01652176.2013.799791.

Barkema HW, Schukken YH, Lam TJ, Beiboer ML, Wilmink H, Benedictus G, Brand A 1998: Incidence of clinical mastitis in dairy herds grouped in three categories by bulk milk somatic cell counts. *Journal of Dairy Science* **81** 411-419. http://dx.doi.org/10.3168/jds.S0022-0302(98)75591-2.

Barnum DA, Newbould FH 1961: The use of the California mastitis test for the detection of bovine mastitis. *Canadian Veterinary Journal* **2** 83-90.

Bates D, Mächler M, Bolker B, Walker S 2015: Fitting linear mixed-effects models using lme4. *Journal of Statistical Software* **67** 1 - 48. http://dx.doi.org/10.18637/jss.v067.i01.

Bauer MS, Damschroder L, Hagedorn H, Smith J, Kilbourne AM 2015: An introduction to implementation science for the non-specialist. *BMC Psychology* **3** 32. http://dx.doi.org/10.1186/s40359-015-0089-9.

Bauman CA, Barkema HW, Dubuc J, Keefe GP, Kelton DF 2018: Canadian National Dairy Study: Herd-level milk quality. *Journal of Dairy Science* **101** 2679-2691. http://dx.doi.org/10.3168/jds.2017-13336.

Bauzad M, Yuliati FN, Prahesti KI, Malaka R 2019: Total plate count and *Escherichia coli* in raw buffalo milk in Curio district Enrekang Regency: *Earth and Environmental Science*. IOP Publishing, 1-7.

Beheshti R, Eshratkhah B, Shayegh J, Ghalehkandi JG, Dianat V, Valiei K 2011: Prevalence and etiology of subclinical mastitis in buffalo of the Tabriz region, Iran. *American Journal of Science* **7** 642-645.

Bennetzen EH, Smith P, Porter JR 2016: Decoupling of greenhouse gas emissions from global agricultural production: 1970-2050. *Global Change Biology* **22** 763-781. http://dx.doi.org/10.1111/gcb.13120.

Bergonier D, de Crémoux R, Rupp R, Lagriffoul G, Berthelot X 2003: Mastitis of dairy small ruminants. *Veterinary Research* **34** 689-716. http://dx.doi.org/10.1051/vetres:2003030.

Berhanu L, Gume B, Kassa T, Dadi LS, Tegegne D, Getnet M, Bediru H, Getaneh A, Suleman S, Mereta ST 2021: Microbial quality of raw cow milk and its predictors along the dairy value chain in Southwest Ethiopia. *International Journal of Food Microbiology* **350** 109228. http://dx.doi.org/10.1016/j.ijfoodmicro.2021.109228.

Berhe G, Wasihun AG, Kassaye E, Gebreselasie K 2020: Milk-borne bacterial health hazards in milk produced for commercial purpose in Tigray, northern Ethiopia. *BMC Public Health* **20** 894. http://dx.doi.org/10.1186/s12889-020-09016-6.

Beuckelaere L, De Visscher A, Souza FN, Meyer E, Haesebrouck F, Piepers S, De Vliegher S 2021: Colonization and local host response following intramammary *Staphylococcus chromogenes* challenge in dry cows. *Veterinary Research* **52** 137. http://dx.doi.org/10.1186/s13567-021-01007-8.

Bhaduri S, Buchanan RL, Phillips JG 1995: Expanded response surface model for predicting the effects of temperatures, pH, sodium chloride contents and sodium nitrite concentrations on the growth rate of *Yersinia enterocolitica*. *Journal of Applied Bacteriology* **79** 163-170. http://dx.doi.org/10.1111/j.1365-2672.1995.tb00930.x.

Bhandari S, Subedi D, Tiwari BB, Shrestha P, Shah S, Al-Mustapha AI 2021: Prevalence and risk factors for multidrug-resistant Escherichia coli isolated from subclinical mastitis in the western Chitwan region of Nepal. *Journal of Dairy Science* **104** 12765-12772. http://dx.doi.org/10.3168/jds.2020-19480.

Bhanot V, Chaudhri SS, Bisla RS, Singh H 2012: Retrospective study on prevalence and antibiogram of mastitis in cows and buffalo of eastern Haryana. *Indian Journal of Animal Research* **46** 160-163.

Bhutia PS, Bansal BK, Gupta DK, Singh RS, Uppal SK 2019: Bacterial isolation of milk samples submitted from clinical mastitis buffalo during 2007 to 2016. *Tropical Animal Health and Production* **51** 1551-1557. http://dx.doi.org/10.1007/s11250-019-01846-w.

Bilal M, Iqbal M, Muhammad G, Avais M, Sajid M 2004: Factors affecting the prevalence of clinical mastitis in buffalo around Faisalabad district (Pakistan). *International Journal of Agriculture And Biology* **6**

Biswas D, Hanif sM, Rana A, Anower AKM 2020: A study on udder health management practices, reproductive disorders and subclinical mastitis in buffalo herds in coastal region of Bangladesh. *Turkish Journal of Agriculture-Food Science and*

Technology **8** 1662-1667. http://dx.doi.org/10.24925/turjaf.v8i8.1662-1667.3416.

Blagitz MG, Souza FN, Batista CF, Diniz SA, Azevedo LFF, Silva MX, Haddad JPA, Heinemann MB, Cerqueira MMOP, Della Libera AMMP 2015: Flow cytometric analysis: Interdependence of healthy and infected udder quarters. *Journal of Dairy Science* **98** 2401-2408. http://dx.doi.org/https://doi.org/10.3168/jds.2014-8727.

Bobbo T, Ruegg PL, Stocco G, Fiore E, Gianesella M, Morgante M, Pasotto D, Bittante G, Cecchinato A 2017: Associations between pathogen-specific cases of subclinical mastitis and milk yield, quality, protein composition, and cheese-making traits in dairy cows. *Journal of Dairy Science* **100** 4868-4883. http://dx.doi.org/10.3168/jds.2016-12353.

Bombade K, Kamboj A, Alhussien MN, Mohanty AK, Dang AK 2018: Diurnal variation of milk somatic and differential leukocyte counts of Murrah buffalo as influenced by different milk fractions, seasons and parities. *Biological Rhythm Research* **49** 151-163. http://dx.doi.org/10.1080/09291016.2017.1345472.

Borena BM, Gurmessa FT, Gebremedhin EZ, Sarba EJ, Marami LM 2023: *Staphylococcus aureus* in cow milk and milk products in Ambo and Bako towns, Oromia, Ethiopia: prevalence, associated risk factors, hygienic quality, and antibiogram. *International Journal of Microbiology* http://dx.doi.org/10.1007/s10123-022-00317-x.

Borghese A 2005: *Buffalo production and research*. Food and Agriculture Organization (FAO), Rome, Italy. 67.

Borriello G, Lucibelli MG, De Carlo E, Auriemma C, Cozza D, Ascione G, Scognamiglio F, Iovane G, Galiero G 2012: Characterization of enterotoxigenic *E. coli* (ETEC), Shiga-toxin producing *E. coli* (STEC) and necrotoxigenic *E. coli* (NTEC) isolated from diarrhoeic Mediterranean water buffalo calves (*Bubalus bubalis*). *Research in Veterinary Science* **93** 18-22. http://dx.doi.org/10.1016/j.rvsc.2011.05.009.

Boss R, Cosandey A, Luini M, Artursson K, Bardiau M, Breitenwieser F, Hehenberger E, Lam T, Mansfeld M, Michel A, Mösslacher G, Naskova J, Nelson S, Podpečan O, Raemy A, Ryan E, Salat O, Zangerl P, Steiner A, Graber HU 2016: Bovine *Staphylococcus aureus*: Subtyping, evolution, and zoonotic transfer. *Journal of Dairy Science* **99** 515-528. http://dx.doi.org/10.3168/jds.2015-9589.

Bulla T, Rana Y, Sharma A, Beniwal B 2006: Prevalence of subclinical mastitis in Murrah buffalo. *Haryana Veterinarian* **45** 53-56.

Bustin SA, Benes V, Garson JA, Hellemans J, Huggett J, Kubista M, Mueller R, Nolan T, Pfaffl MW, Shipley GL, Vandesompele J, Wittwer CT 2009: The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. *Clinical Chemistry* **55** 611-622. http://dx.doi.org/10.1373/clinchem.2008.112797.

Caine LA, Nwodo UU, Okoh AI, Ndip RN, Green E 2014: Occurrence of virulence genes associated with diarrheagenic *Escherichia coli i*solated from raw cow's milk from two commercial dairy farms in the Eastern Cape Province, South Africa. *International Journal of Environmental Research and Public Health* **11** 11950-11963. http://dx.doi.org/10.3390/ijerph111111950.

Cameron M, Barkema HW, De Buck J, De Vliegher S, Chaffer M, Lewis J, Keefe GP 2017: Identification of bovine-associated coagulase-negative staphylococci by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry using a direct transfer protocol. *Journal of Dairy Science* **100** 2137-2147. http://dx.doi.org/10.3168/jds.2016-12020.

Cappelli G, Di Vuolo G, Gerini O, Noschese R, Bufano F, Capacchione R, Rosini S, Limone A, De Carlo E 2021: Italian tracing system for water buffalo milk and processed milk products. *Animals* **11** http://dx.doi.org/10.3390/ani11061737.

Carlson ZE, McPhillips LJ, Stowell RR, Erickson GE, Drewnoski M, MacDonald JC 2023: Evaluation of growth performance, carcass characteristics, and methane and CO2 emissions of growing and finishing cattle raised in extensive or partial-intensive cow-calf production systems. *Journal of Animal Science* **101** http://dx.doi.org/10.1093/jas/skac368.

Caruso C, Prato R, Ingravalle F, Vecchio D, Sciarra A, Ternavasio M, Ceccarelli L, Martucciello A, Galiero G, De Carlo E, Masoero L 2016: Prevalence of antibodies against Bubaline herpesvirus (BuHV-1) among Mediterranean water buffalo (*Bubalus bubalis*) with implications in buffalo trade. *Veterinary Quarterly* **36** 184-188. http://dx.doi.org/10.1080/01652176.2016.1205236.

Catozzi C, Cuscó A, Lecchi C, Talenti A, Martucciello A, Cappelli G, Bonastre AS, Francino O, Ceciliani F 2019: Short communication: Intra- and inter-individual milk microbiota variability in healthy and infected water buffalo udder quarters. *Journal of Dairy Science* **102** 7476-7482. http://dx.doi.org/10.3168/jds.2019-16352.

Catozzi C, Sanchez Bonastre A, Francino O, Lecchi C, De Carlo E, Vecchio D, Martucciello A, Fraulo P, Bronzo V, Cuscó A, D'Andreano S, Ceciliani F 2017: The microbiota of water buffalo milk during mastitis. *PLoS One* **12** e0184710. http://dx.doi.org/10.1371/journal.pone.0184710.

CCKP 2021a: Monthly climatology of mean-temperature and precipation in Bangladesh from 1991-2020. Climate Change Knowledge Portal, Accessed on 06 March 2023. https://climateknowledgeportal.worldbank.org/country/bangladesh.

CCKP 2021b: Monthly climatology of mean-temperature and precipation in Ethiopia from 1991-2020. Climate change knowledge portal, Accessed on 06 March 2023. https://www.climatestotravel.com/climate/ethiopia.

CDC 2020: Foodborne germs and illnesses. National center for emerging and zoonotic infectious diseases (NCEZID), Division of foodborne, waterborne, and environmental diseases (DFWED), Centers for Disease Control and Phererevention, *1600 Clifton Road Atlanta, GA 30329-4027 USA*. Accessed on 12 August 2022. https://www.cdc.gov/foodsafety/foodborne-germs.html.

CDC 2021: Zoonotic diseases. National center for emerging and zoonotic infectious diseases (NCEZID), Division of foodborne, waterborne, and environmental diseases (DFWED), Centers for Disease Control and Phererevention, *1600 Clifton Road Atlanta, GA 30329-4027 USA*. Accessed on 12 August 2022. https://www.cdc.gov/onehealth/basics/zoonotic-diseases.html.

Ceciliani F, Audano M, Addis MF, Mitro N, Lecchi C, Ghaffari MH, Albertini M, De Carlo E, Vecchio D, Di Vuolo G, Cappelli G, Tangorra F, Piccinini R, Bronzo V, Caruso D 2022: Changes in the lipidome of water buffalo milk during

intramammary infection by non-aureus Staphylococci. *Scientific Reports* **12** 9665. http://dx.doi.org/10.1038/s41598-022-13400-0.

Census 2010: Census of Agriculture 2008: Structure of Agricultural Holdings and Livestock Population. Bangladesh Bureau of Statistics, Statistics Division, Ministry of Planning, Government of The People's Republic of Bangladesh, *Dhaka, Bangladesh*. Accessed on 20 October 2022. http://www.fao.org/fileadmin/templates/ess/ess_test_folder/World_Census_Agriculture/Country_info_2010/Reports/Reports_3/BGD_ENG_REP_2008.pdf.

Cerva C, Bremm C, Reis EM, Bezerra AV, Loiko MR, Cruz CE, Cenci A, Mayer FQ 2014: Food safety in raw milk production: risk factors associated to bacterial DNA contamination. *Tropical Animal Health and Production* **46** 877-882. http://dx.doi.org/10.1007/s11250-014-0580-y.

Chambers JV 2002: The microbiology of raw milk: *Dairy Microbiology Handbook*. 3rd Edition. John Wiley & Sons Inc, New York, NY. 39–90.

Chandra S, Oberoi P, Bhakat M, Yogi R, Yadav A, Singh P, Kumar A 2016: Effect of dietary supplementation of poly-herbal mixture and butyric acid on milk production, milk quality and somatic cell counts of postpartum Murrah buffalo. *Indian Journal of Animal Research* **51** http://dx.doi.org/10.18805/ijar.9646.

Charaya G, Kumar A, Sharma A, Hoel P, Kumar A, Kumar T, Kumar P, Gupta S, Mittal D 2015: Comparative efficacy of tylosin, enrofloxacin and ceftriaxone in treatment of buffalo suffering from mastitis. *Haryana Veterinarian* **34** 143-146.

Charaya G, Sharma A, Kumar A, Singh M, Goel P 2014: Pathogens isolated from clinical mastitis in Murrah buffalo and their antibiogram. *Veterinary World* **7** 980-985.

Chavoshi M, Husaini J 2012: Buffalo subclinical mastitis bacterial pathogens in Iran. *International Proceedings of Chemical, Biological and Environmental Engineering (IPCBEE),* 143-146.

Choudhary BB, Sirohi S 2019: Sensitivity of buffalo (*Bubalus bubalis*) to heat stress. *Journal of Dairy Research* **86** 399-405. http://dx.doi.org/10.1017/S0022029919000773.

Chye FY, Abdullah A, Ayob MK 2004: Bacteriological quality and safety of raw milk in Malaysia. *Food Microbiology* **21** 535-541. http://dx.doi.org/10.1016/j.fm.2003.11.007.

Cimmino F, Catapano A, Villano I, Di Maio G, Petrella L, Traina G, Pizzella A, Tudisco R, Cavaliere G 2023: Invited review: Human, cow, and donkey milk comparison: Focus on metabolic effects. *Journal of Dairy Science* http://dx.doi.org/10.3168/jds.2022-22465.

Cobirka M, Tancin V, Slama P 2020: Epidemiology and classification of mastitis. *Animals (Basel)* **10** http://dx.doi.org/10.3390/ani10122212.

Condas LAZ, De Buck J, Nobrega DB, Carson DA, Roy JP, Keefe GP, DeVries TJ, Middleton JR, Dufour S, Barkema HW 2017: Distribution of non-aureus staphylococci species in udder quarters with low and high somatic cell count, and clinical mastitis. *Journal of Dairy Science* **100** 5613-5627. http://dx.doi.org/10.3168/jds.2016-12479.

Constable PD, Hinchcliff KW, Done SH, Grünberg W 2016: Diagnosis of Bovine Mastitis: *Veterinary medicine: a textbook of the diseases of cattle, horses, sheep, pigs and goats*. 11th Edition. Elsevier Health Sciences, 3251 Riverport Lane St. Louis, Missouri 63043, United States. 1914-1921.

Coroian A, Coroian CO, Vodnar DC, Trif M 2010: Study of the main microbiological traits in Romanian buffalo milk. *International Journal of the Bioflux Society* **2** 92-98.

Cortinhas CS, Botaro BG, de Macedo SN, Dos Santos MV 2018: Herd characteristics and management practices associated with bulk tank milk quality of dairy herds in southeastern Brazil. *Tropical Animal Health and Production* **50** 1605-1610. http://dx.doi.org/10.1007/s11250-018-1601-z.

Costa A, Neglia G, Campanile G, De Marchi M 2020: Milk somatic cell count and its relationship with milk yield and quality traits in Italian water buffalo. *Journal of Dairy Science* **103** 5485-5494. http://dx.doi.org/10.3168/jds.2019-18009.

Cremonesi P, Cortimiglia C, Picozzi C, Minozzi G, Malvisi M, Luini M, Castiglioni B 2016: Development of a droplet digital polymerase chain reaction for rapid and simultaneous identification of common foodborne pathogens in soft cheese. *Frontiers in Microbiology* **7** http://dx.doi.org/10.3389/fmicb.2016.01725.

Cremonesi P, Pisani LF, Lecchi C, Ceciliani F, Martino P, Bonastre AS, Karus A, Balzaretti C, Castiglioni B 2014: Development of 23 individual TaqMan® real-time PCR assays for identifying common foodborne pathogens using a single set of amplification conditions. *Food Microbiology* **43** 35-40. http://dx.doi.org/10.1016/j.fm.2014.04.007.

Dang AK, Kapila S, Tomar P, Singh C 2007: Relationship of blood and milk cell counts with mastitic pathogens in Murrah buffalo. *Italian Journal of Animal Science* **6** 821-824. http://dx.doi.org/10.4081/ijas.2007.s2.821.

Darwish SF, Asfour HA, Allam HA 2015: Incidence of *Yersinia enterocolitica* and *Yersinia pseudotuberculosis* in raw milk samples of different animal species using conventional and molecular methods. *Alexandria Journal of Veterinary Sciences* **44** 174-185. http://dx.doi.org/10.5455/ajvs.176360

De Barros LD, Garcia JL, Bresciani KDS, Cardim ST, Storte VS, Headley SA 2020: A review of toxoplasmosis and Neosporosis in water buffalo (*Bubalus bubalis*). *Frontiers in Veterinary Science* **7** 455. http://dx.doi.org/10.3389/fvets.2020.00455.

De Buyser M-L, Dufour B, Maire M, Lafarge V 2001: Implication of milk and milk products in food-borne diseases in France and in different industrialised countries. *International Journal of Food Microbiology* **67** 1-17. http://dx.doi.org/https://doi.org/10.1016/S0168-1605(01)00443-3.

De Silva SASD, Kanugala KANP, Weerakkody NS 2016: Microbiological quality of raw milk and effect on quality by implementing good management practices. *Procedia Food Science* **6** 92-96. http://dx.doi.org/10.1016/j.profoo.2016.02.019.

De Vries A, Kaylegian KE, Dahl GE 2020: MILK Symposium review: Improving the productivity, quality, and safety of milk in Rwanda and Nepal. *Journal of Dairy Science* **103** 9758-9773. http://dx.doi.org/10.3168/jds.2020-18304.

Deb GK, Nahar TN, Duran PG, Presicce GA 2016: Safe and sustainable traditional production: the water buffalo in Asia. *Frontiers in Environmental Science* **4** 1-7. http://dx.doi.org/10.3389/fenvs.2016.00038.

Derks M, Hogeveen H, Kooistra SR, van Werven T, Tauer LW 2014: Efficiency of dairy farms participating and not participating in veterinary herd health management programs. *Preventive Veterinary Medicine* **117** 478-486. http://dx.doi.org/10.1016/j.prevetmed.2014.10.008.

DerSimonian R, Laird N 1986: Meta-analysis in clinical trials. *Controlled Clinical Trials* **7** 177-188. http://dx.doi.org/10.1016/0197-2456(86)90046-2.

Desmarchelier P, Fegan N 2016: Pathogens in milk: *Escherichia coli*: *Reference Module in Food Science*. Elsevier Health Sciences, 3251 Riverport Lane St. Louis, Missouri 63043, United States.

Dhakal I 2006: Normal somatic cell count and subclinical mastitis in Murrah buffalo. *Journal of Veterinary Medicine Series A* **53** 81-86.

Dhakal IP, Dhakal P, Koshihara T, Nagahata H 2007: Epidemiological and bacteriological survey of buffalo mastitis in Nepal. *The Journal of Veterinary Medical Science* **69** 1241-1245.

Dhakal IP, Nagahata H 2018: Evaluation of mastitis related measures & their applications to classify buffalo milk in Chitwan, Nepal. *Journal of Agricultural Science and Technology* **8** 99-111. http://dx.doi.org/10.17265/2161-6256/2018.02.006.

Dhakal IP, Neupane M, Nagahata H 2008: Evaluation of direct and indirect measures of quarter milk from crossbred buffalo. *Journal of Animal Science* **79** 628-633. http://dx.doi.org/10.1111/j.1740-0929.2008.00573.x.

Diriba K, Awulachew E, Diribsa K 2021: The prevalence of *Listeria* species in different food items of animal and plant origin in Ethiopia: a systematic review and meta-analysis. *European Journal of Medical Research* **26** 60. http://dx.doi.org/10.1186/s40001-021-00532-8.

Disassa N, Sibhat B, Mengistu S, Muktar Y, Belina D 2017: Prevalence and antimicrobial susceptibility pattern of *E. coli O157:H7* isolated from traditionally marketed raw cow milk in and around Asosa town, western Ethiopia. *Veterinary Medicine International* **2017** 7581531. http://dx.doi.org/10.1155/2017/7581531.

DLS 2021: Livestock economy at a glance. Department of Livestock Services, Division of Livestock Statistics, Ministry of Fisheries and Livestock, *Farmgate, Dhaka, Bangladesh*. Accessed on 26 May 2022. http://dls.portal.gov.bd/sites/default/files/files/dls.portal.gov.bd/files/a7c2c046_864e_41c5_adcc_c9858ebc7887/2021-08-18-05-41-28ed36a96d0db342b627f416f5d1f97a.pdf.

DLS 2022: Contribution of livestock and poultry in the national economy of Bangladesh (2021-22). Department of Livestock Services, Division of Livestock Statistics, Ministry of Fisheries and Livestock, *Farmgate, Dhaka, Bangladesh*. Accessed on 1st January 2023. http://dls.portal.gov.bd/sites/default/files/files/dls.portal.gov.bd/page/ee5f4621_fa3a_40ac_8bd9_898fb8ee4700/2022-07-18-03-43-37d18965a6458cda3c542ab146480962.pdf.

Dohoo I, Martin SW, Stryhn H 2010a: Mixed models for discrete data: *Veterinary Epidemiologic Research*. 2nd Edition. VER Inc., Charlottetown, Prince Edward Island, Canada. 587-591.

Dohoo I, Martin SW, Stryhn H 2010b: Sampling: *Veterinary Epidemiologic Research*. 2nd Edition. VER Inc., Charlottetown, Prince Edward Island, Canada. 36-37.

Dufour S, Fréchette A, Barkema HW, Mussell A, Scholl DT 2011: Invited review: effect of udder health management practices on herd somatic cell count. *Journal of Dairy Science* **94** 563-579. http://dx.doi.org/10.3168/jds.2010-3715.

El-Ashker M, Gwida M, Monecke S, El-Gohary F, Ehricht R, Elsayed M, Akinduti P, El-Fateh M, Maurischat S 2020: Antimicrobial resistance pattern and virulence profile of *S. aureus* isolated from household cattle and buffalo with mastitis in Egypt. *Veterinary Microbiology* **240** 108535. http://dx.doi.org/10.1016/j.vetmic.2019.108535.

El-Gazzar FE, Marth EH 1992: Salmonellae, salmonellosis, and dairy foods: a review. *Journal of Dairy Science* **75** 2327-2343. http://dx.doi.org/10.3168/jds.S0022-0302(92)77993-4.

El-Razik KAA, Arafa AA, Hedia RH, Ibrahim ES 2017: Tetracycline resistance phenotypes and genotypes of coagulase-negative staphylococcal isolates from bubaline mastitis in Egypt. *Veterinary World* **10** 702-710. http://dx.doi.org/10.14202/vetworld.2017.702-710.

El-Salam A, Mohamed H, El-Shibiny S 2011: A comprehensive review on the composition and properties of buffalo milk. *Dairy Science and Technology* **91** 663-699. http://dx.doi.org/10.1007/s13594-011-0029-2.

El-Wishy AB 2007: The postpartum buffalo. II. Acyclicity and anestrus. *Animal Reproduction Science* **97** 216-236. http://dx.doi.org/10.1016/j.anireprosci.2006.03.003.

El-Zamkan MA, Hameed KG 2016: Prevalence of *Campylobacter jejuni* and *Campylobacter coli* in raw milk and some dairy products. *Veterinary World* **9** 1147-1151. http://dx.doi.org/10.14202/vetworld.2016.1147-1151.

El Nahas SM, El Kasas AH, Abou Mossallem AA, Abdelhamid MI, Warda M 2017: A study on IL8RB gene polymorphism as a potential immuno-compromised adherent in exaggeration of parenteral and mammo-crine oxidative stress during mastitis in buffalo. *Journal of Advanced Research* **8** 617-625. http://dx.doi.org/10.1016/j.jare.2017.07.002.

Elahi E, Abid M, Zhang H, Cui W, Ul Hasson S 2018: Domestic water buffalo: Access to surface water, disease prevalence and associated economic losses. *Preventive Veterinary Medicine* **154** 102-112. http://dx.doi.org/10.1016/j.prevetmed.2018.03.021.

Elhaig MM, Selim A 2015: Molecular and bacteriological investigation of subclinical mastitis caused by *Staphylococcus aureus* and *Streptococcus agalactiae* in domestic bovids from Ismailia, Egypt. *Tropical Animal Health and Production* **47** 271-276. http://dx.doi.org/10.1007/s11250-014-0715-1.

Elmoslemany AM, Keefe GP, Dohoo IR, Jayarao BM 2009: Risk factors for bacteriological quality of bulk tank milk in Prince Edward Island dairy herds. Part

2: bacteria count-specific risk factors. *Journal of Dairy Science* **92** 2644-2652. http://dx.doi.org/10.3168/jds.2008-1813.

Elsayed MS, Mahmoud El-Bagoury AE, Dawoud MA 2015: Phenotypic and genotypic detection of virulence factors of *Staphylococcus aureus* isolated from clinical and subclinical mastitis in cattle and water buffaloes from different farms of Sadat city in Egypt. *Veterinary World* **8** 1051-1058. http://dx.doi.org/10.14202/vetworld.2015.1051-1058.

Enger BD, Middleton JR 2021: Letter to the Editor: Comments on "Mammary microbial dysbiosis leads to the zoonosis of bovine mastitis: a One-Health perspective" by Maity and Ambatipudi. *FEMS Microbiology Ecology* **97** http://dx.doi.org/10.1093/femsec/fiab076.

Esposito G, Irons PC, Webb EC, Chapwanya A 2014: Interactions between negative energy balance, metabolic diseases, uterine health and immune response in transition dairy cows. *Animal Reproduction Science* **144** 60-71. http://dx.doi.org/10.1016/j.anireprosci.2013.11.007.

Etikan I, Alkassim R, Abubakar S 2016: Comparision of snowball sampling and sequential sampling technique. *Biometrics and Biostatistics International Journal* **3** 6-7. http://dx.doi.org/10.15406/bbij.2016.03.00055.

Exel CE, Gerritsen K, Spaninks M, Duim B, Koop G, Benedictus L 2023: Association of *Staphylococcus aureus* genotypes with milk or colonization of extramammary sites in Dutch dairy cattle indicates strain variation in reservoirs for intramammary infections. *Research in Veterinary Science* **154** 138-144. http://dx.doi.org/10.1016/j.rvsc.2022.12.010.

Exel CE, Halasa T, Koop G, Steeneveld W, Lam T, Benedictus L, Gussmann M 2022: A stochastic modelling approach to determine the effect of diverse *Staphylococcus aureus* strains on the economic and epidemiological outcomes of mastitis intervention strategies in dairy cattle. *Preventive Veterinary Medicine* **199** 105566. http://dx.doi.org/10.1016/j.prevetmed.2021.105566.

FAOSTAT 2019: Production/Yield quantities of buffalo in world. Accessed on 5 August 2022. https://www.fao.org/faostat/en/#data/QA.

Farooq A, Inayat S, Akhtar M, Mushtaq M 2008: Prevalence of mastitis and antibiotic sensitivity of bacterial isolates recovered from Nili-Ravi buffalo. *Journal of Animal and Plant Sciences* **18** 2-3.

Farrar T 2020: skedastic: Heteroskedasticity diagnostics for linear regression models. *Bellville, South Africa*. Accessed on 10 July 2022. https://github.com/tjfarrar/skedastic.

Faruque M 2000: Identification of the best genotype of buffalo for dairy purpose in Bangladesh and to improve their productivity. *Bangladesh Agricultural Research Council. New Airport Road, Farmgate, Dhaka, Bangladesh*

Faruque MO, Hasnath MA, Siddique NN 1990: Present status of buffalo and their productivity in Bangladesh. *Asian-Australasian Journal of Animal Sciences* **3** 287-292.

Faruque MO, Hossain MI 2007: The effect of feed supplement on the yield and composition of buffalo milk. *Italian Journal of Animal Science* **6** 488-490.

Ferchiou A, Ndiaye Y, Mandour MA, Herman N, Lhermie G, Raboisson D 2023: The marginal abatement cost of antimicrobials for dairy cow mastitis: A bioeconomic optimization perspective. *Veterinary Sciences* **10** http://dx.doi.org/10.3390/vetsci10020092.

Francoz D, Wellemans V, Dupré JP, Roy JP, Labelle F, Lacasse P, Dufour S 2017: Invited review: A systematic review and qualitative analysis of treatments other than conventional antimicrobials for clinical mastitis in dairy cows. *Journal of Dairy Science* **100** 7751-7770. http://dx.doi.org/10.3168/jds.2016-12512.

Fréchette A, Fecteau G, Côté C, Dufour S 2021: Clinical mastitis incidence in dairy cows housed on recycled manure solids bedding: a Canadian cohort study. *Frontiers in Veterinary Science* **8** 742868. http://dx.doi.org/10.3389/fvets.2021.742868.

Freeman MF, Tukey JW 1950: Transformations related to the angular and the square root. *Annals of Mathematical Statistics* **21** 607-611, 605.

Fresán U, Sabaté J 2019: Vegetarian diets: Planetary health and its alignment with human health. *Advances in Nutrition* **10** S380-s388. http://dx.doi.org/10.1093/advances/nmz019.

Frey Y, Rodriguez JP, Thomann A, Schwendener S, Perreten V 2013: Genetic characterization of antimicrobial resistance in coagulase-negative staphylococci from bovine mastitis milk. *Journal of Dairy Science* **96** 2247-2257. http://dx.doi.org/10.3168/jds.2012-6091.

Gao J, Barkema HW, Zhang L, Liu G, Deng Z, Cai L, Shan R, Zhang S, Zou J, Kastelic JP, Han B 2017: Incidence of clinical mastitis and distribution of pathogens on large Chinese dairy farms. *Journal of Dairy Science* **100** 4797-4806. http://dx.doi.org/10.3168/jds.2016-12334.

Geary U, Lopez-Villalobos N, Begley N, McCoy F, O'Brien B, O'Grady L, Shalloo L 2012: Estimating the effect of mastitis on the profitability of Irish dairy farms. *Journal of Dairy Science* **95** 3662-3673. http://dx.doi.org/10.3168/jds.2011-4863.

Gebeyehu A, Taye M, Abebe R 2022: Isolation, molecular detection and antimicrobial susceptibility profile of *Salmonella* from raw cow milk collected from dairy farms and households in southern Ethiopia. *BMC Microbiology* **22** 84. http://dx.doi.org/10.1186/s12866-022-02504-2.

Geletu US, Usmael MA, Ibrahim AM 2022: Isolation, Identification, and Susceptibility Profile of E. coli, Salmonella, and S. aureus in Dairy Farm and Their Public Health Implication in Central Ethiopia. *Veterinary Medicine International* **2022** 1887977. http://dx.doi.org/10.1155/2022/1887977.

Getaneh AM, Gebremedhin EZ 2017: Meta-analysis of the prevalence of mastitis and associated risk factors in dairy cattle in Ethiopia. *Tropical Animal Health and Production* **49** 697-705. http://dx.doi.org/10.1007/s11250-017-1246-3.

Gindonis V, Taponen S, Myllyniemi AL, Pyörälä S, Nykäsenoja S, Salmenlinna S, Lindholm L, Rantala M 2013: Occurrence and characterization of methicillin-resistant staphylococci from bovine mastitis milk samples in Finland. *Acta Veterinaria Scandinavica* **55** 61. http://dx.doi.org/10.1186/1751-0147-55-61.

Giovanni N, Elisa S, Marta C, Rosa F, Loredana C, Alessandra B, Antonio P 2020: Occurrence and characteristics of methicillin-resistant *Staphylococcus aureus*

(MRSA) in buffalo bulk tank milk and the farm workers in Italy. *Food Microbiology* **91** 103509. http://dx.doi.org/10.1016/j.fm.2020.103509.

Godinho FMS, Krug M, Figueiredo RP, Müller A, Jank L, Tomaszewski CA, Hillesheim DR, Kinast É J, Frazzon APG, Motta AS 2020: Microbiological and physicochemical characteristics of buffalo milk used for dairy products in southern Brazil. *Journal of Dairy Research* **87** 463-468. http://dx.doi.org/10.1017/s002202992000093x.

Goma AA, Phillips CJC 2021: The impact of anthropogenic climate change on Egyptian livestock production. *Animals (Basel)* **11** http://dx.doi.org/10.3390/ani11113127.

Guccione J, D'Andrea L, Pesce A, Toni F, Borriello G, Salzano C, Diuccio F, Pascale M, Ciaramella P 2020: Antibiotic dry buffalo therapy: effect of intramammary administration of benzathine cloxacillin against *Staphylococcus aureus* mastitis in dairy water buffalo. *BMC Vet Res* **16** 191. http://dx.doi.org/10.1186/s12917-020-02410-7.

Guha A, Guha R 2012: Comparison of somatic cell count, California mastitis test, chloride test and rennet coagulation time with bacterial culture examination to detect subclinical mastitis in riverine buffalo (Bubalus bubalis). *African Journal of Agricultural Research* **7** 5578-5584.

Gulel GT, Gucukoglu A, Cadirci O, Saka E, Alisarli M 2020: Serotyping and antibiotic resistance of *Listeria monocytogenes* isolated from raw water buffalo milk and milk products. *Journal of Food Science* **85** 2889-2895. http://dx.doi.org/10.1111/1750-3841.15376.

Günther J, Koy M, Berthold A, Schuberth HJ, Seyfert HM 2016: Comparison of the pathogen species-specific immune response in udder derived cell types and their models. *Veterinary Research* **47** 22. http://dx.doi.org/10.1186/s13567-016-0307-3.

Gurler H, Findik A, Sezener MG 2022: Determination of antibiotic resistance profiles and biofilm production of Staphylococcus spp. isolated from Anatolian water buffalo milk with subclinical mastitis. *Polish Journal of Veterinary Sciences* **25** 51-59. http://dx.doi.org/10.24425/pjvs.2022.140840.

Guzman-Hernandez R, Contreras-Rodriguez A, Hernandez-Velez R, Perez-Martinez I, Lopez-Merino A, Zaidi MB, Estrada-Garcia T 2016: Mexican unpasteurised fresh cheeses are contaminated with *Salmonella* spp., non-O157 Shiga toxin producing *Escherichia coli* and potential uropathogenic *E. coli* strains: A public health risk. *International Journal of Food Microbiology* **237** 10-16. http://dx.doi.org/10.1016/j.ijfoodmicro.2016.08.018.

Habib MR, Haque MN, Rahman A, Aftabuzzaman M, Ali MM, Shahjahan M 2017: Dairy buffalo production scenario in Bangladesh: a review. *Asian Journal of Medical and Biological Research* **3** 305-316. http://dx.doi.org/10.3329/ajmbr.v3i3.34518.

Habib MR, Khandakar MMH, Islam MA, Sarkar MM, Alam MK, Rahman MK, Sharmin MM, Hannan MM, Islam MA, Fisheries 2021: Status of the buffalo milk trade and dairy manufacturing business at Bhola district of Bangladesh, and opportunities for buffalo milk products branding. *Research in Agriculture, Livestock and Fisheries* **8** 301-310.

Halasa T, Huijps K, Østerås O, Hogeveen H 2007: Economic effects of bovine mastitis and mastitis management: a review. *Veterinary Quarterly* **29** 18-31. http://dx.doi.org/10.1080/01652176.2007.9695224.

Hamed MI, Ziatoun A 2014: Prevalence of *Staphylococcus aureus* subclinical mastitis in dairy buffalo farms. *International Journal of Livestock Research* **4** 21-28.

Hameed S, Arshad M, Ashraf M, Avais M, Shahid M 2012: Cross-sectional epidemiological studies on mastitis in cattle and buffalo of Tehsil Burewala, Pakistan. *Journal of Animal and Plant Sciences* **22** 371-376.

Hamel J, Zhang Y, Wente N, Krömker V 2020: Non-*S. aureus* staphylococci (NAS) in milk samples: Infection or contamination? *Veterinary Microbiology* **242** 108594. http://dx.doi.org/10.1016/j.vetmic.2020.108594.

Hamid MA, Ahmed S, Rahman MA, Hossain KM 2016a: Status of buffalo production in Bangladesh compared to SAARC countries. *Asian Journal of Animal Sciences* **10** 313-329. http://dx.doi.org/10.3923/ajas.2016.313.329.

Hamid MA, Siddiky MN, Rahman MA, Hossain KM 2016b: Scopes and opportunities of buffalo farming in Bangladesh: A review. *SAARC Journal of Agriculture* **2** 63-77.

Hamiroune M, Berber A, Boubekeur S 2016: Evaluation of the bacteriological quality of raw cow's milk at various stages of the milk production chain on-farms in Algeria. *The Scientific and Technical Review* **35** 925-946. http://dx.doi.org/10.20506/rst.35.3.2580.

Hand KJ, Godkin MA, Kelton DF 2012: Short communication: Bulk milk somatic cell penalties in herds enrolled in Dairy Herd Improvement programs. *Journal of Dairy Science* **95** 240-242. http://dx.doi.org/10.3168/jds.2011-4642.

Harizt AM, Malahubban M, Syed-Hussain SS, Ramanoon SZ, Sadiq MB, Sarbini SR, Hassan MN, Mustafa S, Aziz NAA, Isa NMM, Nating WR, Asut R, Kamaludeen J 2021: Gastrointestinal parasitic infections of buffalo (*Bubalus bubalis*) in Sarawak Borneo: Prevalence, risk factors, and farming practices. *Tropical Biomedicine* **38** 318-326. http://dx.doi.org/10.47665/tb.38.3.072.

Hayashi Y, Shah M, Kumagai H 2014: Changes of milk production and reproductive performance of dairy buffalo in Nepal during recent 15 Years. *Journal of Buffalo Science* **3** 34. http://dx.doi.org/10.6000/1927-520X.2014.03.02.1.

Hegde NG 2019: Buffalo husbandry for sustainable development of small farmers in india and other developing countries. *Asian Journal of Animal and Veterinary Advances* **3** 1-20.

Heredia N, García S 2018: Animals as sources of food-borne pathogens: A review. *Animal Nutrition* **4** 250-255. http://dx.doi.org/10.1016/j.aninu.2018.04.006.

Hoque MN, Talukder AK, Saha O, Hasan MM, Sultana M, Rahman AA, Das ZC 2022: Antibiogram and virulence profiling reveals multidrug resistant *Staphylococcus aureus* as the predominant aetiology of subclinical mastitis in riverine buffalo. *Veterinary Medicine and Science* http://dx.doi.org/10.1002/vms3.942.

Huque K, Khan M 2017: Socio-geographic distribution of livestock and poultry in Bangladesh-A review. *Bangladesh Journal of Animal Science* **46** 65-81.

Hussain A, Ahmad M-u-D, Mushtaq MH, Chaudhry M, Khan MS, Reichel M, Hussain T, Khan A, Nisar M, Khan IA 2018: Prevalence of overall and teatwise mastitis and effect of herd size in dairy buffalo. *Pakistan Journal of Zoology* **50** 1107-1112. http://dx.doi.org/10.17582/journal.pjz/2018.50.3.1107.1112.

Hussain A, Shakoor A, Shahid MA, Numan M, Gulraiz F 2007: Clinical and subclinical *Staphylococcus aureus* mastitis in dairy buffalo: disease characteristics and antibiotic susceptibility profiles of isolates. *International Journal of Agricultural Research, Innovation and Technology* **2** 804-811. http://dx.doi.org/10.3923/ijar.2007.804.811.

Hussain R, Javed MT, Khan A, Muhammad G 2013: Risks factors associated with subclinical mastitis in water buffalo in Pakistan. *Tropical Animal Health and Production* **45** 1723-1729. http://dx.doi.org/10.1007/s11250-013-0421-4.

Hussein HA, Fouad MT, Abd El-Razik KA, Abo El-Maaty AM, D'Ambrosio C, Scaloni A, Gomaa AM 2020: Study on prevalence and bacterial etiology of mastitis, and effects of subclinical mastitis and stage of lactation on SCC in dairy goats in Egypt. *Tropical Animal Health and Production* **52** 3091-3097. http://dx.doi.org/10.1007/s11250-020-02331-5.

Ida JA, Wilson WM, Nydam DV, Gerlach SC, Kastelic JP, Russell ER, McCubbin KD, Adams CL, Barkema HW 2023: Contextualized understandings of dairy farmers' perspectives on antimicrobial use and regulation in Alberta, Canada. *Journal of Dairy Science* **106** 547-564. http://dx.doi.org/10.3168/jds.2021-21521.

Islam J, Islam FR, Jahan IL, Chaudhary PK, Anower AKMM 2019: Assessment of subclinical mastitis in milch animals by different field diagnostic tests in Barishal district of Bangladesh. *Asian-Australasian Journal of Bioscience and Biotechnology* **4** 24-33.

Islam KS, Kabir M H B, Rahman M H, H KM 2016a: Status of buffalo diseases in Bangladesh in relation to casual agents and predisposing factors. *International Journal of Life Sciences and Technology* **9** 44.

Islam M, Kabir S, Rahman M 2016b: Molecular detection and characterization of Staphylococcus aureus isolated from raw milk sold in different markets of Bangladesh. *Bangladesh Journal of Veterinary Medicine* **14** 277-282.

Islam MA, Mondol AS, Boer Ed, Beumer RR, Zwietering MH, Talukder KA, Heuvelink AE 2008: Prevalence and genetic characterization of Shiga toxin-producing *Escherichia coli* isolates from slaughtered animals in Bangladesh. *Applied and Environmental Microbiology* **74** 5414-5421. http://dx.doi.org/doi:10.1128/AEM.00854-08.

Islam MA, Roy S, Nabi A, Solaiman S, Rahman M, Huq M, Siddiquee NA, Ahmed N 2018: Microbiological quality assessment of milk at different stages of the dairy value chain in a developing country setting. *International Journal of Food Microbiology* **278** 11-19. http://dx.doi.org/10.1016/j.ijfoodmicro.2018.04.028.

Islam SKS, Chakma S, Akhter A, Ibrahim N, Sarker NH 2017: Livestock associated epidemiological information profiling in New Sandwip Island (Jahajerchar) of the Meghna estuary, Noakhali using participatory disease searching tool. *Journal of Advanced Veterinary and Animal Research* **4** 267-273. http://dx.doi.org/10.5455/javar.2017.d222.

ISO:4833-1 2013: Microbiology of the food chain–horizontal method for the enumeration of microorganisms–part 1: colony count at 30 degrees c by the pour plate technique. Accessed on 02 July 2022. https://www.iso.org/standard/53728.html.

Jaakkonen A, Castro H, Hallanvuo S, Ranta J, Rossi M, Isidro J, Lindström M, Hakkinen M 2019: Longitudinal study of Shiga toxin-producing *Escherichia coli* and *Campylobacter jejuni* on Finnish Dairy Farms and in Raw Milk. *Applied and Environmental Microbiology* **85** http://dx.doi.org/10.1128/aem.02910-18.

Jabari S, Eslami M, Chaji M, Mohammadabadi T, Bojarpour M 2014: Comparison digestibility and protozoa population of Khuzestan water buffalo and Holstein cow. *Veterinary Research Forum* **5** 295-300.

Jackson A, Green MJ, Kaler J 2022: Fellow cows and conflicting farmers: Public perceptions of dairy farming uncovered through frame analysis. *Frontiers in Veterinary Science* **9** 995240. http://dx.doi.org/10.3389/fvets.2022.995240.

Jackson D, White IR 2018: When should meta-analysis avoid making hidden normality assumptions? *Biometrical Journal* **60** 1040-1058. http://dx.doi.org/https://doi.org/10.1002/bimj.201800071.

Jamali H, Barkema HW, Jacques M, Lavallée-Bourget EM, Malouin F, Saini V, Stryhn H, Dufour S 2018: Invited review: Incidence, risk factors, and effects of clinical mastitis recurrence in dairy cows. *Journal of Dairy Science* **101** 4729-4746. http://dx.doi.org/10.3168/jds.2017-13730.

Javed S, McClure J, Syed MA, Obasuyi O, Ali S, Tabassum S, Ejaz M, Zhang K 2022: Epidemiology and molecular characterization of *Staphylococcus aureus* causing mastitis in water buffalo from the Hazara division of Khyber Pakhtunkhwa, Pakistan. *PLoS One* **17** e0268152. http://dx.doi.org/10.1371/journal.pone.0268152.

Jhambh R, Dimri U, Gopalakrishnan A, Singh M, Chhabra R 2017: Prevalence and risk factors of subclinical mastitis in buffalo at an organized dairy farm in western Haryana. *Haryana Veterinarian* **56** 189-193.

Joshi H, Tanwar R, Chahar A 2013: Prevalence of sub-clinical mastitis in buffalo. *Veterinary Practitioner* **14** 377-378.

Kapila N, Sharma A, Kishore A, Sodhi M, Tripathi PK, Mohanty AK, Mukesh M 2018: Correction: Impact of heat stress on cellular and transcriptional adaptation of mammary epithelial cells in riverine buffalo (*Bubalus Bubalis*). *PLoS One* **13** e0191380. http://dx.doi.org/10.1371/journal.pone.0191380.

Kapila R, Kavadi PK, Kapila S 2013: Comparative evaluation of allergic sensitization to milk proteins of cow, buffalo and goat. *Small Ruminant Research* **112** 191-198. http://dx.doi.org/10.1016/j.smallrumres.2012.11.028.

Käppeli N, Morach M, Zurfluh K, Corti S, Nüesch-Inderbinen M, Stephan R 2019: Sequence types and antimicrobial resistance profiles of *Streptococcus uberis* isolated from bovine mastitis. *Frontiers in Veterinary Science* **6** 234. http://dx.doi.org/10.3389/fvets.2019.00234.

Kashoma IP, Kassem, II, John J, Kessy BM, Gebreyes W, Kazwala RR, Rajashekara G 2016: Prevalence and antimicrobial resistance of campylobacter isolated from

dressed beef carcasses and raw milk in Tanzania. *Microbial Drug Resistance* **22** 40-52. http://dx.doi.org/10.1089/mdr.2015.0079.

Kaur G, Bansal BK, Singh RS, Kashyap N, Sharma S 2018: Associations of teat morphometric parameters and subclinical mastitis in riverine buffalo. *Journal of Dairy Research* **85** 303-308. http://dx.doi.org/10.1017/s0022029918000444.

Kazak F, Yarim GF, Gokceoglu A, Delmecioglu MK, Yarim M 2022: Epidermal growth factor concentration in milk of healthy water buffalo (*Bubalus bubalis*). *Veterinary Research Forum* **13** 593-596. http://dx.doi.org/10.30466/vrf.2021.528757.3167.

Keane OM 2019: Symposium review: Intramammary infections-Major pathogens and strain-associated complexity. *Journal of Dairy Science* **102** 4713-4726. http://dx.doi.org/10.3168/jds.2018-15326.

Kebede T, Afera B, Taddele H, Bsrat A 2014: Assessment of bacteriological quality of sold meat in the butcher shops of Adigrat, Tigray, Ethiopia. *Applied Journal of Hygiene* **3** 38-44.

Keviletsu K, Yadav B 2010: Incidence of mastitis in Sahiwal cattle and Murrah buffalo of a closed organized herd. *The Indian Journal of Animal Sciences* **80** 467-469.

Kh'ng K, Chang CC, Hsu SH 2022: Implications of food and nutrition security on household food expenditure: the case of Malaysia. *Agriculture and Food Security* **11** 30. http://dx.doi.org/10.1186/s40066-022-00367-4.

Khademi P, Ownagh A, Mardani K, Khalili M 2019: Prevalence of *Coxiella burnetii* in milk collected from buffalo (water buffalo) and cattle dairy farms in Northwest of Iran. *Comparative Immunology, Microbiology and Infectious Diseases* **67** 101368. http://dx.doi.org/10.1016/j.cimid.2019.101368.

Khaldi Z, Nafti M, Jilani MT 2022: Baseline composition and microbial quality assessment of raw milk from small ruminants and Maghrebi camels in the oasis area of Tunisia. *Tropical Animal Health and Production* **54** 391. http://dx.doi.org/10.1007/s11250-022-03408-z.

Khan A, Durrani AZ, Yousaf A, Khan JA, Chaudhry M, Khan MA, Habibunnabi, Khan A 2019: Epidemiology of bovine sub-clinical mastitis in pothohar region, Punjab, Pakistan in 2018. *Pakistan Journal of Zoology* **51** 1667-1674. http://dx.doi.org/10.17582/journal.pjz/2019.51.5.1667.1674.

Khan JA, Rathore RS, Ahmad I, Gill R, Husain FM, Arshad M, Alam P, Albalawi T, Al-Kheraif A, Akhtar J, Albarakaty FM, Neyaz LA, Elbanna K, Abulreesh HH 2022: Assessment of foodborne bacterial pathogens in buffalo raw milk using polymerase chain reaction based assay. *Foodborne Pathogens and Disease* **19** 750-757. http://dx.doi.org/10.1089/fpd.2022.0044.

Khan M 2002: Dairy animals/Water Buffalo: *Water buffalo*. Elsevier Health Sciences, 3251 Riverport Lane St. Louis, Missouri 63043, United States.

Khaton R, Hasnat M, Rahman S, Rahman M 2014: Public health safety in relation to microbiological quality of freshly drawn cow's milk in Bangladesh. *Bangladesh Journal of Veterinary Medicine* **12** 231-236.

Khatun MA, Rashid MB, Hygen HO 2016: Climate of Bangladesh. *Bangladesh Meteorological Department Technical Report* 1-158.

Kirkeby C, Zervens L, Toft N, Schwarz D, Farre M, Hechinger S, Halasa T 2019: Transmission dynamics of *Staphylococcus aureus* within two Danish dairy cattle herds. *Journal of Dairy Science* **102** 1428-1442. http://dx.doi.org/10.3168/jds.2018-15106.

Knight-Jones TJ, Hang'ombe MB, Songe MM, Sinkala Y, Grace D 2016: Microbial contamination and hygiene of fresh cow's milk produced by smallholders in western Zambia. *International Journal of Environmental Research and Public Health* **13** http://dx.doi.org/10.3390/ijerph13070737.

Koop G, Nielen M, van Werven T 2009: Bulk milk somatic cell counts are related to bulk milk total bacterial counts and several herd-level risk factors in dairy goats. *Journal of Dairy Science* **92** 4355-4364. http://dx.doi.org/10.3168/jds.2009-2106.

Krishna KSS, Suresh K, Sreenu M, Sundar NS 2014: Prevalence of subclinical hypocalcaemia and subclinical ketosis in buffalo. *Buffalo Bulletin* **33** 107-110.

Krishnamoorthy P, Goudar AL, Suresh KP, Roy P 2021a: Global and countrywide prevalence of subclinical and clinical mastitis in dairy cattle and buffalo by systematic review and meta-analysis. *Research in Veterinary Science* **136** 561-586. http://dx.doi.org/10.1016/j.rvsc.2021.04.021.

Krishnamoorthy P, Suresh KP, Jayamma KS, Shome BR, Patil SS, Amachawadi RG 2021b: An understanding of the global status of major bacterial pathogens of milk concerning bovine mastitis: A systematic review and meta-analysis (Scientometrics). *Pathogens* **10** http://dx.doi.org/10.3390/pathogens10050545.

Krukowski H, Bakuła Z, Iskra M, Olender A, Bis-Wencel H, Jagielski T 2020: The first outbreak of methicillin-resistant *Staphylococcus aureus* in dairy cattle in Poland with evidence of on-farm and intrahousehold transmission. *Journal of Dairy Science* **103** 10577-10584. http://dx.doi.org/10.3168/jds.2020-18291.

Kumar AP 2009: Evaluation of PCR test for detecting major pathogens of bubaline mastitis directly from mastitic milk samples of buffalo. *Tropical Animal Health and Production* **41** 1643-1651. http://dx.doi.org/10.1007/s11250-009-9360-5.

Kumar M, Kumar R, Sharma A, Jain V 2007: Investigations on prevalence and oxidative stress aspects of mastitis in buffalo. *Italian Journal of Animal Science* **6** 978-979.

Kumar P, Ravindra K, Abhishek K, Verma R 2008: Bacteriological examination of mastitis in buffalo. *Progressive Research* **3** 224.

Lam TJ, van Vliet JH, Schukken YH, Grommers FJ, van Velden-Russcher A, Barkema HW, Brand A 1997: The effect of discontinuation of postmilking teat disinfection in low somatic cell count herds. I. Incidence of clinical mastitis. *Veterinary Quarterly* **19** 41-47. http://dx.doi.org/10.1080/01652176.1997.9694738.

Lamey A, Ammar AM, Zaki E, Khairy N, Moshref BS, Refai MK 2013: Virulence factors of *Escherichia coli* isolated from recurrent cases of clinical and subclinical mastitis in buffalo. *Italian Journal of Animal Science* **4** 86-94.

Landin H, Carlsson J, Hennum K-O, Katholm J, Kulkas L, Persson Y, Plym-Forshell K, Rattenborg E, Sandgren C, Solverod L, Whist A 2011: Nordic guidelines for mastitis therapy. *3rd International Symposium,* St. Louis, Missouri, USA.

Lauer Cruz K, de Souza da Motta A 2019: Characterization of biofilm production by Pseudomonas fluorescens isolated from refrigerated raw buffalo milk. *Journal of Food Science and Technology* **56** 4595-4604. http://dx.doi.org/10.1007/s13197-019-03924-1.

LDDP 2019: Union-based livestock and domestic birds' population in Bangladesh: The financial year 2019-20. Livestock and Dairy Development Project, Department of Livestock Services, *Farmgate, Dhaka, Bangladesh*. Accessed on 20 October 2022. http://lddp.portal.gov.bd/sites/default/files/files/lddp.portal.gov.bd/npfblock/3ef283ce_793d_43b5_946f_0ad8b776e791/2022-09-07-07-48-ab440b7208f7eb36419147f82fd001f7.pdf.

Le Loir Y, Baron F, Gautier M 2003: *Staphylococcus aureus* and food poisoning. *Genetics and Molecular Research* **2** 63-76.

Lemma F, Alemayehu H, Stringer A, Eguale T 2021: Prevalence and antimicrobial susceptibility profile of *Staphylococcus aureus* in milk and traditionally processed dairy products in Addis Ababa, Ethiopia. *BioMed Research International* **2021** 5576873. http://dx.doi.org/10.1155/2021/5576873.

Leuenberger A, Sartori C, Boss R, Resch G, Oechslin F, Steiner A, Moreillon P, Graber HU 2019: Genotypes of *Staphylococcus aureus*: On-farm epidemiology and the consequences for prevention of intramammary infections. *Journal of Dairy Science* **102** 3295-3309. http://dx.doi.org/10.3168/jds.2018-15181.

Li N, Richoux R, Boutinaud M, Martin P, Gagnaire V 2014: Role of somatic cells on dairy processes and products: a review. *Dairy Science and Technology* **94** 517-538. http://dx.doi.org/10.1007/s13594-014-0176-3.

Liu D, Lawrence ML, Ainsworth AJ, Austin FW 2005: Comparative assessment of acid, alkali and salt tolerance in *Listeria monocytogenes* virulent and avirulent strains. *FEMS Microbiology Letters* **243** 373-378. http://dx.doi.org/https://doi.org/10.1016/j.femsle.2004.12.025.

Liu H, Meng L, Dong L, Zhang Y, Wang J, Zheng N 2021: Prevalence, antimicrobial susceptibility, and molecular characterization of *Escherichia coli* isolated from raw milk in dairy herds in northern China. *Frontiers in Microbiology* **12** 730656. http://dx.doi.org/10.3389/fmicb.2021.730656.

Locatelli C, Cremonesi P, Caprioli A, Carfora V, Ianzano A, Barberio A, Morandi S, Casula A, Castiglioni B, Bronzo V, Moroni P 2017: Occurrence of methicillin-resistant *Staphylococcus aureus* in dairy cattle herds, related swine farms, and humans in contact with herds. *Journal of Dairy Science* **100** 608-619. http://dx.doi.org/10.3168/jds.2016-11797.

Locatelli C, Piepers S, De Vliegher S, Barberio A, Supré K, Scaccabarozzi L, Pisoni G, Bronzo V, Haesebrouck F, Moroni P 2013: Effect on quarter milk somatic cell count and antimicrobial susceptibility of *Staphylococcus rostri* causing intramammary infection in dairy water buffalo. *Journal of Dairy Science* **96** 3799-3805. http://dx.doi.org/10.3168/jds.2012-6275.

Lopes WD, Monte DFMd, de Leon CMGC, Moura JFPd, Silva NMVd, Queiroga RdCRdE, Gonzaga Neto S, Givisiez PEN, Pereira WE, Oliveira CJBd 2021: Logistic regression model reveals major factors associated with total bacteria and

somatic cell counts in goat bulk milk. *Small Ruminant Research* **198** 106360. http://dx.doi.org/https://doi.org/10.1016/j.smallrumres.2021.106360.

Madhaiyan M, Wirth JS, Saravanan VS 2020: Phylogenomic analyses of the Staphylococcaceae family suggest the reclassification of five species within the genus *Staphylococcus* as heterotypic synonyms, the promotion of five subspecies to novel species, the taxonomic reassignment of five *Staphylococcus* species to *Mammaliicoccus* gen. nov., and the formal assignment of *Nosocomiicoccus* to the family Staphylococcaceae. *The International Journal of Systematic and Evolutionary Microbiology* **70** 5926-5936. http://dx.doi.org/10.1099/ijsem.0.004498.

Maiti S, Neelesh S, Awasthi B 2003: Studies on incidence of subclinical mastitis (SCM) in cattle and buffalo of durg area of Chhattisgarh. *Veterinary Practitioner* **4** 123-124.

Malik MH, Verma HK 2017: Prevalence, economic impact and risk factors associated with mastitis in dairy animals of Punjab. *Indian Journal of Animal Sciences* **87** 1452-1456.

Malmarugan S, Arthanarieswaran M, Suresh P 2010: PCR based detection of coagulase gene encoding *Staphylococcus aureus* isolated from subclinical mastitis cases in buffalo. *The Indian Journal of Veterinary Sciences and Biotechnology* **5** 53-54.

Mansour AS, Wagih GE, Morgan SD, Elhariri M, El-Shabrawy MA, Abuelnaga ASM, Elgabry EA 2017: Detection of *Staphylococcus aureus* enterotoxigenic strains in bovine raw milk by reversed passive latex agglutination and multiplex polymerase chain reaction. *Veterinary World* **10** 843-847. http://dx.doi.org/10.14202/vetworld.2017.843-847.

Marai IFM, Haeeb AAM 2010: Buffalo's biological functions as affected by heat stress - A review. *Livestock Science* **127** 89-109. http://dx.doi.org/10.1016/j.livsci.2009.08.001.

Mason IL 1951: A world dictionary of breeds types and varieties of livestock. *Commonwealth Bureau of Animal Breeding and Genetics* **8** 272.

Medeiros ES, Freitas MF, Saukas TN, Azevedo SS, Pinheiro Junior JW, Brandespim DF, Neto OLdS, Mota RA 2011: Risk factors associated with buffalo mastitis in the Brazilian Northeast. *Brazilian Journal of Veterinary Research* **31** 499-504.

Medhammar E, Wijesinha-Bettoni R, Stadlmayr B, Nilsson E, Charrondiere UR, Burlingame B 2012: Composition of milk from minor dairy animals and buffalo breeds: a biodiversity perspective. *Journal of the Science of Food and Agriculture* **92** 445-474. http://dx.doi.org/10.1002/jsfa.4690.

Mee'aad KA-K, Azhar NH, Ali MA-M 2018: Detection of Î²-lactamases producing Methicillin-Resistant *Staphylococcus aureus* (MRSA). *Al-Qadisiyah Journal of Pure Science* **23** http://dx.doi.org/10.29350/jops.2018.23.2.767.

Mehta BM 2009: 21 - Butter, Butter Oil, and Ghee: *Gourmet and Health-Promoting Specialty Oils*. American Oil Chemists' Society Press, Anand-388 110, Gujarat, India. 527-559.

Merle R, Schröder A, Hamann J 2007: Cell function in the bovine mammary gland: a preliminary study on interdependence of healthy and infected udder quarters.

Journal of Dairy Research **74** 174-179. http://dx.doi.org/10.1017/s002202990600238x.

Mhone TA, Matope G, Saidi PT 2011: Aerobic bacterial, coliform, *Escherichia coli* and *Staphylococcus aureus* counts of raw and processed milk from selected smallholder dairy farms of Zimbabwe. *International Journal of Food Microbiology* **151** 223-228. http://dx.doi.org/10.1016/j.ijfoodmicro.2011.08.028.

Minervino AH, Zava M, Vecchio D, Borghese A 2020: *Bubalus bubalis*: A short story. *Frontiers in Veterinary Science* **7** 570413. http://dx.doi.org/10.3389/fvets.2020.570413.

Minervino AHH, Vecchio D, Jorge AM, Headley SA 2021: Editorial: Buffalo health and production. *Frontiers in Veterinary Science* **8** 810923. http://dx.doi.org/10.3389/fvets.2021.810923.

Mistry TB, Patel ND, Shaikh NM 2015: Bacteriological quality of raw buffalo milk from different villages in Bardoli, Gujarat, India. *International Journal of Current Microbiology and Applied Sciences* **4** 874-884.

Moher D, Shamseer L, Clarke M, Ghersi D, Liberati A, Petticrew M, Shekelle P, Stewart LA, Group P-P 2015: Preferred reporting items for systematic review and meta-analysis protocols (PRISMA-P) 2015 statement. *Systematic Reviews* **4** 1. http://dx.doi.org/10.1186/2046-4053-4-1.

Monnet C, Landaud S, Bonnarme P, Swennen D 2015: Growth and adaptation of microorganisms on the cheese surface. *FEMS Microbiology Letters* **362** 1-9. http://dx.doi.org/10.1093/femsle/fnu025.

Moradi M, Omer AK, Razavi R, Valipour S, Guimarães JT 2021: The relationship between milk somatic cell count and cheese production, quality and safety: A review. *International Dairy Journal* **113** 104884. http://dx.doi.org/https://doi.org/10.1016/j.idairyj.2020.104884.

Morgan AD, Brockhurst MA, Lopez-Pascua LD, Pal C, Buckling A 2007: Differential impact of simultaneous migration on coevolving hosts and parasites. *BMC Evolutionary Biology* **7** 1. http://dx.doi.org/10.1186/1471-2148-7-1.

Moroni P, Nydam DV, Ospina PA, Scillieri-Smith JC, Virkler PD, Watters RD, Welcome FL, Zurakowski MJ, Ducharme NG, Yeager AE, 2018. 8 - Diseases of the teats and udder. In: Peek, S.F., Divers, T.J. (Eds.), Rebhun's diseases of dairy cattle (Third Edition). Elsevier, 389-465.

Moroni P, Pisoni G, Antonini M, Ruffo G, Carli S, Varisco G, Boettcher P 2005a: Subclinical mastitis and antimicrobial susceptibility of *Staphylococcus caprae* and *Staphylococcus epidermidis* isolated from two Italian goat herds. *Journal of Dairy Science* **88** 1694-1704. http://dx.doi.org/https://doi.org/10.3168/jds.S0022-0302(05)72841-1.

Moroni P, Pisoni G, Ruffo G, Boettcher PJ 2005b: Risk factors for intramammary infections and relationship with somatic-cell counts in Italian dairy goats. *Preventive Veterinary Medicine* **69** 163-173. http://dx.doi.org/10.1016/j.prevetmed.2004.10.013.

Moroni P, Sgoifo Rossi C, Pisoni G, Bronzo V, Castiglioni B, Boettcher PJ 2006: Relationships between somatic cell count and intramammary infection in buffalo.

Journal of Dairy Science **89** 998-1003. http://dx.doi.org/10.3168/jds.S0022-0302(06)72165-8.

Mpatswenumugabo JPM, Bebora LC, Gitao GC, Mobegi VA, Iraguha B, Shumbusho B 2019: Assessment of bacterial contamination and milk handling practices along the raw milk market chain in the north-western region of Rwanda. *African Journal of Microbiology Research* **13** 640-648.

Muhammad G, Naureen A, Asi MN, Saqib M, Fazal ur R 2010: Evaluation of a 3% surf solution (surf field mastitis test) for the diagnosis of subclinical bovine and bubaline mastitis. *Tropical Animal Health and Production* **42** 457-464. http://dx.doi.org/10.1007/s11250-009-9443-3.

Mujeeb S, Digraskar SU, Awaz KB 2009: Evaluation of buffalo milk with reference to somatic cell count and antitrypsin. *Veterinary World* **2** 267-268.

Murphy SC 1997: Raw milk bacteria tests: Standard plate count, preliminary incubation count, lab, pasteurization count and coliform count. What do they mean for your farm? *National Mastitis Council,* Madison, WI, New York. 34–41.

Nahar A, Mila FA, Culas RJ, Amin MR 2022: Assessing the factors and constraints for value chain development of dairy food products in Bangladesh. *Heliyon* **8** e10787. http://dx.doi.org/10.1016/j.heliyon.2022.e10787.

Nanda AS, Nakao T 2003: Role of buffalo in the socioeconomic development of rural Asia: Current status and future prospectus. *Animal Science Journal* **74** 443-455. http://dx.doi.org/https://doi.org/10.1046/j.1344-3941.2003.00138.x.

Ndahetuye JB, Artursson K, Båge R, Ingabire A, Karege C, Djangwani J, Nyman AK, Ongol MP, Tukei M, Persson Y 2020a: MILK Symposium review: Microbiological quality and safety of milk from farm to milk collection centers in Rwanda. *Journal of Dairy Scienceis* **103** 9730-9739. http://dx.doi.org/10.3168/jds.2020-18302.

Ndahetuye JB, Twambazimana J, Nyman A-K, Karege C, Tukei M, Ongol MP, Persson Y, Båge R 2020b: A cross sectional study of prevalence and risk factors associated with subclinical mastitis and intramammary infections, in dairy herds linked to milk collection centers in Rwanda. *Preventive Veterinary Medicine* **179** 105007. http://dx.doi.org/10.1016/j.prevetmed.2020.105007.

Neculai-Valeanu AS, Ariton AM 2022: Udder health monitoring for prevention of bovine mastitis and improvement of milk quality. *Bioengineering (Basel)* **9** http://dx.doi.org/10.3390/bioengineering9110608.

Ngassam Tchamba C, Rao AS, Boyen F, Haesebrouck F, Duprez JN, Théron L, Thiry D, Mainil JG 2019: Comparison of quantitative PCR and MALDI-TOF mass spectrometry assays for identification of bacteria in milk samples from cows with subclinical mastitis. *Journal of Applied Microbiology* **127** 683-692. http://dx.doi.org/10.1111/jam.14358.

NMC 2022: Recommended 10-point mastitis control program (International version). National Mastitis Council, *New Prague, MN 56071 USA*. Accessed on 16 October 2022. https://www.nmconline.org/wp-content/uploads/2016/08/RECOMMENDED-MASTITIS-CONTROL-PROGRAM-International.pdf.

Nonnemann B, Lyhs U, Svennesen L, Kristensen KA, Klaas IC, Pedersen K 2019: Bovine mastitis bacteria resolved by MALDI-TOF mass spectrometry. *Journal of Dairy Science* **102** 2515-2524. http://dx.doi.org/10.3168/jds.2018-15424.

Norman H, Cooper T, Ross J 2013: Somatic cell counts of milk from Dairy Herd Improvement herds during 2012. United States Department of Agriculture (USDA), Animal Improvement Programs Laboratory (AIPL) Research Report

Norman H, Miller R, Cooper T 2019: Somatic cell counts of milk from Dairy Herd Improvement herds during 2018. United States Department of Agriculture (USDA), Animal Improvement Programs Laboratory (AIPL) Research Report

Oikonomou G, Bicalho ML, Meira E, Rossi RE, Foditsch C, Machado VS, Teixeira AG, Santisteban C, Schukken YH, Bicalho RC 2014: Microbiota of cow's milk; distinguishing healthy, sub-clinically and clinically diseased quarters. *PLoS One* **9** e85904. http://dx.doi.org/10.1371/journal.pone.0085904.

Okano W, Junior CK, Bogado ALG, Negri Filho LC, Bronkhorst DE, Borges MHF, Junior FAB, dos Santos Diniz M, de Santana EHW, da Silva CB 2015: Relationship between shape of teat and teat tip and somatic cell count (SCC) in dairy cows. *Acta Scientiae Veterinariae* **43** 1-6.

Olde Riekerink RG, Barkema HW, Stryhn H 2007: The effect of season on somatic cell count and the incidence of clinical mastitis. *Journal of Dairy Science* **90** 1704-1715. http://dx.doi.org/10.3168/jds.2006-567.

Ombui JN, Kaburia HF, Macharia JK, Nduhiu G 1994: Coliform counts and *Escherichia coli* in raw commercial milk from dairy farmers in Kiambu District, Kenya. *East African Medical Journal* **71** 635-639.

Paixão MG, Abreu LR, Richert R, Ruegg PL 2017: Milk composition and health status from mammary gland quarters adjacent to glands affected with naturally occurring clinical mastitis. *Journal of Dairy Science* **100** 7522-7533. http://dx.doi.org/10.3168/jds.2017-12547.

Panchal I, Sawhney I, Dang A 2016: Relation between electrical conductivity, dielectric constant, somatic cell count and some other milk quality parameters in diagnosis of subclinical mastitis in Murrah buffalo. *Indian Journal of Dairy Science* **69** 267-271.

Pardo BR, Mendoza-Sánchez G, Nader Filho A, Santos T, Langoni H, Tonhati H, Ferreira E, Ravena D, Oliveira M, Sturion D 2007: Microbiological evaluation of milk samples positive to California mastitis Test in dairy buffalo cows (*Buballus bubalis*). *Italian Journal of Animal Science* **6** 884-887.

Parvej MS, Alam MA, Shono M, Zahan MN, Masuma Parvez MM, Ansari WK, Jowel MS, Uddin MS, Kage-Nakadai E, Rahman MT, Nishikawa Y 2020: Prevalence of virulence genes of diarrheagenic *Escherichia coli* in fecal samples obtained from cattle, poultry and diarrheic patients in Bangladesh. *Japanese Journal of Infectious Diseases* **73** 76-82. http://dx.doi.org/10.7883/yoken.JJID.2019.016.

Pasquini M, Osimani A, Tavoletti S, Moreno I, Clementi F, Trombetta MF 2018: Trends in the quality and hygiene parameters of bulk Italian Mediterranean buffalo (*Bubalus bubalis*) milk: A three year study. *Animal Science Journal* **89** 176-185. http://dx.doi.org/10.1111/asj.12916.

Patbandha TK, Marandi S, Ravikala K, Pathak R, Maharana BR, Murthy KS 2015: Association of milk components with intra-mammary inflammation in Jaffrabadi buffalo. *Veterinary World* **8** 989-993. http://dx.doi.org/10.14202/vetworld.2015.989-993.

Patel R, Kunjadia P, Koringa P, Joshi C, Kunjadiya A 2019: Microbiological profiles in clinical and subclinical cases of mastitis in milking Jafarabadi buffalo. *Research in Veterinary Science* **125** 94-99. http://dx.doi.org/10.1016/j.rvsc.2019.05.012.

Paul O, Chowdhury S, Nath S, Singha S, Eima F, Akter S, Bari S, Debnath N, Biswas P, Hoque M, Rahman M, Hossain D, Khatun M, Islam M, Alam M, Ahmed S, Hossain M, Biswas D, Juli S, Karim M, Sayeed M, Hossain M, Amin M, Huq M, Rahman M, Kamaruddin K, Mansor R, Sivaraman S, Ahmed A, Pathirana I, Fernando P, White E, Derks M, Koop G, Persson Y 2019: Clinical mastitis decision tree: A guideline for field veterinarians, and dairy farmers to improve udder health of dairy cows in Bangladesh. *25th BSVER Annual Scientific Conference,* Bangladesh Agricultural University.

Peeler EJ, Green MJ, Fitzpatrick JL, Morgan KL, Green LE 2000: Risk factors associated with clinical mastitis in low somatic cell count British dairy herds. *Journal of Dairy Science* **83** 2464-2472. http://dx.doi.org/10.3168/jds.S0022-0302(00)75138-1.

Pegolo S, Tessari R, Bisutti V, Vanzin A, Giannuzzi D, Gianesella M, Lisuzzo A, Fiore E, Barberio A, Schiavon E, Trevisi E, Piccioli Cappelli F, Gallo L, Ruegg P, Negrini R, Cecchinato A 2022: Quarter-level analyses of the associations among subclinical intramammary infection and milk quality, udder health, and cheesemaking traits in Holstein cows. *Journal of Dairy Science* **105** 3490-3507. http://dx.doi.org/10.3168/jds.2021-21267.

Pérez-Báez J, Risco CA, Chebel RC, Gomes GC, Greco LF, Tao S, Thompson IM, do Amaral BC, Zenobi MG, Martinez N, Staples CR, Dahl GE, Hernández JA, Santos JEP, Galvão KN 2019: Association of dry matter intake and energy balance prepartum and postpartum with health disorders postpartum: Part II. Ketosis and clinical mastitis. *Journal of Dairy Science* **102** 9151-9164. http://dx.doi.org/10.3168/jds.2018-15879.

Petrovski KR, Heuer C, Parkinson TJ, Williamson NB 2009: The incidence and aetiology of clinical bovine mastitis on 14 farms in Northland, New Zealand. *New Zealand Veterinary Journal* **57** 109-115. http://dx.doi.org/10.1080/00480169.2009.36887.

Petzl W, Zerbe H, Günther J, Yang W, Seyfert HM, Nürnberg G, Schuberth HJ 2008: *Escherichia coli*, but not *Staphylococcus aureus* triggers an early increased expression of factors contributing to the innate immune defense in the udder of the cow. *Veterinary Research* **39** 18. http://dx.doi.org/10.1051/vetres:2007057.

Piccinini R, Miarelli M, Ferri B, Tripaldi C, Belotti M, Daprà V, Orlandini S, Zecconi A 2006: Relationship between cellular and whey components in buffalo milk. *Journal of Dairy Research* **73** 129-133. http://dx.doi.org/10.1017/s0022029905001548.

Pisanu S, Cacciotto C, Pagnozzi D, Puggioni GMG, Uzzau S, Ciaramella P, Guccione J, Penati M, Pollera C, Moroni P, Bronzo V, Addis MF 2019: Proteomic changes in the milk of water buffalo (*Bubalus bubalis*) with subclinical mastitis due to

intramammary infection by *Staphylococcus aureus* and by non-aureus staphylococci. *Scientific Reports* **9** 15850. http://dx.doi.org/10.1038/s41598-019-52063-2.

Pizauro L, Silva D, Santana A, Clemente V, Lara G, Listoni F, Vaz A, Vidal-Martins A, Ribeiro M, Fagliari J 2014: Prevalence and etiology of buffalo mastitis and milk somatic cell count in dry and rainy seasons in a buffalo herd from Analândia, São Paulo State, Brazil. *Arquivo Brasileiro de Medicina Veterinária e Zootecnia* **66** 1703-1710.

Pizauro LJL, de Almeida CC, Soltes GA, Slavic D, de Ávila FA, Zafalon LF, MacInnes JI 2019: Short communication: Detection of antibiotic resistance, mecA, and virulence genes in coagulase-negative *Staphylococcus* spp. from buffalo milk and the milking environment. *Journal of Dairy Science* **102** 11459-11464. http://dx.doi.org/10.3168/jds.2018-15920.

Prack McCormick B, Quiroga MP, Álvarez VE, Centrón D, Tittonell P 2023: Antimicrobial resistance dissemination associated with intensive animal production practices in Argentina: A systematic review and meta-analysis. *Revista Argentina de Microbiología* **55** 25-42. http://dx.doi.org/10.1016/j.ram.2022.07.001.

Prasad A, Kothari N 2021: Cow products: boon to human health and food security. *Tropical Animal Health and Production* **54** 12. http://dx.doi.org/10.1007/s11250-021-03014-5.

Prasad R, Rao ER, Sudhakar K, Gupta BR, Mahender M 2010: Studies on udder and teat measurements as affected by parity and their relationship with milk yield in Murrah buffalo. *Buffalo Bulletin* **29** 194-198.

Preethirani PL, Isloor S, Sundareshan S, Nuthanalakshmi V, Deepthikiran K, Sinha AY, Rathnamma D, Nithin Prabhu K, Sharada R, Mukkur TK, Hegde NR 2015: Isolation, biochemical and molecular identification, and in-vitro antimicrobial resistance patterns of bacteria isolated from bubaline subclinical mastitis in south India. *PLoS One* **10** e0142717. http://dx.doi.org/10.1371/journal.pone.0142717.

Presicce G, Baruselli P, Bifulco G, Bosco A, Braghieri A, Chaiyabutr N, Chanthakhoun V, Cringoli G, Cruz L, Deb G, Rosa G, Gasparrini B, Grasso F, Iannuzzi A, Iannuzzi L, Khejornsart P, Kong I-K, Maurelli M, Mingala C, Zicarelli L 2017: *The Buffalo (Bubalus bubalis) - Production and Research*. Bentham Sci., Sharjah, U.A.E.

Puggioni GMG, Tedde V, Uzzau S, Guccione J, Ciaramella P, Pollera C, Moroni P, Bronzo V, Addis MF 2020: Evaluation of a bovine cathelicidin ELISA for detecting mastitis in the dairy buffalo: Comparison with milk somatic cell count and bacteriological culture. *Research in Veterinary Science* **128** 129-134. http://dx.doi.org/10.1016/j.rvsc.2019.11.009.

Purohit G, Gaur M, Shekher C 2014: Mammary gland pathologies in the parturient buffalo. *Asian Pacific Journal of Reproduction* **3** 322-336.

Putz EJ, Palmer MV, Ma H, Casas E, Reinhardt TA, Lippolis JD 2020: Case report: characterization of a persistent, treatment-resistant, novel Staphylococcus aureus infection causing chronic mastitis in a Holstein dairy cow. *BMC Veterinary Research* **16** 336. http://dx.doi.org/10.1186/s12917-020-02528-8.

Pyörälä S 2003: Indicators of inflammation in the diagnosis of mastitis. *Veterinary Research* **34** 565-578. http://dx.doi.org/10.1051/vetres:2003026.

Pyz-Łukasik R, Paszkiewicz W, Tatara MR, Brodzki P, Bełkot Z 2015: Microbiological quality of milk sold directly from producers to consumers. *Journal of Dairy Science* **98** 4294-4301. http://dx.doi.org/10.3168/jds.2014-9187.

Qayyum A, Arshad U, Yousuf MR, Ahmad N 2018: Effect of breeding method and season on pregnancy rate and embryonic and fetal losses in lactating Nili-Ravi buffalo. *Tropical Animal Health and Production* **50** 555-560. http://dx.doi.org/10.1007/s11250-017-1468-4.

Raeisi M, Khoshbakht R, Ghaemi EA, Bayani M, Hashemi M, Seyedghasemi NS, Shirzad-Aski H 2017: Antimicrobial resistance and virulence-associated genes of *Campylobacter* spp. isolated from raw milk, fish, poultry, and red meat. *Microbial Drug Resistance* **23** 925-933. http://dx.doi.org/10.1089/mdr.2016.0183.

Rahimi E, Doosti A, Ameri M, Kabiri E, Sharifian B 2010: Detection of *Coxiella burnetii* by nested PCR in bulk milk samples from dairy bovine, ovine, and caprine herds in Iran. *Zoonoses Public Health* **57** e38-41. http://dx.doi.org/10.1111/j.1863-2378.2009.01289.x.

Rahimi E, Sepehri S, Dehkordi FS, Shaygan S, Momtaz H 2014: Prevalence of *Yersinia* species in traditional and commercial dairy products in Isfahan province, Iran. *Jundishapur Journal of Microbiology* **7** http://dx.doi.org/10.5812/jjm.9249.

Rahman SM, Islam M, Harun-ur-Rashid M, Siddiki M, Islam M 2019a: Dairy buffalo production under intensive system in semi arid area of Bangladesh. *Buffalo Bulletin* **38** 83-98.

Rahman SR, Islam MN, Harun-ur-Rashid M, Sarker NR, Siddiki MSR, Islam MZ, Islam MA 2019b: Buffalo milk yield, quality, and marketing in different agro-climatic districts of Bangladesh. *Journal of Buffalo Science* **8** 62-67. http://dx.doi.org/10.6000/1927-520X.2019.08.03.2.

Rai P, Swain S, Talreja P 2020: Community acceptance of available milk and assessment of its quality in peri-urban area of southwest Delhi. *Journal of Family and Community Medicine* **45** S31-s34. http://dx.doi.org/10.4103/ijcm.IJCM_367_19.

Rainard P, Riollet C 2006: Innate immunity of the bovine mammary gland. *Veterinary Research* **37** 369-400. http://dx.doi.org/10.1051/vetres:2006007.

Ranjbar R, Safarpoor Dehkordi F, Sakhaei Shahreza MH, Rahimi E 2018: Prevalence, identification of virulence factors, O-serogroups and antibiotic resistance properties of Shiga-toxin producing *Escherichia coli* strains isolated from raw milk and traditional dairy products. *Antimicrobial Resistance and Infection Control* **7** 53. http://dx.doi.org/10.1186/s13756-018-0345-x.

Rathore AK, Sheldrake RF 1977: Teat orifice stretchability associated with teat diameter gradient and milk yield in lactating cows. *Journal of Animal Science* **24** 215-220. http://dx.doi.org/10.1017/S0003356100011685.

Restucci B, Dipineto L, Martano M, Balestrieri A, Ciccarelli D, Russo TP, Varriale L, Maiolino P 2019: Histopathological and microbiological findings in buffalo chronic mastitis: evidence of tertiary lymphoid structures. *Journal of Veterinary Science* **20** e28. http://dx.doi.org/10.4142/jvs.2019.20.e28.

Romera AJ, Bos AP, Neal M, Eastwood CR, Chapman D, McWilliam W, Royds D, O'Connor C, Brookes R, Connolly J, Hall P, Clinton PW 2020: Designing future dairy systems for New Zealand using reflexive interactive design. *Agricultural Systems* **181** 102818. http://dx.doi.org/10.1016/j.agsy.2020.102818.

Rosa NM, Penati M, Fusar-Poli S, Addis MF, Tola S 2022: Species identification by MALDI-TOF MS and gap PCR-RFLP of non-aureus *Staphylococcus*, *Mammaliicoccus*, and *Streptococcus* spp. associated with sheep and goat mastitis. *Veterinary Research* **53** 84. http://dx.doi.org/10.1186/s13567-022-01102-4.

Rowe SM, Godden SM, Royster E, Timmerman J, Crooker BA, Boyle M 2019: Cross-sectional study of the relationships among bedding materials, bedding bacteria counts, and intramammary infection in late-lactation dairy cows. *Journal of Dairy Science* **102** 11384-11400. http://dx.doi.org/10.3168/jds.2019-17074.

Ruegg PL 2017: A 100-Year Review: Mastitis detection, management, and prevention. *Journal of Dairy Science* **100** 10381-10397. http://dx.doi.org/https://doi.org/10.3168/jds.2017-13023.

Saadullah M 2012: Buffalo production and constraints in Bangladesh. *The Journal of Animal and Plant Sciences* **22** 221-224.

Sah K, Karki P, Shrestha RD, Sigdel A, Adesogan AT, Dahl GE 2020: MILK Symposium review: Improving control of mastitis in dairy animals in Nepal. *Journal of Dairy Science* **103** 9740-9747. http://dx.doi.org/10.3168/jds.2020-18314.

Sahin A, Yildirim A, Ulutas Z, E U 2017: The effects of stage of lactation, parity and calving season on somatic cell counts in Anatolian water buffalo. *Indian Journal of Animal Research* **51** 35-39.

Sahin A, Yıldırım A, Ulutaş Z 2016: Effect of various environmental factors and management practices on somatic cell count in the raw milk of anatolian buffalo. *Pakistan Journal of Zoology* **48** 325-332.

Sahoo G, More T, Singh VK 1998: A comparative study on certain enzymes of the granulocyte from different ruminant species. *Comparative Immunology, Microbiology and Infectious Diseases* **21** 319-325. http://dx.doi.org/10.1016/s0147-9571(97)00029-5.

Sahoo N, Kumar P, Bhushan B, Bhattacharya T, Sharma A, Dayal S, Pankaj DP, Sahoo M 2010: PCR-SSCP of serum lysozyme gene (Exon-III) in riverine buffalo and its association with lysozyme activity and somatic cell count. *Asian Australasian Journal of Animal Sciences* **23** 993. http://dx.doi.org/10.5713/ajas.2010.90323.

Salvador RT, Beltran JM, Abes NS, Gutierrez CA, Mingala CN 2012: Short communication: Prevalence and risk factors of subclinical mastitis as determined by the California mastitis test in water buffalo (*Bubalis bubalis*) in Nueva Ecija, Philippines. *Journal of Dairy Science* **95** 1363-1366. http://dx.doi.org/10.3168/jds.2011-4503.

Salvador RT, Garcia AAA, Abes NS, Mingala CN 2013: Correlation of California mastitis test and somatic cell count on milk of water buffalo cows in the Philippines. *Tropical Agriculture* **90** 153-159.

Samad M 2020: A systematic review of research findings on buffalo health and production published during the last six decades in Bangladesh. *Journal of*

Veterinary Medical and One Health Research **2** 1-62. http://dx.doi.org/10.36111/jvmohr.2020.2(1).0016.

Sanaa M, Poutrel B, Menard JL, Serieys F 1993: Risk factors associated with contamination of raw milk by *Listeria monocytogenes* in dairy farms. *Journal of Dairy Science* **76** 2891-2898. http://dx.doi.org/https://doi.org/10.3168/jds.S0022-0302(93)77628-6.

Sannino M, Faugno S, Crimaldi M, Di Francia A, Ardito L, Serrapica F, Masucci F 2018: Effects of an automatic milking system on milk yield and quality of Mediterranean buffalo. *Journal of Dairy Science* **101** 8308-8312. http://dx.doi.org/10.3168/jds.2017-14157.

Santos AS, Lima DCV, Abad ACA, Oliveira PRF, Silva JGD, Moura GS, Silva ATF, Amorim VDS, Costa MMD, Mota RA 2020: Antimicrobial resistance profile of non-aureus staphylococci isolates from buffalo, goat and sheep mastitis in the Northeast region of Brazil. *Journal of Dairy Research* **87** 290-294. http://dx.doi.org/10.1017/s0022029920000771.

Sargeant JM, Schukken YH, Leslie KE 1998: Ontario bulk milk somatic cell count reduction program: progress and outlook. *Journal of Dairy Science* **81** 1545-1554. http://dx.doi.org/10.3168/jds.S0022-0302(98)75720-0.

Schröder J, Glaub A, Schneider J, Trost E, Tauch A 2012: Draft genome sequence of *Corynebacterium bovis* DSM 20582, which causes clinical mastitis in dairy cows. *Journal of Bacteriology* **194** 4437. http://dx.doi.org/10.1128/jb.00839-12.

Schukken YH, González RN, Tikofsky LL, Schulte HF, Santisteban CG, Welcome FL, Bennett GJ, Zurakowski MJ, Zadoks RN 2009: CNS mastitis: nothing to worry about? *Veterinary Microbiology* **134** 9-14. http://dx.doi.org/10.1016/j.vetmic.2008.09.014.

Schukken YH, Günther J, Fitzpatrick J, Fontaine MC, Goetze L, Holst O, Leigh J, Petzl W, Schuberth HJ, Sipka A, Smith DG, Quesnell R, Watts J, Yancey R, Zerbe H, Gurjar A, Zadoks RN, Seyfert HM 2011: Host-response patterns of intramammary infections in dairy cows. *Veterinary Immunology and Immunopathology* **144** 270-289. http://dx.doi.org/10.1016/j.vetimm.2011.08.022.

Schwarz DGG, de Sousa Júnior PF, Saraiva da Silva L, Polveiro RC, de Oliveira JF, Faria MPO, Marinho G, de Oliveira RP, Moreira MAS 2021: Spatiotemporal distribution and temporal trends of brucellosis and tuberculosis in water buffalo (*Bubalus bubalis*) in Brazil. *Preventive Veterinary Medicine* **193** 105417. http://dx.doi.org/10.1016/j.prevetmed.2021.105417.

Selim A, Kelis K, AlKahtani MDF, Albohairy FM, Attia KA 2022: Prevalence, antimicrobial susceptibilities and risk factors of Methicillin resistant Staphylococcus aureus (MRSA) in dairy bovines. *BMC Veterinary Research* **18** 293. http://dx.doi.org/10.1186/s12917-022-03389-z.

Sentitula, Yadav BR, Kumar R 2012: Incidence of staphylococci and streptococci during winter in mastitic milk of sahiwal cow and Murrah buffalo. *Indian Journal of Microbiology* **52** 153-159. http://dx.doi.org/10.1007/s12088-011-0207-1.

Serraino A, Florio D, Giacometti F, Piva S, Mion D, Zanoni RG 2013: Presence of *Campylobacter* and *Arcobacter* species in in-line milk filters of farms authorized

to produce and sell raw milk and of a water buffalo dairy farm in Italy. *Journal of Dairy Science* **96** 2801-2807. http://dx.doi.org/10.3168/jds.2012-6249.

Seyoum ET, Woldetsadik DA, Mekonen TK, Gezahegn HA, Gebreyes WA 2015: Prevalence of *Listeria monocytogenes* in raw bovine milk and milk products from central highlands of Ethiopia. *Journal of Infection in Developing Countries* **9** 1204-1209. http://dx.doi.org/10.3855/jidc.6211.

Shaban AK, Mohamed RH, Zakaria AM, Baheeg EM 2022: Detection of foot-and-mouth disease virus in raw milk in Menofia Governorate and its effect on reproductive hormones and physiochemical properties of milk. *Veterinary World* **15** 2202-2209. http://dx.doi.org/10.14202/vetworld.2022.2202-2209.

Shailja, Singh M 2002: Post milking teat dip effect on somatic cell count, milk production and composition in cows and buffalo. *Asian-Australasian Journal of Animal Sciences* **15** 1517-1522. http://dx.doi.org/10.5713/ajas.2002.1517.

Sharif A, Ahmad T 2007: Prevalence of severity of mastitis in buffalo in district Faisalabad (Pakistan). *The Journal of Agricultural Science* **3** 34-36.

Sharif A, Ahmad T, Bilal MQ, Yousaf A, Muhammad G, Pansota F, Biology 2007: Estimation of milk lactose and somatic cells for the diagnosis of sub-clinical mastitis in dairy buffalo. *International Journal of Agriculture and Biology* **9** 267-270.

Sharma A, Sindhu N 2007: Occurrence of clinical and subclinical mastitis in buffalo in the State of Haryana (India). *Italian Journal of Animal Science* **6** 965-967.

Sharma N, K M, Sharma K 2007: Prevalence, etiology and antibiogram of microorganisms associated with sub-clinical mastitis in buffalo in durg, Chhattisgarh state (India). *International Journal of Dairy Science* **2** 145-151. http://dx.doi.org/10.3923/ijds.2007.145.151.

Sharma N, Rho GJ, Hong YH, Kang TY, Lee HK, Hur T-Y, Jeong DK 2012: Bovine mastitis: an Asian perspective. *Asian Journal of Animal and Veterinary Advances* **7** 454-476. http://dx.doi.org/10.3923/ajava.2012.454.476.

Sharma N, Singh NK, Bhadwal MS 2011: Relationship of somatic cell count and mastitis: An overview. *Asian-Australasian Journal of Animal Sciences* **24** 429-438. http://dx.doi.org/10.5713/ajas.2011.10233.

Siddiky M, Faruque M 2018: Buffalo for dairying in South Asia: potential, challenges and way forward. *SAARC Journal of Agriculture* **15** 227-239. http://dx.doi.org/https://doi.org/10.3329/sja.v15i2.35167.

Sindhu N, Anshu S, Vikas N, Jain V 2009: Occurrence of subclinical mastitis in cows and buffalo at an organised farm. *Haryana Veterinarian* **48** 85-87.

Singh M, Sharma A, Mittal D, Charaya G 2014: Prevalence and characterization of coagulase negative staphylococci associated with buffalo mastitis. *Indian Journal of Comparative Microbiology, Immunology and Infectious Diseases* **35** 67-72.

Singha S, Ericsson CD, Chowdhury S, Nath SC, Paul OB, Hoque MA, Boqvist S, Persson Y, Rahman MM 2021a: Occurrence and aetiology of subclinical mastitis in water buffalo in Bangladesh. *Journal of Dairy Research* **88** 314-320. http://dx.doi.org/10.1017/s0022029921000698.

Singha S, Koop G, Ceciliani F, Derks M, Hoque MA, Hossain MK, Howlader MMR, Rahman MM, Khatun M, Boqvist S, Persson Y 2023: The prevalence and risk factors of subclinical mastitis in water buffalo (*Bubalis bubalis*) in Bangladesh. *Research in Veterinary Science* **158** 17-25. http://dx.doi.org/10.1016/j.rvsc.2023.03.004.

Singha S, Koop G, Persson Y, Hossain D, Scanlon L, Derks M, Hoque MA, Rahman MM 2021b: Incidence, etiology, and risk factors of clinical mastitis in dairy cows under semi-tropical circumstances in Chattogram, Bangladesh. *Animals* **11** http://dx.doi.org/10.3390/ani11082255.

Singha S, Persson Y, Datta PK, Ceciliani F, Rahman M 2021c: Traditional buffalo milk chain in Bangladesh. IDF Animal Health Report, Report, International Dairy Federation, *Boulevard Auguste Reyers, 1030 Brussels, Belgium*. Accessed on 07 October 2022.

Sinha MK, Thombare NN, Mondal B 2014: Subclinical mastitis in dairy animals: incidence, economics, and predisposing factors. *Scientific World Journal* **2014** 523984. http://dx.doi.org/10.1155/2014/523984.

Smistad M, Vatne MK, Sølverød L, Dean KR 2022: Sensitivity and specificity of bacterial culture, qPCR, and somatic cell count for detection of goats with Staphylococcus aureus intramammary infection using Bayesian latent class models. *Preventive Veterinary Medicine* **209** 105793. http://dx.doi.org/10.1016/j.prevetmed.2022.105793.

Smith EM, Green LE, Medley GF, Bird HE, Dowson CG 2005: Multilocus sequence typing of *Staphylococcus aureus* isolated from high-somatic-cell-count cows and the environment of an organic dairy farm in the United Kingdom. *Journal of Clinical Microbiology* **43** 4731-4736. http://dx.doi.org/10.1128/jcm.43.9.4731-4736.2005.

Sohel MMH, Amin MR, Fisheries 2015: Identification of types of buffalo available in Kanihari buffalo pocket of Mymensingh district. *Research in Agriculture Livestock and Fisheries* **2** 109-115.

Sonnier JL, Karns JS, Lombard JE, Kopral CA, Haley BJ, Kim SW, Van Kessel JAS 2018: Prevalence of *Salmonella enterica*, *Listeria monocytogenes*, and pathogenic *Escherichia coli* in bulk tank milk and milk filters from US dairy operations in the National Animal Health Monitoring System Dairy 2014 study. *Journal of Dairy Science* **101** 1943-1956. http://dx.doi.org/10.3168/jds.2017-13546.

Sordillo LM 2016: Nutritional strategies to optimize dairy cattle immunity. *Journal of Dairy Science* **99** 4967-4982. http://dx.doi.org/10.3168/jds.2015-10354.

Srinivasan P, Jagadeswaran D, Manoharan R, Giri T, Balasubramaniam G, Balachandran P 2013: Prevalence and etiology of subclinical mastitis among buffalo (*Bubalus bubalus*) in Namakkal, India. *Pakistan Journal of Biological Sciences* **16** 1776-1780.

Stetca G, Mocuta N, Chindris V, Coldea TE, Morea A 2015: Microbiological analysis on Romanian dairy products obtained from buffalo milk. *Bulletin of University of Agricultural Sciences and Veterinary Medicine Cluj-Napoca-Food Science and Technology* **72** 279-280. http://dx.doi.org/10.15835/buasvmcn-fst:11546.

Stevens M, Piepers S, Supré K, De Vliegher S 2018: Antimicrobial consumption on dairy herds and its association with antimicrobial inhibition zone diameters of non-aureus staphylococci and *Staphylococcus aureus* isolated from subclinical mastitis. *Journal of Dairy Science* **101** 3311-3322. http://dx.doi.org/10.3168/jds.2017-13365.

Sultana Z 2018: Animal breeding policies and strategies in South Asia. Accessed on 02 July 2022. https://www.sac.org.bd/archives/publications/Animal%20Breeding%20Policies%20and%20Strategies.pdf.

Suriyasathaporn W, Schukken YH, Nielen M, Brand A 2000: Low somatic cell count: a risk factor for subsequent clinical mastitis in a dairy herd. *Journal of Dairy Science* **83** 1248-1255. http://dx.doi.org/10.3168/jds.S0022-0302(00)74991-5.

Svennesen L, Nielsen SS, Mahmmod YS, Krömker V, Pedersen K, Klaas IC 2019: Association between teat skin colonization and intramammary infection with *Staphylococcus aureus* and *Streptococcus agalactiae* in herds with automatic milking systems. *Journal of Dairy Science* **102** 629-639. http://dx.doi.org/10.3168/jds.2018-15330.

Talukder AA, Rahman HH, Mahmud SMJ, Alam F, Dey SK 2016: Isolation, identification and resistance pattern of microorganisms associated with mastitis in buffalo. *Bangladesh Journal of Microbiology* **30** 1-5. http://dx.doi.org/10.3329/bjm.v30i1-2.28445.

Tassew H, Abdissa A, Beyene G, Gebre-Selassie S 2010: Microbial flora and food borne pathogens on minced meat and their susceptibility to antimicrobial agents. *Ethiopian-Journal of Health Sciences* **20** 137-143. http://dx.doi.org/10.4314/ejhs.v20i3.69442.

Terzi Gulel G, Gucukoglu A, Cadirci O, Saka E, Alisarli M 2020: Serotyping and antibiotic resistance of Listeria monocytogenes isolated from raw water buffalo milk and milk products. *Journal of Food Science* **85** 2889-2895. http://dx.doi.org/10.1111/1750-3841.15376.

Thomas CS, Svennersten-Sjaunja K, Bhosrekar MR, Bruckmaier RM 2004: Mammary cisternal size, cisternal milk and milk ejection in Murrah buffalo. *The Journal of Dairy Research* **71** 162-168. http://dx.doi.org/10.1017/s0022029904000081.

Tigabu E, Asrat D, Kassa T, Sinmegn T, Molla B, Gebreyes W 2015: Assessment of risk factors in milk contamination with *Staphylococcus aureus* in urban and peri-urban small-holder dairy farming in central Ethiopia. *Zoonoses Public Health* **62** 637-643. http://dx.doi.org/https://doi.org/10.1111/zph.12199.

Tiwari B, Subedi D, Bhandari S 2022: Prevalence and risk factors of Staphylococcal subclinical mastitis in dairy animals of Chitwan, Nepal. *Journal of Pure and Applied Microbiology* http://dx.doi.org/10.22207/JPAM.16.2.67.

Tiwari R, Sharma M, Singh B 2007: Major problems of small holder buffalo production on-farms in India. *The Indian Journal of Animal Sciences* **77**

Tiwari U, Cummins E, Valero A, Walsh D, Dalmasso M, Jordan K, Duffy G 2015: Farm to fork quantitative risk assessment of *Listeria monocytogenes* contamination in raw and pasteurized milk cheese in Ireland. *Risk Analysis* **35** 1140-1153. http://dx.doi.org/10.1111/risa.12332.

Tolosa T, Verbeke J, Ayana Z, Piepers S, Supré K, De Vliegher S 2015: Pathogen group-specific risk factors for clinical mastitis, intramammary infection and blind quarters at the herd, cow and quarter level in smallholder dairy farms in Jimma, Ethiopia. *Preventive Veterinary Medicine* **120** 306-312. http://dx.doi.org/10.1016/j.prevetmed.2015.05.001.

Tong SC, Siow LF, Tang TK, Lee YY 2022: Plant-based milk: Unravel the changes of the antioxidant index during processing and storage - a review. *Critical Reviews in Food Science and Nutrition* 1-19. http://dx.doi.org/10.1080/10408398.2022.2143477.

Traversari J, van den Borne BHP, Dolder C, Thomann A, Perreten V, Bodmer M 2019: Non-aureus staphylococci species in the teat canal and milk in four commercial Swiss dairy herds. *Frontiers in Veterinary Science* **6** 186. http://dx.doi.org/10.3389/fvets.2019.00186.

Triwulanningsih E, Praharani L 2006: Buffaloes in Indonesia. Proceedings of the International Seminar on Reproductive Biotechnology for Buffalo, Bogor, Indonesia. 116-120.

Troendle JA, Tauer LW, Gröhn YT 2017: Optimally achieving milk bulk tank somatic cell count thresholds. *Journal of Dairy Science* **100** 731-738. http://dx.doi.org/10.3168/jds.2016-11578.

Tsuji T 2019: Traditional water buffalo milk production and consumption in southeast Asia. *SAARC Journal of Agriculture* **15** 227-239. http://dx.doi.org/10.3329/sja.v15i2.35167.

Turutoglu H, Hasoksuz M, Ozturk D, Yildirim M, Sagnak S 2009: Methicillin and aminoglycoside resistance in Staphylococcus aureus isolates from bovine mastitis and sequence analysis of their mecA genes. *Veterinary Research Communications* **33** 945-956. http://dx.doi.org/10.1007/s11259-009-9313-5.

Uddin MK, Mintoo AA, Awal TM, Kondo M, Kabir A 2016: Characterization of buffalo milk production system in Bangladesh. *Bangladesh Journal of Animal Sciencekha* **45** 69-77. http://dx.doi.org/10.3329/bjas.v45i1.27492.

Uddin MM, Sultana MN, Ndambi OA, Shawabkeh OA, Hemme T, Peters KJ 2011: Milk production trends and dairy development in Bangladesh. *Outlook on Agriculture* **40** 263-271.

Urer EK, Aslantaş Ö, Tek E, Yılmaz MA, Ergün Y 2023: Antimicrobial susceptibility and biofilm forming ability of staphylococci from subclinical buffalo mastitis. *Journal of Dairy Research* 1-4. http://dx.doi.org/10.1017/s0022029923000080.

Valckenier D, Piepers S, De Visscher A, De Vliegher S 2020: The effect of intramammary infection in early lactation with non-aureus staphylococci in general and *Staphylococcus chromogenes* specifically on quarter milk somatic cell count and quarter milk yield. *Journal of Dairy Science* **103** 768-782. http://dx.doi.org/10.3168/jds.2019-16818.

Valckenier D, Piepers S, Schukken YH, De Visscher A, Boyen F, Haesebrouck F, De Vliegher S 2021: Longitudinal study on the effects of intramammary infection with non-aureus staphylococci on udder health and milk production in dairy heifers. *Journal of Dairy Science* **104** 899-914. http://dx.doi.org/10.3168/jds.2020-18685.

Van den Crommenacker-Konings L, Van Dam P, Everts R, Shittu A, Nielen M, Lam T, Koop G 2021: Dynamics of intramammary infections in suckler ewes during early lactation. *Journal of Dairy Science* **104** 5979-5987. http://dx.doi.org/10.3168/jds.2020-19865.

Vega RS, Del Barrio AN, Sangel PP, Katsube O, Canaria JC, Herrera JV, Lapitan RM, Orden EA, Fujihara T, Kanai Y 2010: Eating and rumination behaviour in Brahman grade cattle and crossbred water buffalo fed on high roughage diet. *Animal Science Journal* **81** 574-579. http://dx.doi.org/10.1111/j.1740-0929.2010.00784.x.

Verraes C, Vlaemynck G, Van Weyenberg S, De Zutter L, Daube G, Sindic M, Uyttendaele M, Herman L 2015: A review of the microbiological hazards of dairy products made from raw milk. *International Dairy Journal* **50** 32-44. http://dx.doi.org/10.1016/j.idairyj.2015.05.011.

Viçosa GN, Moraes PM, Yamazi AK, Nero LA 2010: Enumeration of coagulase and thermonuclease-positive *Staphylococcus* spp. in raw milk and fresh soft cheese: An evaluation of Baird-Parker agar, Rabbit Plasma Fibrinogen agar and the Petrifilm™ Staph Express count system. *Food Microbiology* **27** 447-452. http://dx.doi.org/10.1016/j.fm.2009.12.007.

Viechtbauer W 2010: Conducting meta-analyses in R with the metafor package. *Journal of Statistical Software* **1** 1-48. http://dx.doi.org/10.18637/jss.v036.i03.

Vu-Khac H, Cornick NA 2008: Prevalence and genetic profiles of Shiga toxin-producing *Escherichia coli* strains isolated from buffalo, cattle, and goats in central Vietnam. *Veterinary Microbiology* **126** 356-363. http://dx.doi.org/10.1016/j.vetmic.2007.07.023.

Vyas D, Nelson CD, Bromfield JJ, Liyanamana P, Krause M, Dahl GE 2020: MILK Symposium review: Identifying constraints, opportunities, and best practices for improving milk production in market-oriented dairy farms in Sri Lanka. *Journal of Dairy Science* **103** 9774-9790. http://dx.doi.org/10.3168/jds.2020-18305.

Waak E, Tham W, Danielsson-Tham ML 2002: Prevalence and fingerprinting of *Listeria monocytogenes* strains isolated from raw whole milk in farm bulk tanks and in dairy plant receiving tanks. *Applied and Environmental Microbiology* **68** 3366-3370. http://dx.doi.org/10.1128/aem.68.7.3366-3370.2002.

Wang Z, Wu Y, Shi L, Cui L, Li X, He C, Yang L, Li X 2020: Evaluation and mining the applicable methods of roughage digestibility determination for buffalo (*Bubalus bubalis*). *Tropical Animal Health and Production* **52** 2639-2646. http://dx.doi.org/10.1007/s11250-020-02292-9.

White DG, Harmon RJ, Matos JE, Langlois BE 1989: Isolation and identification of coagulase-negative *Staphylococcus* species from bovine body sites and streak canals of nulliparous heifers. *Journal of Dairy Science* **72** 1886-1892. http://dx.doi.org/10.3168/jds.S0022-0302(89)79307-3.

WHO 2022: Food safety. World Health Organization, *Geneva, Switzerland*. Accessed on 12 August 2022. https://www.who.int/news-room/fact-sheets/detail/food-safety.

Worldbank 2022: Livestock and dairy development project. World Bank Group, *1818 H Street, NW Washington, DC 20433 USA*. Accessed on 10 January, 2023. https://projects.worldbank.org/en/projects-operations/project-detail/P161246.

Wuytack A, De Visscher A, Piepers S, Haesebrouck F, De Vliegher S 2020: Fecal non-aureus Staphylococci are a potential cause of bovine intramammary infection. *Veterinary Research* **51** 32. http://dx.doi.org/10.1186/s13567-020-00761-5.

Xu J, Tan X, Zhang X, Xia X, Sun H 2015: The diversities of staphylococcal species, virulence and antibiotic resistance genes in the subclinical mastitis milk from a single Chinese cow herd. *Microbial Pathogenesis* **88** 29-38. http://dx.doi.org/10.1016/j.micpath.2015.08.004.

Yadav P, Yadav B, Swain DK, Anand M, Yadav S, Madan AK 2021: Differential expression of miRNAs and related mRNAs during heat stress in buffalo heifers. *Journal of Thermal Biology* **97** 102904. http://dx.doi.org/10.1016/j.jtherbio.2021.102904.

Yang BZ, Zhang C 2006: Buffalo crossbreeding in China. *Asian Buffalo Magazine* **3** 4-10.

Yigit A, Cinar M, Macun HC, Ozenc E, Kabakçi R, Yazici E, Kalender H, Bastan A, Salar S 2018: Total oxidant and antioxidant activities in milk with various somatic cell count intervals during discrete cow and buffalo lactation periods. *Journal of Dairy Research* **71** 72-77.

Zapata-Salas R, Guarín JF, Ríos-Osorio LA 2022: Udder health, conceptual construct, and uses of the term: A systematic review from 1962 to 2019. *Veterinary World* **15** 855-869. http://dx.doi.org/10.14202/vetworld.2022.855-869.

Zhang X, Ahmad MJ, An Z, Niu K, Wang W, Nie P, Gao S, Yang L 2022: Relationship between somatic cell counts and mammary gland parenchyma ultrasonography in buffalo. *Frontiers in Veterinary Science* **9** 842105. http://dx.doi.org/10.3389/fvets.2022.842105.

Zhang Y, Colli L, Barker JSF 2020: Asian water buffalo: domestication, history and genetics. *Animal Genetics* **51** 177-191. http://dx.doi.org/https://doi.org/10.1111/age.12911.

Zigo F, Vasil M, Ondrašovičová S, Výrostková J, Bujok J, Pecka-Kielb E 2021: Maintaining optimal mammary gland health and prevention of mastitis. *Frontiers in Veterinary Science* **8** 607311. http://dx.doi.org/10.3389/fvets.2021.607311.

APPENDICES

APPENDICES

12.1 Questionnaire for udder health (farm level variables)

Survey questionnaire for assessing risk factors of SCM in buffaloes in intensive and free ranging system Bangladesh

Objective:

Identification of risk factors associated with subclinical mastitis in Buffalo

Questionnaire sheet ID [ ]

Declaration: I have answered all the questions in the interview sheet and I have full consent about the information given. Best of my Knowledge, the information given by me is correct and can be used in research. If necessary the researcher can contact me for further information or vice versa in future.

==========

Interviewee signature

Name of the Interviewer: ……

Farm/Bathan ID:…… **Date:** …../…../ 20…..

1. Participant information

1.1 Name of the Interviewee:			1.2 Mobile no:	
1.3 Gender:	☐ Male	☐ Female	1.3.1 Age:	
1.4 Title of Interviewee:	☐ Owner	☐ Manager	☐ Worker	☐ Other ……..
1.5 Education:	☐ Illiterate	☐ Primary	☐ Secondary	☐ Graduation
1.6 Farm owner's communication with staffs	☐ Good contact with the staffs ☐ Irregularly come to see the farm ☐ Scarcely come to the farm ☐ Others *(Please specify)*:……			

2. Demographic features of farm/Bathan

2.1 Farm / Bathan name:		
2.2 Farm zone: ☐ Coastal ☐ Semi-coastal ☐ River basin ☐ Inland ☐ Others (*Please specify*): …… Coastal: Permanently stay in the coastal area Semi-costal: Partly the animals stay in coastal area and partly in home River basin: The animals remains in riverine area Inland: The animals remain in fresh water area not connected to river		
2.3 Farm Location		
2.3.1 Village/Ward:		
2.3.2 Upazila:	2.3.3 District:	
2.4 GPS co-ordinates		
2.4.1. Latitude (Degree-minute):	2.4.2. Longitude (Degree-minute):	
2.4.3. Elevation from sea level (meters):		
2.5 Temperature Humidity Index	**2.5.1 Time of observation:**	
2.5.2 Temperature: ……°C	2.5.3 Relative Humidity: ……..%	
2.5.4 THI score for Heat stress	☐ Comfort ☐ Mild stress ☐ Severe stress ☐ Very severe stress	

2.6 Total number of Buffalo:			
2.7 Composition of population: Total number of animals in a particular category			
☐ Milk Buffalo:	☐ Dry Cow:	☐ Bull:	
☐ Heifer:	☐ Calf:	☐ Pregnant:	
2.8 Type of Buffalo:	☐ Swamp type	☐ River type	☐ Both
2.9. What are the available breeds in your farm	☐ Indigenous ☐ Murrah ☐ Nili-Ravi ☐ Albino ☐ Surti ☐ Jaffrabadi ☐ Mediterranean ☐ others…… ……		
2.9.1. If cross breed what are the breeds	……		

3. Rearing System

3.1 Rearing type	☐ Free ranging/bathan ☐ Household ☐ Commercial/intensive ☐ Semi-intensive ☐ Semi-bathan Free ranging/bathan: They always stay out of home and permanently stay in grazing land Household/backyard: If the buffaloes are raised in home and also carried to grazing land Commercial/intensive: If the buffaloes are raised intensively and never allowed to grazing land Semi-intensive: If the buffaloes are raised intensively and have some free space to move, also have access to wallowing area outside. Semi-bathan: They are reared both in house and in free-ranging area.
3.2 Types of ranging area used	☐ Muddy field ☐ Wallowing in water ☐ Both ☐ Never
3.3 If wallowing kind of wallowing water	☐ Pond water ☐ River water ☐ Sea water ☐ Others ……
3.3.1 Please specify duration of wallowing:	☐ <6hr ☐ 6-12hr ☐ 12-18hr ☐ >18hr
3.3.2 Does the frequency of wallowing change during the year depending on the season?	☐ Yes ☐ No
3.3.3 If yes, please specify duration of season	☐ Summer…… ☐ Winter……. ☐ Rainy…….. ☐ Others ……
3.4 Is wallowing mandatory for buffalo	☐ Yes ☐ No
3.5 If there is no wallowing facility, what they usually do?	☐ Spray water ☐ Others……
3.5.1 If yes, Source of that water facility?	☐ Tube-well ☐ Deep tube well ☐ Pond water ☐ River water ☐ Others ……..
3.6.1 pH of wallowing water (0-14)	
3.6.2 Temperature of wallowing water	
3.6.3 TDS (Total dissolved solids) of wallowing water	
3.6.4 Hygienic score of wallowing water	☐ Excellent = Water is clear and water interface is excellent ☐ Good = Water is cloudy and interface is moderately dirty ☐ Poor = Water is very cloudy and interface is very dirty

4. Bathan/Semi-bathan rearing system

4.1. Do you move your herd from pasture land during winter season for long period?	☐ Yes	☐ No
4.2 Do you have any temporary shelter for herd at bathan?	☐ Yes	☐ No
4.3 Sources of drinking water	☐ Deep Tubewell ☐ Tube-well ☐ Pond water ☐ Sea water ☐ River water ☐ Others……	
4.4 Parameters on drinking water	pH of water (0-14):…….. Temperature of water:…….. TDS of water:……	

5. Commercial/Semi-intensive/ Household farm

5.1. Does the farm have any protected boundary?	☐ Yes	☐ No
5.2. Does the farm have any bedding material?	☐ Yes ☐ No	
5.2.1. Kind of bedding material?	☐ Straw ☐ Rubber mattress ☐ Others…….. ☐ No bedding materials	
5.2.2. Scoring of particular bedding	☐ Excellent (Clean and dry animal lies in completely dry place) ☐ Good (Wet and 20% covered with mud) ☐ Fair (Dirty and more than 20% covered with mud)	
5.3. Sources of drinking water	☐ Deep well ☐ Tube-well ☐ Pond water ☐ Sea water ☐ River water ☐ Others……	
5.3.1. Parameters on drinking water	pH of water (0-14):……. Temperature of water:……. TDS of water:……	
5.3.2 How they have facility to drink water?	☐ Individual	☐ In group
5.3.3 If in group, how many buffaloes in a group drinking water? (if more than 50 buffaloes, they will be divided into groups) (at least 25) at least 2 groups will be considered	…… ……	
5.4.1. Do you have a water trough?	☐ Yes ☐ No	
5.4.2 If yes, how often do you clean water trough?	☐ Weekly ☐ Monthly ☐ Never ☐ Others(Please specify) ……	
5.4.3 Hygienic score of water trough?	☐ Excellent ☐ Good ☐ Poor Excellent (Water clear and no evidence of crusts of dirt or decay, Clean water trough) Good (Water clear but contains wastes, Partly discolored trough) Poor (Water colored, such as brown, green, red, Moldy water trough)	
5.5 Availability of Water	☐ Ad-libitum ☐ Scarcity of water	

5.5.1 If water is scarce, when/ how much is available per day?	……
5.6 Are different types of animals/birds kept in same house?	☐ Yes ☐ No
5.6.1 If yes, which animals?	……
5.7. Where do they dispose the manure?	☐ Adjacent to the farm ☐ Away from farm
5.8. Is there a drainage facility?	☐ Yes ☐ No
5.8.1 If yes, score of the drainage facility?	☐ Excellent (Cleaned daily) ☐ Good (Cleaned weekly) ☐ Poor (Cleaned monthly / more)
5.9 How is the floor made of?	☐ Soil/ Muddy floor ☐ Sand ☐ Concrete ☐ Bricks ☐ Others (Specify)……..
5.9.1. If concrete floor, daily frequency of cleaning floor?	☐ Once ☐ Twice ☐ Thrice ☐ Others:……
5.9.2 Score of the floor in terms of cleanliness?	☐ Excellent =Dry and clean floor (not more than 10% area covered with dung) ☐ Good = Intermediate level of cleanliness (between 10%- 50% area covered with dung) ☐ Fair = Dirty floor (more than 50% area covered with dung) **Time** **Observation time:**…… **Time elapsed after last cleaning (minutes)**: ……
5.9.3. Score of the floor in terms of condition of walking surface?	☐ Excellent = Non-slippery and non-cracked on the floor surface ☐ Good= not more than 20% of floor is slippery and non-cracked all surfaces on which animal walk ☐ Poor = Smooth and slippery, More than 8% Cracked,
5.9.4. Do they disinfect the floor?	☐ Yes ☐ No
5.9.4.1. If yes, how often they disinfect the floor?	☐ Daily ☐ Weekly ☐ Monthly ☐ Others *(Please specify)*……
5.9.4.2. If disinfection is applied, what kinds of disinfectants are used? (specify name pls.)	……
5.10. Does the farm have free space for exercising or walking	☐ Yes ☐ No

6. Feeding management

6.1 Feeding system?	☐ Stall feeding ☐ Grazing ☐ Both
6.2 What type of feed is provided to your Buffaloes?	☐ Roughage ☐ Concentrate ☐ Both ☐ Other……
6.2.1 Amount of each kind of feed for lactating animals?	☐ Roughages: …… ☐ Concentrates…… ☐ Others……
6.2.2. Frequency of feeding per day? (Grazers)	☐ Graze all day ☐…… Graze half day ☐……Others……

6.2.3. Frequency of feeding per day? (Non-grazers)	☐ Once ☐ Twice ☐ Thrice ☐ Others……
6.3 Do they provide any other feed supplement? (unconventional feed) *locally available feeds	……..……..……..……..
6.4. How do you feed your buffalo?	☐ Single buffalo ☐ Split up between groups ☐ All buffaloes together ☐ Others……..……..
6.5. Do you provide formulated ration?	☐ Yes ☐ No
a. To whom you provide that ration?	☐ Calves ☐ Heifer ☐ Cow ☐ Others
b. Who formulated that ration?	☐ Self ☐ Vet ☐ Feed company ☐ Specialist
c. Do you provide any vitamin mineral supplement in feed (pls. specify)?	☐ Yes ☐ No If yes.....

7. Sources of Animal

7.1 What are the sources of animals? ☐ Own stock ☐ Purchased ☐ Both
7.2 How many buffaloes purchased over last 12 months: ☐
7.3 If purchase milk buffalo, what are the purchase considerations?
☐ Health condition ☐ Udder conformation ☐ Breed ☐ Milk production history
☐ Parturition history ☐ Body conformation ☐ Low price ☐ Hooves shape:…….
☐ Wide hind quarter ☐ Others (*Please specify*):
……

8. Record keeping

8.1 Do you keep any farm records?	☐ Yes ☐ No
8.1.1 If yes, what types of records you record?	☐ Disease ☐ Breeding ☐ Milk production ☐ Mortality ☐ Treatment ☐ Prevention ☐ Others……..
8.2 Mention type of keeping tool.	☐ Log book ☐ Computer ☐ Others.....
8.3 What clinical signs/ diseases did you notice in the herd in last 12 months	……..

9. Mastitis related records	
9.1. How many buffalo had clinical mastitis in last 12 months?	……
9.2. Any buffalo culled due to mastitis in last 12 months?	☐ Yes ☐ No
9.3. Any buffalo died in your farm in last 12 months?	☐ Yes ☐ No
9.4 How many calves died in last 12 months?	……
9.5. How many adult buffalo died in last 12 months?	……

10. Udder Hygiene

10.1 Do you wash your buffaloes before milking?	☐ Yes ☐ No
10.1.1 If yes, which types of water do you use for bathing?	☐ River ☐ Pond ☐ Tubewell
10.2. Do you need to control the buffalo during milking	☐ Yes …… ☐ No

10.3. Does the milker wash udder before milking?	☐ Yes ☐ No
10.4. Do you let the udder be dried before milking?	☐ Yes ☐ No
10.4.1. If yes, how do you dry the udder?	☐ Towel ☐ Tissue ☐ Others
10.5 Do you practice teat dipping?	☐ Yes, Everyday ☐ Often ☐ Not usually ☐ Never
10.5.1. If yes, what are the dipping materials you use? ……	
10.6. What do you use to stimulate the udder before milking?	☐ Calf suckling ☐ Hand ☐ Warm water ☐ Warm cloth ☐ Others.....
10.7 Do you practice fore-striping?	☐ Yes ☐ No
10.8 What types of milking procedure do you practice in your farm?	☐ Hand milking ☐ Machine milking
10.8.1. If hand milking, which method do you follow? ***Please see in illustration**	☐ Full hand ☐ Stripping ☐ Knuckling ☐ Others……..... Full hand milking: Grasping the teat with all the five fingers and pressing it against the palm. Stripping: Firmly holding the teat between the thumb and fore finger and drawing it down the length of the teat and at the same time pressing it to cause the milk to flow down in a stream. Knuckling: Bend their thumb against the teat. The method is known as knuckling.
10.9. Frequency of milking of Buffalo/day	☐ Once ☐ Twice ☐ Others
10.10 Score of udder hygiene:	☐ Excellent (Washing udder, Pre & post dipping, use of towel) ☐ Good (Washing udder, Pre/ post dipping) ☐ Fair (Washing udder) ☐ Poor (Don't wash the udder)
10.10. Milking is done by:	☐ Same person ☐ Multiple persons
10.10.1 Score of milkers hygiene?	☐ Excellent = Milkers use antiseptics and wash hands ☐ Good = Milkers only wash hand ☐ Poor = Milkers don't wash hand
10.11. Do you use any antiseptics before and after milking?	☐ Yes ☐ No
10.11.1. If yes, which antiseptics?	……
10.12 Do you usually offer feeds after milking? (To keep the animals in standing position)	☐ Yes ☐ No
10.13. Does the milker milk the clinical mastitis affected animal at last?	☐ Yes ☐ No

11. Udder Health

11.1. Bulk milk somatic cell count:	……/µL of milk
11.2 How many clinical mastitic animals you have right now?	……
11.3 Which signs of clinical mastitis you observed?	☐ Swollen udder ☐ Redness of udder ☐ Painful udder ☐ Abnormal milk ☐ Loss of appetite ☐ Depressed ☐ Dehydration ☐ Fever ☐ Rumination ☐

	Others.....
11.4 Other udder related diseases you noticed in your farm in last 12 months.	Udder edema/ Buffalo pox/ Ulcerative mammilitis/Wart/abscess/Hematoma/Udder rot/Blood in milk/Rupture of suspensory ligament of udder/Prepubic tendon rupture
11.5. What is the udder related symptoms encountered in the last 12 months in your farm?	……

12. Therapeutic & preventive management

12.1 Does the farm animal get veterinary service?	☐ Yes ☐ No
12.1.1 If yes, How often do you get veterinary service?	☐ On call ☐ weekly ☐ Monthly ☐ rarely ☐ Others (specify):……
12.1.2 If yes, who gives you the service?	☐ Private Vet. ☐ Govt. Vet. ☐ VFA ☐ Farmers himself ☐ Others.....
12.2 Kind of drugs you used to treat clinical mastitis cases?	☐ Antibiotics …… ☐ Other drugs: ……....
12.3. Do you know about drug withdrawal period of antibiotics?	☐ Yes ☐ No
12.3.1. What do you do with the milk after following antibiotics treatment?	……
12.4.1. What do you do with the milk from clinical mastitis affected animals?	☐ Drink ☐ Discard ☐ Sell ☐ Others:……
12.5. Do you provide vaccines to the animals?	☐ Yes ☐ No
12.5.1. If yes, which vaccines do you use in your farm?	☐ FMD ☐ HS ☐ BQ ☐ Anthrax ☐ Others.....
12.6. Do you use any anthelmintics in your farm?	☐ Yes ☐ No
12.6.1. If yes, how often do you deworm buffaloes?	☐ 4 month ☐ Yearly ☐ Others……
12.6.2. Do you use same strategy for calf	☐ Yes ☐ No
12.6.3. How often do you deworm calves	……
12.6.2. Which anthelmintic do you use?	……
12.7. Do you use any herbal or traditional treatment for clinical mastitis?	☐ Yes ☐ No
12.7.1 If yes, name of those?	……
12.8. Are there any quarantine facilities for new animals? * If they keep the newly purchased animals separately for a period of at least 3-4 weeks to observe manifestation of any disease.	☐ Yes ☐ No
12.9. Do you have isolation shed for sick animals? * If Sick animals are separated from healthy animals and should be treated.	☐ Yes ☐ No

12.2 Questionnaire for Subclinical mastitis (animal and quarter level variables)

Information from individual mastitis affected cow: (Animal level risk factors)

Questionnaire ID:	Farm/Bathan ID:	Animal Id:	Date: ...../...../ 20.....

1. Month of the observation: .....
2. Season of the observation: ☐ Summer ☐ Autumn ☐ Winter ☐ Spring
3. Affected quarter and CMT score in mastitis affected cow?
   ☐ FR: ☐ 1 ☐ 2 ☐ 3 ☐ 4 ☐ 5
   ☐ FL: ☐ 1 ☐ 2 ☐ 3 ☐ 4 ☐ 5
   ☐ RR: ☐ 1 ☐ 2 ☐ 3 ☐ 4 ☐ 5
   ☐ RL: ☐ 1 ☐ 2 ☐ 3 ☐ 4 ☐ 5
   *FR(front right) FL (front left) RR (rear right) RL (rear left)
4. **Type of mastitis in the animal?** ☐ Clinical ☐ Sub-clinical ☐ Normal/ Control
5. Position of teats? ☐ Symmetrical ☐ Directed backward ☐ Directed inward ☐ others.....
6. Teat shape:
   FL ☐ Funnel shaped ☐ Bottle shaped ☐ Cylindrical shaped
   FR ☐ Funnel shaped ☐ Bottle shaped ☐ Cylindrical shaped
   RL ☐ Funnel shaped ☐ Bottle shaped ☐ Cylindrical shaped
   RR ☐ Funnel shaped ☐ Bottle shaped ☐ Cylindrical shaped
7. Visible lesions in teat (teat end callosity**):**
   FL ☐ Yes ☐ No
   FR ☐ Yes ☐ No
   RL ☐ Yes ☐ No
   RR ☐ Yes ☐ No
8. Blocked quarters due to previous clinical mastitis:
   ☐ FR(front right) ☐ FL (front left) ☐ RR (rear right) ☐ RL (rear left)
9. If yes, what are those (**Teat cheilitis/ chaps/ warts/ bovine herpes mammalities**)? .....
10. Any congenital anomalies in teats? ☐ Yes ☐ No
    14.1. If yes, mention the anomalies .....
11. Breed: ☐ Indigenous ☐ Crossbred ☐ others.....
    If crossbred, please specify: .....
12. Age (Number of rings in the horn): .....years
13. Parity: ☐ 1 ☐ 2 ☐ 3 ☐ 4 ☐ 5 ☐ Others: .....
14. Lactation stage in days:.....days
15. Pregnancy? ☐ Pregnant ☐ Non-pregnant
16. Average milk yield: (famers observation in an average for this buffalo):..... litres
17. BCS: ☐ 1 ☐ 2 ☐ 3 ☐ 4 ☐ 5 ☐ 6 ☐ 7 ☐ 8 ☐ 9 (See the illustration)
    1. **Definitely emaciated**: Ribs and bone structures visible; physically weak; difficulty in walking; no presence of fat in sight or pressure.
    2. **Emaciated**: Similar but not physically weak.
    3. **Very skinny**: No visible fat in ribs/ brisket; muscles in spinous process and hind quarter is evident.
    4. **Scrawny**: Ribs and bony eminences easily visible with absence of fat evident on palpation. The individual muscles on the hindquarter are still visible.
    5. **Sufficient nutrition**: The first ribs are covered, while the last 2 or 3 ribs are easily evident. The triangle formed by the iliac, ischial tuberosity and coxomedoral joint is evident and the muscular masses are concave.
    6. **Discrete state of nutrition**: Fat deposit in ribs but absent in brisket; tuberosity triangle is still evident; mascular margin is straight; spinous process is evedent; absence of fat in tail.
    7. **Good level of nutrition**: Slight deposition of fat in brisket region; 1cm fat deposit over ribs; mascular mass is convex; invisible spinous and transverse process; base of tail is full.
    8. **Fat**: Brisket is full; Bony projections show fat deposits; back becomes square in shape with fat; 1-2 cm fat over last 3-4 ribs; excessive accumulation of fat in tail and no visible dimple.
    9. **Very fat**: Very square rear. Particularly pronounced breast tip extended by fat. Large fat deposits on the bony projections and at the base of the tail. Big neck. At least 3-4 cm fat on the last 3 ribs. Line of demarcation very evident on the spine.

18. Udder symmetry? ☐ Symmetric ☐ Asymmetric
19. Udder shape: ☐ Cup/Pendulous ☐ Round/Globular ☐ Bowl
20. Cleanliness of the hind quarter? ☐ Excellent ☐ Good ☐ Poor
    * Overall cleanlines of udder along with hind legs
21. Did you notice any udder related diseases in this animal before?
    ☐ Yes ☐ No
    If, yes
    ☐ Udder edema ☐ Buffalo pox ☐ Wart ☐ Ulcerative mammilitis ☐ Hematoma
    ☐ Udder rot ☐ Blood in milk ☐ Rupture of suspensory ligament of udder
    ☐ Prepubic tendon rupture ☐ Abscess
22. Did the animal have history of any other previous diseases? ☐ Yes ☐ No
    If yes, …….
23. Previous history of lameness? ☐ Yes ☐ No
24. Previous history of clinical mastitis in last 12 months? ☐ Yes ☐ No
25. History of reproductive disease in last 12 months? ☐ Yes ☐ No
26. History of abortion in last 12 months? ☐ Yes ☐ No
27. History of calf mortality in last 12 months? ☐ Yes ☐ No
28. Did you use any antibiotics (Trade name) in previous disease treatment: …….
29. Did you vaccinate this cow? ☐ Yes ☐ No
30. If yes, which vaccine you used? ☐ FMD ☐ HS ☐ BQ ☐ Anthrax
    ☐ Others.....
31. Which milking system do you follow? ☐ Machine ☐ Hand
32. If Hand milking, then milking done by? ☐ Same milker ☐ Multiple milker
33. Experience of the milkers? ☐ Old ☐ New ☐ Both

Clinical mastitis

34. If clinical mastitis, duration of the illness: …….. days
35. Do you practice forestripping before milking? ☐ Yes ☐ No
36. Which clinical signs did you observe?
    ☐ Swollen udder ☐ Redness of udder ☐ Painful udder ☐ Abnormal milk ☐ Loss of appetite ☐ Depressed ☐ Dehydration ☐ Fever ☐ Rumination ☐ others.....
37. If treated which antibiotic you administered? (trade name)……
38. Average daily milk yield: a) 1 week before mastitis... Litre a) 1 week after mastitis:.....Litre

Sub-Clinical mastitis

39. Do you have any idea about sub-clinical mastitis? ☐ Yes ☐ No
40. If yes, do you practice CMT for detection of SCM? ☐ Yes ☐ No
41. If the CMT is positive what do you do?.....

Is there anything more you would like to add?

.....

THANK YOU FOR PARTICIPATING IN THE RESEARCH ☺

We are planning to conduct workshops with buffalo herders, government representatives, and other relevant stakeholders to understand the requirements of all stakeholders to ensure sustainable buffalo farming in Bangladesh.

Would you like to take part in next research?

.....

12.3 Questionnaire for milk quality (farm level variables)

Survey questionnaire for assessing risk factors associated with contamination level of buffalo milk chain at Noakhali, Bangladesh.

Objectives:

1) Identification of different practices at various nodes of buffalo milk value chain in Noakhali, Bangladesh
2) Assessing the level of contamination of milk/milk products associated with various practices along the buffalo milk value chain in Noakhali, Bangladesh

Sample ID:	**BMSCC:**	**Date: …./…../2020**

A) Study unit: Household/ Bathan

1. General information:

1.1. Name of the Interviewee:			1.2 Mobile no:		
1.3. Gender and Age	☐ Male	☐ Female	☐ Age:		
1.4. Location	Village:	Union:	Upazilla:		
1.5. GPS coordinates : Latitude (Degree): Longitude (Degree): Elevation from the sea level:					
1.6. Title of Interviewee:	☐ Owner	☐ Manager	☐ Other ……..		
1.7. Educational status:	☐ Illiterate	☐ Primary	☐ Secondary	☐ Graduation	
1.8: Composition of household/bathan	No. of Lactating	No of dry	No of heifers	No of calves	Average milk yield (Farm) per day (Litres)
1.9. Selling price of milk(BDT/Litre)					
1.10. Where do you sell your milk?	On spot sell from farm/ middle man/ milk collection centre				
1.11. How you sell your milk?	☐By own shop	☐ Contract basis to middle man	☐ Contract basis to		

2. Source and type of sample

2.1. Source of milk	☐ Household	☐ Bathan	☐ Semi-bathan
2.3.Farm bulk milk somatic Cell Count (BMSCC)			
2.5 Time of sample collection			

3. Milk Container

3.1. Types of container use	☐ Aluminum ☐ Plastic ☐ Others…….
3.2. What do you use to clean the milk container?	☐Hot water ☐Tube well water ☐ Tube well water with detergent ☐ Pond water ☐Others
3.3. Frequency of cleaning of milk container (per day)?	☐ Once ☐ Twice ☐ Thrice
3.4. Cleanliness score of milk container	☐ Excellent (no greasiness and dirt inside and outside the container) ☐ Good(No greasiness and dirt inside the container) ☐ Poor(greasiness and dirt present)
3.5. Do you use brush or something like that during cleaning container	☐ Yes ☐ No
3.6. Do you dry the container after cleaning	☐ Yes ☐ No
3.7. If yes, how you dry the container?	☐ Sun/air dry ☐ Using cloth ☐ Using tissue paper
3.8. Do you keep the container upside down during drying?	☐ Yes ☐ No
3.9. Do you keep the bulk milk container open during each milking?	☐ Yes ☐ No
3.10. If no, what do you use for covering?	
3.11. Do you use anything for sieving milk after milking into the BM container?	☐Yes ☐No
3.12. If yes, what do you use?	☐ Cloth ☐ Plastic sieve ☐ Other…

4. Milker's and Buffalo hygiene

4.1. Who does milk the buffalo cows?	☐ Owner ☐ worker ☐ Others……..
4.2. Do you wash buffalo before milking?	☐ Yes ☐ No
4.3. If yes, which type of water do you use for bathing?	☐ River ☐ Pond ☐ Tube well
4.4. Does the milker wash udder before milking?	☐ Yes ☐ No

4.5. Does the milker dry the udder before milking?	☐ Yes ☐ No
4.6. If yes, how they dry the udder?	☐ Wait for air dry ☐ Using individual cloth ☐ Using common cloth ☐ Others……
4.7. Does the milker wash hand before milking?	☐ Yes ☐ No
4.8. Score of milker's hygiene?	☐ 1=Excellent=Milkers use antiseptic and wash hand ☐ 2=Good=Milkers only wash hand ☐ 3=Poor=Milkers don't wash
4.9. Score of udder hygiene	☐ 1=Excellent=Udder is clean and dried ☐ 2=Good=Udder is clean but not dry ☐ 3=Poor=Udder is not clean enough
4.10. Does any buffalo affected with clinical mastitis (either changes in milk, udder, systematic weakness)?	☐ Yes ☐ No
4.11. Do you mix the mastitis milk with normal milk?	☐ Yes ☐ No

5. Storage:

5.1. Milk storage at home	☐ Using lid ☐ Without lid
5.2. Storage time (milking to shifting for sell)?	☐……Min ☐……. Hour
5.3. How do you store milk at home?	☐ Room temp ☐ Cold storage ☐ Freezer

E.Some generic question to farmers:

9.1 Do you face any problem during milking of your animal?	☐ Yes	☐ No
9.2. Do you face any difficulty to store milk at home before shifting?	☐ Yes	☐ No
9.3. Do you have proper transportation facility to shift the milk to shop?	☐ Yes	☐ No
9.4. Do you get the proper price by selling milk?	☐ Yes	☐No

12.4 Questionnaire for milk quality (middleman level variables)

B) Milk collection point(Trader shipment-at entry)

6. Milk transportation

Sample ID:	Date: …../…./2020

Name of person: …….. Mobile No: ……..	Type of trader: Whole seller curd producer/ Occasional retail curd producer/ Middleman/ Family consumer/ Other……
Latitude:	Longitude:
6.1. Transport milk sample through(by boat/bicycle/both/others….)	
6.1.1. Time (Milking to arrival to market/ hand over to middle man)…… min/ hour	
6.2. Types of container use	☐ Aluminum ☐ Plastic ☐ Others……
6.3. Covering material during transport	☐ Cloth ☐ Plastic plate ☐ Aluminum plate ☐ Banana leaves
6.4. Travel time to collection point (Walking time/ time by vehicle)	☐…….min ☐……hour
6.5. Do you use any material inside the container to prevent milk spoilage during transportation?	☐Yes ☐No
6.6. If yes, what you use?	☐ Leaves ☐ Ice cubes ☐ Others……
6.7 Trading experience of milk transporter	☐ Yes ☐ No
6.8. Nature of milk composition?	☐ Mixed milk (cow and buffalo) ☐ Buffalo milk
6.9. Frequency of cleaning milk container per day?	☐ Once ☐ Twice ☐ Thrice
6.10. Do you clean the milk container after each shipment?	☐Yes ☐No
6.11. What do you use to clean the milk container?	☐Hot water ☐Tube well water ☐ Tube well water with detergent ☐ Pond water ☐Others
6.12. Cleanliness score of milk container	☐ Excellent (no greasiness and dirt inside and outside the container) ☐ Good(No greasiness and dirt inside the container) ☐ Poor(greasiness and dirt present)
6.13. Do you use brush or something like that during cleaning container?	☐ Yes ☐ No
6.14. Do you dry the container after cleaning?	☐ Yes ☐ No
6.15.If yes, how you dry the container?	☐ Sun/air dry ☐ Using cloth ☐ Using tissue paper
6.16. Do you keep the container upside down during drying?	☐ Yes ☐ No

12.5 Questionnaire for milk quality (milk collection center level variables)

C) Selling point (After mixing-couple of hours)

Sample ID:	Date: …../…./2020

Name of person: …….. Mobile No: ……..	Type of trader: Whole seller curd producer/ Occasional retail curd producer/ Middleman/ Family consumer/ Others ……
Latitude:	Longitude:
Time (Milking to arrival to market/ hand over to middle man)…… min/ hour	Travel time to collection point (Walking time/ time by vehicle)…… min/ hour
Nature of milk composition?	☐ Mixed milk (cow and buffalo) ☐ Buffalo milk
Types of container use at selling point	☐ Aluminum ☐ Plastic ☐ Others……
Milk storage at shop	☐ Using lid ☐ Without lid
Milk kept in cold storage/freeze	☐ Yes ☐ No
Duration of milk kept at shop	☐…….min ☐……. Hour ☐……. Day

Sample ID:	Date: …../…./2020

Name of person: …….. Mobile No: ……..	Type of trader: Whole seller curd producer/ Occasional retail curd producer/ Middleman/ Family consumer/ Others ……
Latitude:	Longitude:
Time (Milking to arrival to market/ hand over to middle man)…… min/ hour	Travel time to collection point (Walking time/ time by vehicle)…… min/ hour
Nature of milk composition?	☐ Mixed milk (cow and buffalo) ☐ Buffalo milk
Types of container use at selling point	☐ Aluminum ☐ Plastic ☐ Others……
Milk storage at shop	☐ Using lid ☐ Without lid
Milk kept in cold storage/freeze	☐ Yes ☐ No
Duration of milk kept at shop	☐…….min ☐……. Hour ☐……. Day

12.6 Questionnaire for milk quality (milk products level variables)

D) Milk product

Sample ID:	Date: …../…../2020

Name of person: …….. Mobile No: ……..	
Latitude:	Longitude:
8.1. Types of the product	☐ Curd ☐ Ghee ☐ Sweet ☐ Other
8.2. Where it is made?	☐ At shop ☐ At households ☐ Other…….
8.3. Storage time	☐……. day ☐……Min ☐……. Hour
8.4. Containers used	☐ Earthen pot ☐ Plastic container ☐ Other
8.5 Type of seller?	☐ Retail seller ☐ Whole seller
8.6 Source of milk purchase	☐ Own shop ☐ Buy milk from others
8.7 Nature of milk composition?	☐ Mixed milk (cow and buffalo) ☐ Buffalo milk
8.8 Milk used	☐ Boiled ☐ Unboiled

Sample ID:	Date: …../…../2020

Name of person: …….. Mobile No: ……..	
Latitude:	Longitude:
8.1. Types of the product	☐ Curd ☐ Ghee ☐ Sweet ☐ Other
8.2. Where it is made?	☐ At shop ☐ At households ☐ Other…….
8.3. Storage time	☐……. day ☐……Min ☐……. Hour
8.4. Containers used	☐ Earthen pot ☐ Plastic container ☐ Other
8.5 Type of seller?	☐ Retail seller ☐ Whole seller
8.6 Source of milk purchase	☐ Own shop ☐ Buy milk from others
8.7 Nature of milk composition?	☐ Mixed milk (cow and buffalo) ☐ Buffalo milk
8.8 Milk used	☐ Boiled ☐ Unboiled

SUPPLEMENTARY

12.7 Supplementary Tables

Supplementary Table 4.1. A list of 71 Excluded full-text studies with reasons

Title	Author	Year	Journal	Rejection reasons
Mastitis in housed dairy buffalo: incidence, etiology, clinical finding, antimicrobial sensitivity and different medical treatment against *E. coli* mastitis.	Abd-Elrahman, A.H	2013	Life Sci.J.	CM prevalence
Observations on sub-clinical mastitis in buffalo-cows with emphasis on measuring of milk electrical resistance for its early detection.	Ahmed, W. M.	2008	Global Vet.	Individual prevalence data not available
Characterization of Staphylococcus aureus isolates from buffalo, bovine, ovine, and caprine milk samples collected in Rio de Janeiro State, Brazil.	Aires-de-Sousa M	2007	Appl.Environ. Microbiol.	Full-text English not-available
Hematology, bacteriology and antibiotic resistance in milk of water buffalo with subclinical mastitis.	Al-Dujaily	2019	Onl.J.Vet.Res.	Animal level prevalence
Ultrasonographic diagnosis of subclinical mastitis in buffalo (*Bubalus bubalis*)	Al-Galil, A. S. A. A.	2016	Research Opinions in Animal and Veterinary Sciences	Experimental trial
Bacteriology of Mastitis in Buffalo in Tehsil Samundri of District Faisalabad, Pakistan	Ali, L.	2008	Pakistan Vet. J.	Individual prevalence data not available
Study on clinical mastitis in buffalo caused by Staphylococcal species.	Baloch, H.	2013	Pakistan J.Agric.Agric. Engineering, Veterinary Sciences	Animal level prevalence
Studies on mastitis in buffalo caused by bacterial species.	Baloch, H.	2013	Pak. J. Agric. Agric. Eng. Vet. Sci.	CM prevalence
Status of mastitis and allied udder problems in buffalo in Punjab State.	Bansal, B. K.	2004	Intas.Polivet.	Individual prevalence data not available

Bacterial isolation of milk samples submitted from clinical mastitis buffalo during 2007 to 2016	Bhutia, P. S.	2019	Trop. Anim. Health Prod.	Composite milk sample
Factors affecting the prevalence of clinical mastitis in buffalo around Faisalabad District (Pakistan).	Bilal, M. Q.	2004	Int.J. Agric.Biol.	CM prevalence
The emergence of Coagulase Positive Methicillin Resistant Staphylococcus Aureus Isolated from Buffalo Mastitis Milk Samples	Biswas	2018	Explor. Anim. Medical Res.	Individual prevalence data not available
Distribution of Contagious and Environmental Mastitis Agents Isolated from Milk Samples Collected from Clinically Health Buffalo Cows Between Brazilian Dry and Rainy Seasons of the Year	Bonini Pardo, R.	2007	Italian J. Anim. Sci.	Same population group used in different studies
Comparison of different routes of administration of antibiotic therapy for sub-clinical mastitis in buffalo.	Bulla, T. R.	2005	Haryana Veterinarian	Experimental trial
In vitro sensitivity of different organisms isolated from subclinical mastitis of buffalo.	Bulla, T. R.	2005	Vet. Pract.	Individual prevalence data not available
Simultaneous identification by multiplex PCR of major Prototheca spp. isolated from bovine and buffalo intramammary infection and bulk tank	Capra E	2014	Lett. Appl.Microbial.	Bulk milk
Somatic cell count and mastitis causing pathogens isolation in water buffalo (Bubalus bubalis) [Contagem de célulassomáticas e isolamento de agentescausadores de mastiteembúfalas (Bubalus bubalis)]	Carvalho L.B.	2007	Arq. Bras. Med. Vet. Zootec.	Full-text English not-available
Short communication: Intra- and inter-individual milk microbiota variability in healthy and infected water buffalo udder quarters	Catozzi C	2019	J. Dairy Sci.	Experimental trial
The microbiota of water buffalo milk during mastitis	Catozzi, C	2017	Plos One	Individual prevalence data not available
Pathogens isolated from clinical mastitis in Murrah buffalo and their antibiogram	Charaya, G.	2014	Vet. World	CM prevalence
Comparative efficacy of tylosin, enrofloxacin and ceftriaxone in treatment of buffalo suffering from mastitis.	Charaya, G.	2015	Haryana Veterinarian	CM prevalence
Application of generalized estimating equations (GEE) and random effect	Chaudhary, J. K.	2017	Indian J. Dairy Sci.	Individual prevalence

model for estimation of occurrence and non-occurrence of mastitis In cattle and buffalo				data not available
Prevalence of mastitis in cows, sheep and buffalo in five localities from Sibiu County.	Cirstea, M.	2010	Lucrari Stiintifice - Medicina Veterinara, Universitate a De Stiinte Agricole Si Medicina Veterinara "Ion Ionescu De La Brad" Iasi	Prevalence data from mixed species
Characterization of Staphylococcus spp. strains in milk from buffalo with mastitis in Brazil: the need to identify to species level to avoid misidentification	Coimbra-e-Souza	2017	Arq. Bras. Med. Vet. Zootec.	Individual prevalence data not available
Mastitis occurrence in buffalo (Bubalus bubalis) extensively farmed in the state of Para, Brazil [Ocorrência de mastite em búfalas (Bubalus bubalis) criada sem sistema extensivo no estado do Pará, Brasil]	da Silva e Silva N.	2014	Bioscience	Full-text English not-available
Some coagulase negative Staphylococcus spp. isolated from buffalo can be misidentified as Staphylococcus aureus by phenotypic and Sa442 PCR methods	de Almeida CC	2018	BMCRes. Notes	Individual prevalence data not available
Epidemiological and bacteriological survey of buffalo mastitis in Nepal.	Dhakal, I. P.	2007	J. Vet. Med. Sci.	CM prevalence
Antimicrobial resistance pattern and virulence profile of S. aureus isolated from household cattle and buffalo with mastitis in Egypt	El-Ashker	2020	Vet. Microbiol.	Individual prevalence data not available
Staphylococci in cattle and buffalo with mastitis in Dakahlia Governorate, Egypt	El-Ashker	2015	J. Dairy Sci.	Prevalence data from mixed species
Acute coliform mastitis in buffalo (Bubalus bubalis): Clinical findings and treatment outcomes	El-Khodery	2008	Trop. Anim. Health Prod.	Animal level prevalence
Phenotypic and genotypic detection of virulence factors of Staphylococcus aureus isolated from clinical and subclinical mastitis in cattle and water buffalo from different farms of Sadat City in Egypt	Elsayed	2012	Vet. World	Cultured composite milk sample

Biofilm formation, hemolysin production and antimicrobial susceptibilities of Staphylococcus aureus isolated from the mastitis milk of buffalo in Udaipur, India.	Gayatri S.	2017	Int.J. Vet. Sci.	Unknown sample source
Subclinical mastitis in cattle and buffalo and its impact on somatic cell count and milk composition.	Ghosh, C. P.	2004	Indian J. Dairy Sci.	Prevalence data from mixed species
Prevalence and etiology of subclinical mastitis in dairy buffalo versus dairy cows from Transylvania, Romania.	Giupana, R. M.	2016	Lucrari Stiintifice - Universitate a De Stiinte Agricole A Banatului Timisoara, Medicina Veterinara	Prevalence data from mixed species
Clinical outcomes and molecular genotyping of Staphylococcus aureus isolated from milk samples of dairy primiparous Mediterranean buffalo (Bubalus bubalis)	Guccione J	2014	J. Dairy Sci.	Composite milk sample
Comparison of α1-antitrypsin, α1-acid glycoprotein, fibrinogen and NOx as indicator of subclinical Mastitis in Riverine Buffalo (Bubalus bubalis)	Guha A.	2013	Asian Australas. J. Biosci. Biotechnol.	Individual prevalence data not available
Phenotypic characterization, biofilm production and antimicrobial resistance of Staphylococcus spp. Isolates from cattle and buffalo mastitis	Guimaraes, Gabriela		Pesqui. Vet. Bras.	
Effect of parity and calving month on average daily milk yield, mastitis rate on major milk components in Iraqi buffalo.	Ibrahim, W. I.	2019	Biochem.Cel l. Arch.	Individual prevalence data not available
Comparative Prevalence of Streak Canal and Intramammary Microorganisms and their Contemporaneous Association in a Dairy Cow and Buffalo Herd Lacking Mastitis Control Program	Javed	2015	Int.J.Agric and Biol.	Individual prevalence data not available
Comparative Study on the Incidence of Mastitis During Different Parities in Cows and Buffalo	Jingar, S. C.	2014	Indian J. Anim. Res.	CM risk factor
Correlation between the California Mastitis Test (CMT) and somatic cells count on milk from Murrah buffalo cows [Correlação entre o California Mastitis Test (CMT) e a contagem de célulassomáticas (CCS) do leite de búfalasMurrah]	Jorge A.M.	2005	Brazilian J. Anim. Sci.	Full-text English not-available

Investigation On Clinical Mastitis in Cattle and Buffalo in The Western Hills Of Nepal.	Joshi, H. D.	1997	Vet. Review	Same population group used in different studies
Quarter-wise comparative prevalence of mastitis in buffalo and crossbred cows.	Khan, A. Z.	2005	Pakistan Vet. J.	Prevalence data from mixed species
Evaluation of PCR test for detecting major pathogens of bubaline mastitis directly from mastitic milk samples of buffalo.	Kumar A.P	2009	Japan	
Molecular surveillance of putative virulence factors and antibiotic resistance in Staphylococcus aureus isolates recovered from intra-mammary infections of river buffalo	Kumar R	2011	Microb.Pathog.	Individual prevalence data not available
Effect on quarter milk somatic cell count and antimicrobial susceptibility of *Staphylococcus rostri* causing intramammary infection in dairy water buffalo	Locatelli C	2013	J.Dairy. Sci.	Individual prevalence data not available
Some studies on E-coli mastitis in cattle and buffalo.	Mahmoud, A. K. A.	2015	Alexandria Journal of Veterinary Sciences	Prevalence data from mixed species
Status of sub-clinical mastitis and associated risk factors in Indian water buffalo in Doaba region of Punjab, India.	Manpreet Kaur	2015	Indian J. Dairy Sci.	Individual prevalence data not available
Antimicrobial resistance of Staphylococcus spp. isolates from cases of mastitis in buffalo in Brazil.	Medeiros, E. S.	2011	J.Vet. Diag. Invest.	Individual prevalence data not available
Mastitis in buffalo herds and their susceptibility to antimicrobial.	Mesquita, A. A.	2017	Pubvet	Full-text English not-available
Relationships between somatic cell count and intramammary infection in buffalo	Moroni P	2006	J. Dairy Sci.	Repeated sample from the same animal
Impact of livestock hygiene education programs on mastitis in smallholder water buffalo (Bubalus bubalis) in Chitwan, Nepal	Ng, Linda	2010	Prev. Vet. Med.	Animal level prevalence
An Evaluation of Subclinical Mastitis During Lactation in Anatolian Buffalo	Oezenc, Erhan	2008	TURJAF	Individual prevalence

				data not available
Use of the Modified Whiteside and California Mastitis Test on the diagnosis of buffalo subclinical mastitis related to microbiological exam.	Oliveira, M. V. V.	2004	CienciaAnimal	Full-text English not-available
Phenotypic, antimicrobial susceptibility profile and virulence factors of Klebsiella pneumoniae isolated from buffalo and cow mastitic milk	Osman KM	2014	Pathog. Glob. Health	Composite milk sample
Mastitis in dairy buffalo and cattle in Egypt due to Clostridium perfringens: prevalence, incidence, risk factors and costs	Osman, K. M.	2009	Revue Scientifique Et Technique-Office International Des Epizooties	Prevalence data from mixed species
Genetic diversity and methicillin resistance of Staphylococcus aureus originating from buffalo with mastitis in Iran	Panahi,	2019	Indian J. Comp. Microbiol. Immunol. and Infect. Dis.	Individual prevalence data not available
Relation between electrical conductivity, dielectric constant, somatic cell count, and other milk quality parameters in diagnosing subclinical mastitis in Murrah buffalo.	Panchal et al.	2016	Indian J. Dairy Sci.	Cross-sectional
Prospects of controlling sub-clinical mastitis in cattle and buffalo through the use of trisodium citrate.	Pankaj D	2017	Indian Dairyman	Composite milk sample
Metagenomic approach to study the bacterial community in clinical and subclinical mastitis in buffalo	Patel	2017	Meta Gene	Individual prevalence data not available
Microbiological profiles in clinical and subclinical cases of mastitis in milking Jafarabadi buffalo	Patel	2019	Res. Vet. Sci.	Individual prevalence data not available
Relationship between cellular and whey components in buffalo milk	Piccinini R	2006	J. Dairy Res.	Experimental trial
Short communication: Detection of antibiotic resistance, mecA, and virulence genes in coagulase-negative Staphylococcus spp. from buffalo milk and the milking environment	Pizauro LJL	2019	J. Dairy Sci.	Individual prevalence data not available
Short communication: Prevalence and risk factors of subclinical mastitis as	Salvador, R. T	2012	J. Dairy Sci.	Animal level prevalence

determined by the California Mastitis Test in water buffalo (*Bubalis bubalis*) in Nueva Ecija, Philippines				
Incidence and pattern of antibiotic resistance of Staphylococcus aureus isolated from clinical and subclinical mastitis in cattle and buffalo	Sharma L.	2015	Asian J.Anim. Sci.	Individual prevalence data not available
Comparative Efficacy of Screening Tests for Subclinical Mastitis in Buffalo.	Shrirame, K. R.	1997	Indian J. Vet. Res.	Same population group used in different studies
Subclinical Mastitis in Buffalo in Different Herd Sizes and Milking Management Systems	Thomas, Cs	2005	Mastitis in Dairy Production: Current Knowledge and Future Solutions	Same population group used in different studies
Species identification and antimicrobial susceptibility profile of bacteria causing subclinical mastitis in buffalo	Vasquez-Garcia	2017	Pesqui. Vet. Bras.	Composite milk sample
Profile of antimicrobial susceptibility in strains of Gram-positive cocci, negative catalase, isolated from buffalo subclinical mastitis.	Vianni, MCE	2003	Pesqui. Vet. Bras.	Full-text English not-available
Studies on comparative incidence of sub-clinical and clinical mastitis and in vitro antibiotic susceptibility of isolates from Holstein-Friesian and Jersey cows and buffalo.	Zahid, I. A.	2004	Pakistan Vet. J.	Prevalence data from mixed species
Environmental organisms as risk factors in the occurrence of mastitis in dairy buffalo with suggested methods of control. A field study	Zaki M.M.	2010	Global Vet.	CM risk factor

Supplementary Table 4.2 Quality assessment of the eligible studies using the NHLBI tool for observational studies

Author, year	Was the research question or objective in this paper clearly stated?	Was the study population specified and defined?	Were all the subjects selected or recruited from the same or similar populations (including the same time period)? Were inclusion and exclusion criteria for being in the study prespecified and applied uniformly to all participants?	Random selection of population	Was a sample size justification, power description, or variance and effect	Were the outcome measures (dependent variables) clearly defined, valid, reliable, and implemented consistently across all study participants?	Average overall assessment score	**Overall assessment***	**Grading based on overall estimates**	**Grade ranges**	**N (Number of publications)**
Ahmad, R. 2001	2	2	3	1	1	3	2.0	Fair	Good	2.5-3	3
Ahmed, H. et al., 2018	3	3	2	1	1	3	2.2	Fair	Fair	1.5-2.4	52
Algammal, A.M. et al., 2020	3	3	3	1	1	3	2.3	Fair	Poor	1-1.4	0
Ali, M. et al., 2011	3	2	3	1	1	3	2.2	Fair			
Ali, T. et al., 2014	3	2	3	1	1	3	2.2	Fair			
Aliul, H et al., 2020	3	2	3	1	1	3	2.3	Fair			
AL-Saadi, B.Q.H. et al., 2015	3	3	3	1	1	3	2.2	Fair			
Anirban, G. et al., 2010	3	2	3	1	1	3	1.8	Fair			
Anirban, G. et al., 2013	3	1	2	1	1	3	2.0	Fair			
Awad, NM et al., 2019	3	2	3	1	1	3	2.3	Fair			
Bachaya, H. et al., 2005	3	2	2	1	1	3	2.3	Fair			
Baloch, H. et al., 2016	3	3	3	1	1	3	2.2	Fair			
Baloch, H. et al., 2018	3	3	3	1	1	3	2.3	Fair			
Beheshti, R. et al., 2011	3	3	2	1	1	3	1.8	Fair			
Bhanot, V. et al., 2012	3	3	3	1	1	3	2.3	Fair			
Biswas, D. et al., 2020	3	2	3	1	1	1	2.2	Fair			

Bulla, T. et al., 2006	2	2	2	1	1	3	2.3	Fair
Chavoshi, M. et al., 2012	3	2	3	2	1	3	2.0	Fair
Dhakal, I. et al., 2006	3	3	2	1	1	3	2.2	Fair
Elhaig, M.M. et al., 2014	3	3	3	1	1	3	2.3	Fair
El-Razik, K.A.A. et al., 2017	3	1	3	1	1	3	1.8	Fair
Farooq, A. et al., 2008	3	3	2	1	1	3	2.3	Fair
Guha, A. et al., 2012	3	3	3	1	1	3	2.0	Fair
Hamed, M.I. et al., 2014	2	2	2	1	1	3	2.5	Good
Hameed, S. et al., 2012	3	3	2	2	1	3	2.5	Good
Hussain, A. et al., 2007	3	2	2	1	1	3	2.0	Fair
Hussain, A. et al., 2018	3	3	3	2	1	3	2.3	Fair
Hussain, R. et al., 2013	3	3	3	2	1	3	1.8	Fair
Islam, J. et al., 2019	3	3	1	1	1	3	2.5	Good
Jhambh, R. et al., 2017	3	3	3	1	1	3	2.2	Fair
Joshi, H. et al., 2013	2	2	2	1	1	3	2.2	Fair
Kaur, G. et al., 2018	3	3	3	2	1	3	1.7	Fair
Kumar, M. et al., 2007	3	2	3	1	1	3	1.8	Fair
Kumar, P. et al., 2008	2	2	2	1	1	2	2.2	Fair
Lamey, A. et al., 2013	3	2	2	1	1	3	2.2	Fair
Maiti, S. et al., 2003	2	2	2	1	1	3	2.2	Fair
Malmarugan, S. et al., 2010	3	2	2	2	1	3	2.0	Fair
Medeiros, E.S. et al., 2011	3	2	3	1	1	3	2.0	Fair
Muhammad, G. et al., 2010	3	2	3	1	1	3	2.3	Fair
Pardo, B.R. et al., 2007	3	2	2	1	1	3	2.2	Fair

Patbandha, T.K. et al., 2015	3	3	3	1	1	3	2.2	Fair
Pizauro, L. et al., 2014	3	3	2	1	1	3	2.3	Fair
Preethirani, P.L. et al., 2015	3	2	3	1	1	3	2.3	Fair
Puggioni, G.M.G. et al., 2020	3	3	2	1	1	3	1.8	Fair
Salvador, R.T. et al., 2013	3	3	3	1	1	3	2.2	Fair
Sentitula. et al., 2012	3	3	3	1	1	3	2.0	Fair
Sharif, A. et al., 2007	3	2	1	1	1	3	2.0	Fair
Sharif, A. et al., 2007	3	2	2	2	1	3	2.0	Fair
Sharma, A. et al., 2007	3	2	2	1	1	3	2.0	Fair
Sharma, N. et al., 2007	3	2	2	1	1	3	2.3	Fair
Sindhu, N. et al., 2009	3	2	2	1	1	3	2.0	Fair
Singh, M. et al., 2014	3	2	2	1	1	3	2.0	Fair
Srinivasan, P. et al., 2013	3	3	3	1	1	3	2.0	Fair

Supplementary Table 4.3 Descriptive characteristics of the 53 eligible studies on subclinical buffalo mastitis included in the meta-analysis

Title	Journal	Author	Country	Study design	Total quarters tested
Studies on mastitis among dairy buffalo.	Pakistan Vet. J.	Ahmad (2001)	Pakistan	Cross-sectional	2,279
Subclinical mastitis in dairy cattle and buffalo among small holders in Egypt: Prevalence and evidence of virulence of Escherichia coli causative agent.	Trop. Biomed.	Ahmed et al. (2018)	Egypt	Cross-sectional	682
Antimicrobial resistance profiles, virulence and Enterotoxins-Determinant genes of MRSA isolated from subclinical bovine mastitis in Egypt.	Pathogens	Algammal et al. (2020)	Egypt	Cross-sectional	200
Prevalence of sub clinical mastitis in dairy buffalo of Punjab, Pakistan.	J. Anim. Plant Sci.	Ali et al. (2011)	Pakistan	Cross-sectional	2,400
Effect of management practices and animal age on incidence of mastitis in Nili Ravi buffalo.	Trop. Anim. Health Prod.	Ali et al. (2014)	Pakistan	Cross-sectional	1,560
Investigation of prevalence and risk factors of subclinical mastitis of dairy buffalo at Bhola district of Bangladesh.	Asian J. Med. Biol. Res.	Aliul et al. (2021)	Bangladesh	Cross-sectional	800
Some physiological factors influence somatic cell counts and milk contents associated with mastitis in Iraqi buffalo.	World J. Pharma. Res.	AL-Saadi and AL-Shakh (2015)	Iraq	Cross-sectional	320
Assessment of chemical and electrolyte profile as an indicator of subclinical mastitis in riverine buffalo (*Bubalus bubalis*).	Haryana Veterinarian	Anirban et al. (2010)	India	Cross-sectional	1,798
Evaluation of milk biochemical constituents as indicators of subclinical	Buffalo J.	Anirban et al. (2013)	India	Cross-sectional	2,048

mastitis in Murrah buffalo (*Bubalus bubalis*).					
Prevalence, etiology and antibiogram of mastitis in buffalo in Sohag governorate.	Assiut Vet.Med.J.	Awad (2019)	Egypt	Cross-sectional	418
Subclinical mastitis in buffalo in Attock district of Punjab (Pakistan).	Pakistan Vet. J.	Bachaya et al. (2005)	Pakistan	Cross-sectional	1,200
Effect of diverse factors on the frequency of clinical and subclinical mastitis in Kundi buffalo of Sindh, Pakistan.	Pakistan J.Zool.	Baloch et al. (2018)	Pakistan	Case-control	840
Prevalence and risk factors associated with sub-clinical mastitis in Kundhi buffalo.	J. Basic Appl. Sci.	Baloch et al. (2016)	Pakistan	Cross-sectional	840
Prevalence and etiology of subclinical mastitis in buffalo of the Tabriz region, Iran.	J. American Sci.	Beheshti et al. (2011)	Iran	Cross-sectional	201
Retrospective study on prevalence and antibiogram of mastitis in cows and buffalo of eastern Haryana.	Indian J.Anim. Res.	Bhanot et al. (2012)	India	Retrospective cross-sectional	2,411
A study on udder health management practices, reproductive disorders and subclinical mastitis in buffalo herds in coastal region of Bangladesh.	TURJAF	Biswas et al. (2020)	Bangladesh	Cross-sectional	280
Prevalence of subclinical mastitis in Murrah buffalo.	Haryana Veterinarian	Bulla et al. (2006)	India	Cross-sectional	239
Buffalo subclinical mastitis bacterial pathogens in Iran.	IPCBEE	Chavoshi and Husaini (2012)	Iran	Cross-sectional	400
Normal somatic cell count and subclinical mastitis in Murrah buffalo.	J. Vet. Med.	Dhakal (2006)	Nepal	Cross-sectional	200
Molecular and bacteriological investigation of subclinical mastitis caused by Staphylococcus aureus and *Streptococcus agalactiae* in	Trop. Anim. Health Prod.	Elhaig and Selim (2015)	Egypt	Cross-sectional	368

domestic bovids from Ismailia, Egypt.					
Tetracycline resistance phenotypes and genotypes of coagulase-negative staphylococcal isolates from bubaline mastitis in Egypt.	Vet. World	El-Razik et al. (2017)	Egypt	Cross-sectional	81
Prevalence of mastitis and antibiotic sensitivity of bacterial isolates recovered from Nili-Ravi buffalo.	J. Anim. Plant Sci.	Farooq et al. (2008)	Pakistan	Cross-sectional	800
Comparison of somatic cell count, California mastitis test, chloride test and rennet coagulation time with bacterial culture examination to detect subclinical mastitis in riverine buffalo (*Bubalus bubalis*).	African J. Agric. Res.	Guha and Guha (2012)	India	Cross-sectional	2,452
Prevalence of Staphylococcus aureus subclinical mastitis in dairy buffalo farms.	Int.J. Livest. Res.	Hamed and Ziatoun (2014)	Egypt	Cross-sectional	956
Cross-sectional epidemiological studies on mastitis in cattle and buffalo of Tehsil Burewala, Pakistan	J. Anim. Plant Sci.	Hameed et al. (2012)	Pakistan	Cross-sectional	1,528
Clinical and subclinical *Staphylococcus aureus* mastitis in dairy buffalo: Disease characteristics and antibiotic susceptibility profiles of isolates.	Int.J. Agric. Res.	Hussain et al. (2007)	Pakistan	Cross-sectional	560
Prevalence of overall and teat wise mastitis and effect of herd size in dairy buffalo.	Pakistan J. Zool.	Hussain et al. (2018)	Pakistan	Cross-sectional	4,144
Risks factors associated with subclinical mastitis in water buffalo in Pakistan.	Trop. Anim. Health Prod.	Hussain et al. (2013)	Pakistan	Cross-sectional	2,202
Assessment of subclinical mastitis in milch animals by different field diagnostic tests in the Barishal district of Bangladesh.	Asian Australas. J. Biosci. Biotechnol.	Islam et al. (2019)	Banglades h	Cross-sectional	160

Prevalence and risk factors of subclinical mastitis in buffalo at an organized dairy farm in western Haryana.	Haryana Veterinarian	Jhambh et al. (2017)	India	Cross-sectional	864
Prevalence of sub-clinical mastitis in Buffalo.	Vet. Pract.	Joshi et al. (2013)	India	Cross-sectional	200
Associations of teat morphometric parameters and subclinical mastitis in riverine buffalo.	J. Dairy Res.	Kaur et al. (2018)	India	Cross-sectional	324
Investigations on the prevalence and oxidative stress aspects of mastitis in buffalo.	Italian J. Anim. Sci.	Kumar et al. (2007)	India	Cross-sectional	424
Bacteriological examination of mastitis in buffalo.	Progress. Res.	Kumar et al. (2008)	India	Cross-sectional	442
Virulence factors of Escherichia coli isolated from recurrent cases of clinical and subclinical mastitis in buffalo.	Int.J. Microbiol. Res.	Lamey et al. (2013)	Egypt	Cross-sectional	840
Studies on the incidence of subclinical mastitis (SCM) in cattle and buffalo of the durg area of Chhattisgarh.	Vet. Pract.	Maiti et al. (2003)	India	Cross-sectional	611
PCR based detection of coagulase gene encoding Staphylococcus aureus isolated from subclinical mastitis cases in buffalo.	Indian J. Field Vet.	Malmarugan et al. (2010)	India	Cross-sectional	88
Risk factors associated with buffalo mastitis in the Brazilian Northeast.	Pesqui. Vet. Bras.	Medeiros et al. (2011)	Brazil	Cross-sectional	1,996
Evaluation of a 3% surf solution (surf field mastitis test) to diagnose subclinical bovine and bubaline mastitis.	Trop. Anim. Health Prod.	Muhammad et al. (2010)	Pakistan	Cross-sectional	400
Microbiological evaluation of milk samples positive to California Mastitis Test in dairy buffalo cows (*Bubalus bubalis*).	Italian J. Anim. Sci.	Pardo et al. (2007)	Brazil	Cross-sectional	734

Association of milk components with intra-mammary inflammation in Jaffrabadi buffalo.	Vet. World	Patbandha et al. (2015)	India	Cross-sectional	1,516
Prevalence and etiology of buffalo mastitis and milk somatic cell count in dry and rainy seasons in a buffalo herd from Analândia, São Paulo State, Brazil.	Arq. Bras. Med. Vet. Zootec.	Pizauro et al. (2014)	Brazil	Cross-sectional	1,042
Isolation, biochemical and molecular identification, and in-vitro antimicrobial resistance patterns of bacteria isolated from bubaline subclinical mastitis in South India.	Plos One	Preethirani et al. (2015)	India	Cross-sectional	190
Evaluation of a bovine cathelicidin ELISA for detecting mastitis in the dairy buffalo: Comparison with milk somatic cell count and bacteriological culture.	Res. Vet. Sci.	Puggioni et al. (2020)	Italy	Cross-sectional	618
Correlation of California mastitis test and somatic cell count on the milk of water buffalo cows in the Philippines.	Trop. Agric.	Salvador et al. (2013)	Philippines	Cross-sectional	751
Incidence of Staphylococci and Streptococci during winter in mastitic milk of Sahiwal Cow and Murrah Buffalo.	Indian J.Microbial.	Sentitula et al. (2012)	India	Cross-sectional	57
Prevalence of severity of mastitis in buffalo in district Faisalabad (Pakistan).	J. Agric. Soc. Sci.	Sharif and Ahmad (2007)	Pakistan	Cross-sectional	400
Estimation of milk lactose and somatic cells for the diagnosis of sub-clinical mastitis in dairy buffalo.	Int.J. Agric. Biol.	Sharif et al. (2007)	Pakistan	Cross-sectional	400
Occurrence of clinical and subclinical mastitis in buffalo in the State of Haryana (India).	Italian J. Anim. Sci.	Sharma and Sindhu (2007)	India	Cross-sectional	5,707

Prevalence, etiology and antibiogram of microorganisms associated with sub-clinical mastitis in Buffalo in Drug, Chhattisgarh State (India).	Int.J. Dairy Sci.	Sharma et al. (2007)	India	Cross-sectional	1,962
Occurrence of subclinical mastitis in cows and buffalo at an organized farm.	Haryana Veterinarian	Sindhu et al. (2009)	India	Cross-sectional	228
Prevalence and characterization of coagulase-negative staphylococci associated with buffalo mastitis.	Indian J. Comp. Microbiol. Immunol. and Infect. Dis.	Singh et al. (2014)	India	Cross-sectional	253
Prevalence and etiology of subclinical mastitis among buffalo (*Bubalus bubalus*) in Namakkal, India.	Pakistan J. Biol. Sci.	Srinivasan et al. (2013)	India	Cross-sectional	824

Supplementary Table 4.4 The subclinical mastitis detection threshold of quarter prevalence defined by 38 eligible studies for particular detection tests

Author year	Country	Mastitis diagnosis tests**	The threshold used for examining quarters for SCM				
			Healthy	Subclinical			
1. Algammal, A.M. 2020	Egypt	CMT	Negative	Trace	1	2	3
2. Islam, J. 2019***	Bangladesh	CMT	Not given				
3. Patbandha, T.K. 2015	India	CMT	Negative	1	2	3	
4. Elhaig, M.M. 2015	Egypt	CMT	Negative	Trace	1	2	3
5. Dhakal, I. 2006	Nepal	CMT	Negative	Trace	1	2	3
6. Muhammad, G. 2010	Pakistan	CMT	Negative	Trace	1	2	3
7. Preethirani, P.L. 2015***	India	CMT	Not given				
8. Pizauro, L. 2014***	Brazil	CMT	Not given				
9. Salvador, R.T. 2013***	Philippines	CMT	Not given				
10. Ali, T. 2014	Pakistan	CMT	Negative	Trace	1	2	3
11. Guha, A. 2012	India	CMT	Negative	Trace	1	2	3
12. Chavoshi, M. 2012	Iran	CMT	Negative	Trace	1	2	3
13. Kumar, P. 2008	India	CMT	Not given				
14. Pardo, B.R. 2007	Brazil	CMT	Negative	Trace	1	2	3
15. Baloch, H. 2016	Pakistan	CMT	Negative	1	2	3	4
16. Ahmed, H. 2018	Egypt	CMT	Negative	Trace	1	2	3
17. Hussain, R. 2013	Pakistan	CMT	Negative	Trace	1	2	3
18. Lamey, A. 2013	Egypt	CMT	Negative	1	2	3	4
19. Beheshti, R. 2011	Iran	CMT	Negative	Trace	1	2	3
20. Ahmad, R. 2001	Pakistan	CMT	Negative	Trace	1	2	3
21. Awad, A. M. 2019	Egypt	CMT	Negative	Trace	1	2	3
22. Maiti, S. 2003	India	MCMT	Negative	Trace	1	2	3
23. Sharma, N. 2007	India	MCMT	Negative	Trace	1	2	3

24. Kaur, G. 2018	India	SCC	<400,000	>400,000			
25. Anirban, G. 2010	India	SCC	<200,000	>200,000			
26. Anirban, G. 2013	India	SCC	<200,000	>200,000			
27. Puggioni, G.M.G. 2020	Italy	SCC	<200,000	>200,000			
28. Kumar, M. 2007	India	SCC	<500,000	>500,000			
29. Singh, M. 2014	India	SCC	<500,000	>500,000			
30. Jhambh, R. 2017	India	SCC	<50,000	>50,000			
31. Sindhu, N. 2009	India	SCC	<500,000	>500,000			
32. Bulla, T. 2006	India	SCC	<500,000	>500,000			
33. Hussain, A. 2007***	Pakistan	SFMT	Not given				
34. Sharif, A. 2007	Pakistan	SFMT	Negative	Trace	1	2	3
35. Bachaya, H. 2005	Pakistan	SFMT	Negative	1	2	3	4
36. Hameed, S. 2012***	Pakistan	SFMT	Not given				
37. Farooq, A. 2008***	Pakistan	SFMT	Not given				
38. Ali, M. 2011***	Pakistan	WST	Not given				

** Tests Employed (CMT = California mastitis test; MCMT = Modified California mastitis test; SFMT = Surf field mastitis test; WST = White side test; SCC = Somatic cell count)

*** Only positive or negative test results were interpreted but differential scoring for subclinical mastitis was not defined

Supplementary Table 5.1 List of the individual farm with the total number of buffalo and quarters tested for detection of subclinical mastitis using CMT in the Bagerhat and Noakhali region of Bangladesh

FID	Rearing system	Total buffalo	Numbers of lactating buffalo	Numbers of buffalo tested	Numbers of quarters tested
1	Semi-intensive	487	66	50	195
2	Household	7	2	2	8
3	Bathan	6	1	1	4
4	Semi-intensive	41	7	7	28
5	Household	5	2	1	4
6	Household	7	2	2	8
7	Household	5	2	1	4
8	Bathan	55	8	3	12
9	Bathan	200	12	-	-
10	Household	3	1	1	4
11	Household	4	3	1	4
12	Household	5	2	2	8
13	Household	3	1	1	4
14	Household	6	2	1	4
15	Household	5	2	2	8
16	Household	2	1	1	4
Total		**841**	**114**	**76**	**299**

Supplementary Table 6.1 Relevant data on-farm size (buffalo heads), number of lactating buffalo, number of buffalo sampled, and bulk milk somatic cell count for each of 248 selected farms

Farm ID	Total number of buffalo per farm	Total number of lactating buffalo per farm	Number of buffalo sampled	Bulk milk somatic cell count per mL of milk
1	5	1	1	52,000
2	6	3	1	77,000
3	4	2	1	110,000
4	8	1	1	596,000
5	5	1	1	190,000
6	9	3	1	NA
7	8	3	1	262,000
8	22	4	1	528,000
9	3	1	1	537,000
10	26	4	2	529,000
11	2	2	2	64,000
12	23	3	2	47,000
13	9	3	2	122,000
14	21	2	2	405,000
15	2	2	2	394,000
16	11	2	2	264,000
17	6	3	2	123,000
18	7	2	2	110,000
19	5	2	2	78,000
20	19	4	2	431,000
21	30	4	2	609,000
22	7	2	2	478,000
23	10	3	2	774,000
24	6	2	2	189,000
25	9	2	2	195,000
26	7	2	2	362,000
27	7	3	2	49,000
28	17	4	2	115,000
29	9	2	2	421,000

30	23	4	2	671,000
31	24	4	2	336,000
32	2	2	2	NA
33	4	2	2	165,000
34	15	3	2	168,000
35	8	2	2	105,000
36	9	3	2	95,000
37	9	3	2	118,000
38	6	2	2	172,000
39	6	2	2	244,000
40	5	2	2	267,000
41	24	3	2	170,000
42	24	4	2	111,000
43	4	2	2	151,000
44	3	2	2	137,000
45	4	2	2	50,000
46	5	2	2	80,000
47	2	2	2	198,000
48	5	2	2	147,000
49	5	2	2	107,000
50	12	2	2	370,000
51	8	2	2	89,000
52	28	21	2	101,000
53	14	4	2	38,000
54	2	2	2	1,008,000
55	2	2	2	286,000
56	15	6	2	344,000
57	2	2	2	334,000
58	6	2	2	102,000
59	12	4	2	235,000
60	10	3	2	282,000
61	9	2	2	707,000
62	6	3	2	459,000
63	8	2	2	280,000
64	22	6	2	858,000

65	2	2	2	287,000
66	5	2	2	204,000
67	8	2	2	336,000
68	6	2	2	446,000
69	45	10	2	267,000
70	32	5	2	435,000
71	15	3	2	351,000
72	10	7	2	442,000
73	63	20	3	138,000
74	48	17	3	201,000
75	43	3	3	118,000
76	18	4	3	170,000
77	22	4	3	307,000
78	21	6	3	117,000
79	10	3	3	149,000
80	8	4	3	136,000
81	14	7	3	NA
82	27	8	3	89,000
83	14	3	3	78,000
84	23	5	3	151,000
85	31	3	3	288,000
86	65	16	3	516,000
87	22	11	3	260,000
88	22	3	3	279,000
89	3	3	3	106,000
90	55	13	3	480,000
91	19	3	3	302,000
92	6	3	3	332,000
93	7	3	3	328,000
94	14	5	3	355,000
95	12	3	3	113,000
96	19	6	3	453,000
97	23	7	3	521,000
98	80	9	3	822,000
99	17	3	3	NA

100	70	8	3	673,000
101	16	5	3	NA
102	17	3	3	318,000
103	3	3	3	NA
104	15	4	3	192,000
105	14	3	3	91,000
106	5	3	3	444,000
107	12	7	3	193,000
108	10	5	3	73,000
109	13	5	3	318,000
110	7	3	3	324,000
111	20	5	3	118,000
112	17	5	3	78,000
113	24	3	3	131,000
114	10	3	3	156,000
115	14	4	3	87,000
116	12	3	3	118,000
117	8	3	3	203,000
118	10	3	3	157,000
119	17	3	3	142,000
120	6	3	3	72,000
121	9	3	3	297,000
122	7	3	3	128,000
123	36	9	3	93,000
124	35	8	3	143,000
125	3	3	3	170,000
126	3	3	3	167,000
127	46	3	3	207,000
128	3	3	3	147,000
129	3	3	3	103,000
130	3	3	3	42,000
131	25	7	3	157,000
132	28	3	3	136,000
133	3	3	3	38,000
134	3	3	3	70,000

135	9	3	3	276,000
136	17	5	3	80,000
137	8	3	3	107,000
138	7	3	3	206,000
139	14	6	3	141,000
140	10	3	3	242,000
141	15	6	3	259,000
142	18	5	3	423,000
143	7	3	3	315,000
144	15	3	3	369,000
145	9	3	3	322,000
146	11	3	3	198,000
147	30	10	3	322,000
148	25	3	3	305,000
149	12	3	3	611,000
150	90	8	3	886,000
151	24	6	3	317,000
152	9	4	3	1,213,000
153	9	3	3	629,000
154	8	3	3	653,000
155	3	3	3	691,000
156	18	3	3	168,000
157	50	17	3	262,000
158	13	3	3	135,000
159	27	5	3	438,000
160	20	3	3	351,000
161	22	7	4	367,000
162	17	4	4	271,000
163	18	4	4	216,000
164	33	9	4	137,000
165	22	8	4	145,000
166	60	10	4	194,000
167	32	10	4	87,000
168	4	4	4	196,000
169	45	11	4	589,000

170	39	7	4	150,000
171	14	5	4	851,000
172	32	11	4	279,000
173	24	6	4	270,000
174	60	6	4	268,000
175	44	7	4	36,000
176	14	4	4	37,000
177	12	4	4	124,000
178	13	7	4	392,000
179	43	20	4	155,000
180	15	6	4	105,000
181	12	7	4	168,000
182	18	8	4	107,000
183	13	6	4	287,000
184	27	11	4	215,000
185	54	25	4	216,000
186	89	24	4	312,000
187	12	4	4	115,000
188	11	4	4	125,000
189	28	4	4	72,000
190	60	20	4	465,000
191	13	5	4	267,000
192	17	4	4	193,000
193	16	6	4	281,000
194	18	5	4	184,000
195	12	4	4	318,000
196	22	4	4	351,000
197	52	24	4	781,000
198	27	12	4	516,000
199	74	20	4	781,000
200	33	7	4	819,000
201	22	4	4	192,000
202	30	11	5	86,000
203	20	10	5	367,000
204	12	6	5	135,000

205	26	9	5	106,000
206	25	10	5	262,000
207	24	5	5	76,000
208	44	16	5	240,000
209	37	17	5	207,000
210	37	19	5	170,000
211	28	5	5	110,000
212	22	10	5	503,000
213	18	5	5	274,000
214	46	20	5	279,000
215	38	19	5	95,000
216	45	21	5	178,000
217	49	22	5	36,000
218	5	5	5	83,000
219	44	20	5	73,000
220	42	18	5	212,000
221	28	11	5	319,000
222	44	10	5	295,000
223	30	11	5	441,000
224	30	13	5	104,000
225	20	6	5	314,000
226	22	5	5	88,000
227	21	6	5	362,000
228	21	5	5	387,000
229	25	8	5	147,000
230	80	18	5	124,000
231	6	5	5	855,000
232	39	20	5	727,000
233	23	19	5	737,000
234	33	7	5	604,000
235	50	10	5	184,000
236	47	14	5	171,000
237	33	14	5	240,000
238	28	5	5	437,000
239	80	12	5	316,000

240	100	12	5	278,000
241	24	12	5	604,000
242	14	5	5	414,000
243	50	20	5	371,000
244	43	15	9	215,000
245	50	13	11	335,000
246	187	15	15	296,000
247	278	35	31	304,000
248	205	43	41	691,000

Supplementary Table 6.2 Univariable mixed-effects logistic regression analysis of subclinical mastitis (defined as CMT score ≥2) regressed against quarter level variables (P ≤ 0.3) of 3,491 quarters of 880 buffalo in 248 buffalo farms expressed as odds ratio (OR), 95 % confidence interval (95 % CI), and *P* values.

Variable name	Categories	N (quarter)	SCM Positive (P/N %)	Odds ratio (95 % CI)	*P*
Front-hind quarter position	Hindquarters	1,744	442 (25)	Reference	0.012
	Front quarters	1,747	494 (28)	1.3 (1.1 to 1.6)	
Left-Right quarter position	Right quarters	1,746	441 (25)	Reference	0.006
	Left quarters	1,745	495 (28)	1.3 (1.1 to 1.6)	
Rearing system	Free-ranging	641	157 (24)	Reference	0.004
	Semi-intensive	814	196 (24)	1.0 (0.5 to 1.9)	
	Semi-free-ranging	1,461	369 (25)	1.0 (0.6 to 1.8)	
	Household	308	96 (31)	1.8 (0.8 to 4.1)	
	Intensive	267	118 (44)	6.5 (2.2 to 19.2)	
Teat shape	Cylindrical	2,684	671 (25)	Reference	0.002
	Bottle	445	120 (27)	1.3 (0.9 to 2.0)	
	Funnel	326	124 (38)	2.2 (1.4 to 3.3)	

Supplementary Table 6.3 Univariable mixed-effects logistic regression analysis of subclinical mastitis (defined as at least one of the quarters has CMT ≥2) regressed against buffalo level variables (P ≤ 0.3) of 880 buffalo in 248 buffalo farms expressed as odds ratio (OR), 95 % confidence interval (95 % CI), and *P* values.

Variable name	Categories	N (buffalo)	SCM Positive (P/N %)	OR (95 % CI)	*P*
Rearing system	Semi-intensive	204	94 (46)	Reference	0.022
	Semi-free-ranging	366	172 (47)	1.0 (0.7 to 1.5)	
	Free-ranging	164	82 (50)	1.2 (0.8 to 1.8)	
	Household	77	45 (58)	1.7 (0.9 to 2.9)	
	Intensive	69	49 (71)	3.1 (1.5 to 6.3)	
Age (years)	>6 to 8	201	91 (45)	Reference	0.088
	>8 to 20	351	167 (48)	1.2 (0.8 to 1.7)	
	2.5 to 6	313	174 (56)	1.5 (1.0 to 2.2)	
Breed	Indigenous	457	217 (47)	Reference	0.220
	Crossbreed	416	221 (53)	1.2 (0.9 to 1.6)	
Stage of lactation	3 or less	262	123 (47)	Reference	0.329
	3.1 to 6	436	215 (49)	1.2 (0.9 to 1.7)	
	6.1 to 9	107	58 (54)	1.5 (0.9 to 2.5)	
	9.1 to 20	50	28 (56)	1.5 (0.8 to 2.8)	
Average milk yield	3L or less	659	315 (48)	Reference	0.079
	>3.1L	138	81 (59)	1.5 (1.0 to 2.3)	
Udder symmetry	Symmetrical	579	264 (46)	Reference	<0.001
	Asymmetrical	282	166 (59)	1.8 (1.3 to 2.4)	
History of the previous CM	No	845	417 (49)	Reference	0.018
	Yes	31	24 (77)	3.1 (1.2 to 7.8)	
History of abortion	No	856	425 (50)	Reference	0.059
	Yes	21	16 (76)	2.8 (1.0 to 8.2)	
Number of milkers on the farm	Multiple persons	411	184 (45)	Reference	0.006
	Single person	460	254 (55)	1.5 (1.1 to 2.0)	

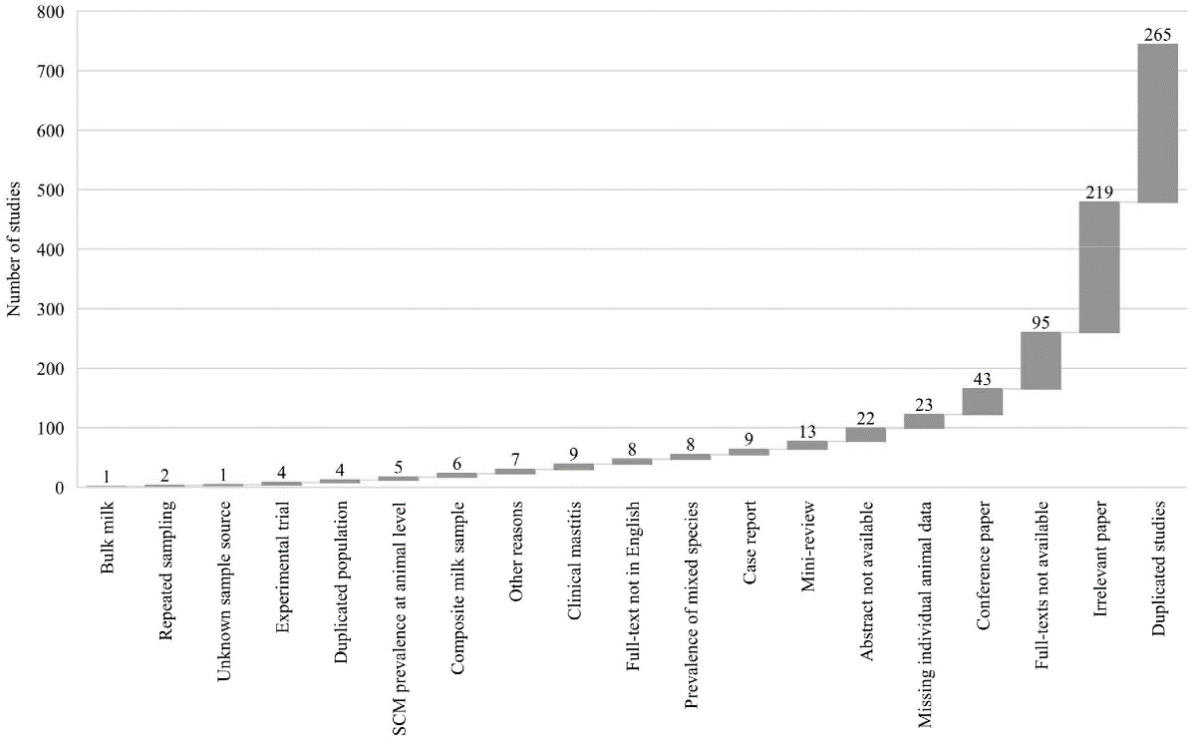
Supplementary Table 6.4 Univariable linear regression analysis of log10-transformed bulk milk somatic cell count regressed against the farm level variables (P ≤ 0.3) in 242 buffalo farms in Bangladesh expressed as geometric mean BMSCC, coefficient (β), 95 % confidence interval (95 % CI), and *P* values.

Variables	Categories	N (farms)	Geometric Mean BMSCC (cells per mL)	*β*	95 % CI	*P*
Type of rearing system	Semi-free-ranging	103	200,062	Reference		0.104
	Free-ranging	48	209124	0.02	-0.09 to 0.13	
	Semi-intensive	54	217,231	0.04	-0.07 to 0.14	
	Household	33	273,311	0.14	0.01 to 0.26	
	Intensive	4	412,120	0.31	-0.005 to 0.63	
Farm area	Costal	34	179,595	Reference		<0.001
	River basin	130	181,945	0.007	-0.1 to 0.1	
	Inlands	33	262,488	0.17	0.02 to 0.31	
	Semi-costal	40	359,579	0.30	0.2 to 0.4	
	Islands	5	391,254	0.34	0.06 to 0.6	
Season	Winter	77	172,390	Reference		<0.001
	Rainy	3	235,333	0.17	-0.17 to 0.51	
	Summer	72	279,889	0.17	0.08 to 0.27	
	Spring	88	371,659	0.32	0.23 to 0.41	
	Autumn	2	513,000	0.52	0.11 to 0.93	
Communication with the employees	Irregularly come to see the farm	35	169,831	Reference		0.048
	No employees	119	205,757	0.08	-0.04 to 0.20	
	Good contact with the staff	68	250,534	0.17	0.04 to 0.30	
	Scarcely come to meet the staff	11	280,420	0.22	0.003 to 0.43	
District	Mymensingh	8	109,691	Reference		<0.001
	Moulvibazar	37	128,102	0.07	-0.13 to 0.27	
	Chattogram	41	156,181	0.15	-0.05 to 0.35	
	Jamalpur	30	167,165	0.18	-0.02 to 0.39	
	Rajshahi	39	224,819	0.31	0.11 to 0.51	
	Dhaka	1	296,000	0.43	-0.12 to 0.98	
	Bhola	39	312,075	0.45	0.25 to 0.65	
	Noakhali	45	412,528	0.57	0.38 to 0.77	
	Laxmipur	2	481,128	0.64	0.23 to 1.05	
Buffalo type	Swamp type	23	124,163	Reference		<0.001
	River type	179	232,891	0.27	0.13 to 0.41	
	Mixed type	24	246,164	0.30	0.11 to 0.48	
Wallowing water source	River	82	173,087	Reference		<0.001
	Combination of sources	13	199,154	0.06	-0.1 to 0.2	
	Tube-well	17	220,651	0.11	-0.05 to 0.3	
	Deep Tube-well			0.13	-0.006 to 0.3	
	Pond	39	338,724	0.29	0.2 to 0.4	
Feeding system	Stall feeding	6	200,228	Reference		0.240

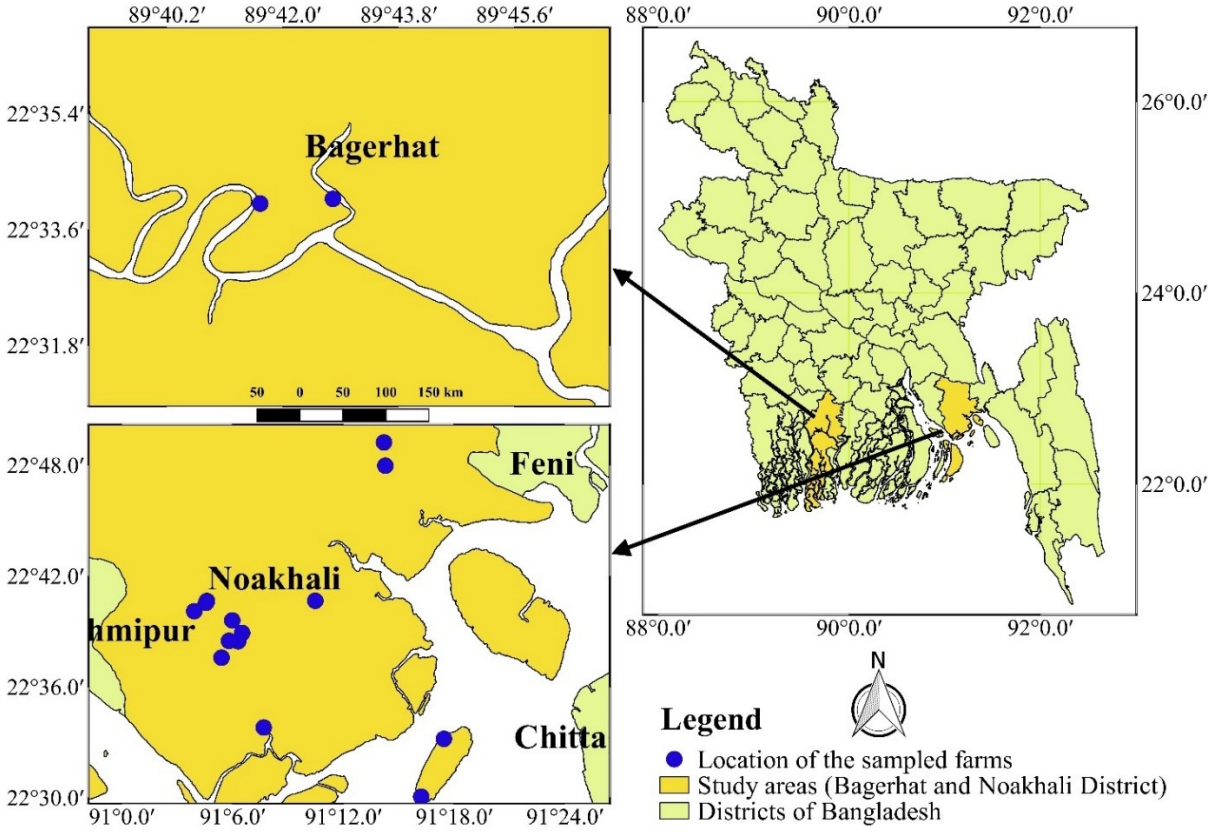
	Mixed feeding system	124	201,821	0.002	-0.26 to 0.27	
	Grazing	109	237,405	0.07	-0.19 to 0.34	
Supplied feed	Concentrates	8	143,326	Reference		0.006
	Roughage and concentrates	76	179,715	0.02	-0.33 to 0.37	
	Straw and unconventional local feed	5	150,552	0.10	-0.13 to 0.33	
	No additional food	29	220,935	0.19	-0.06 to 0.44	
	Roughage	121	254,471	0.25	0.02 to 0.47	
Source of buffalo	Take care on a contract basis	5	81,376	Reference		0.004
	Stock or purchased	133	204,132	0.40	0.12 to 0.68	
	Purchased	20	211,779	0.41	0.11 to 0.72	
	Stock	79	250,770	0.49	0.20 to 0.77	
History of any buffalo mortality with known disease	No	119	201,681	Reference		0.1
	Yes	119	235,035	0.07	-0.02 to 0.1	
Restraining during milking	No	108	187,688	Reference		0.008
	Yes	128	243,152	0.10	0.03 to 0.2	
Fore stripping	Yes	3	70,814	Reference		0.008
	No	236	220,411	0.49	0.13 to 0.85	
Method of hand milking	Full hand	165	203,987	Reference		0.061
	Stripping	58	245,364	0.08	-0.02 to 0.18	
	Knuckling	12	311,487	0.18	-0.004 to 0.37	
Udder hygiene	Excellent	2	104,269	Reference		0.207
	Good	6	163,729	0.19	-0.3 to 0.7	
	Poor	215	216,883	0.32	-0.1 to 0.8	
	Fair	10	296,302	0.45	-0.04 to 0.9	
Score of milkers' hygiene	Excellent	4	114,786	Reference		0.067
	Good	60	195,289	0.23	-0.1 to 0.6	
	Fair	168	232,126	0.30	-0.02 to 0.6	
Offering feed before milking	Yes	101	237,347	Reference		0.005
	No	138	313,544	0.12	0.04 to 0.20	
Having a quarantine facility	Yes	29	120,353	Reference		< 0.001
	No	209	236,499	0.29	0.17 to 0.41	

12.8 Supplementary Figures

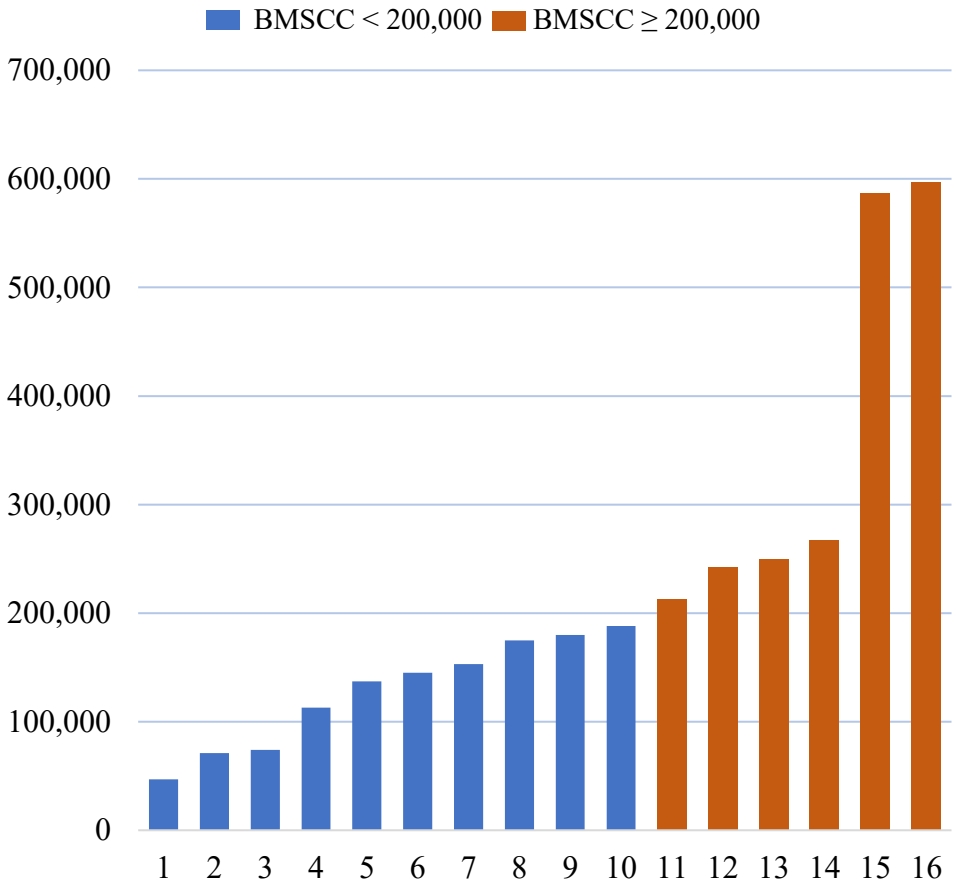
Supplementary Fig 4.1 Description of the studies rejected with reasons



Supplementary Fig 5.1 Location of the 16 buffalo farms in the Noakhali and Bagerhat regions of Bangladesh



Supplementary Fig 5.2 Bulk milk somatic cell count of the 16 buffalo farms in Bangladesh's Noakhali and Bagerhat region. A total of 10 farms had BMSCC < 200,000 cells per mL of milk, and 6 farms had ≥ 200,000 cells per mL of buffalo milk.



PUBLICATIONS

Tropical Animal Health and Production (2023) 55:242
https://doi.org/10.1007/s11250-023-03644-x

REGULAR ARTICLES

Factors influencing somatic cell counts and bacterial contamination in unpasteurized milk obtained from water buffalo in Bangladesh

Shuvo Singha[1,2,3,4] · Fabrizio Ceciliani[1,4] · Md. Mizanur Rahman[3,4] · Mohammad Abdul Mannan[3,4] · Salma Chowdhury[3,4] · Sanjib Chandra Nath[3,4,5] · Ovirup Bhushan Paul[3,4,6] · Ylva Persson[4,7] · Sofia Boqvist[4,8]

Received: 19 January 2023 / Accepted: 30 May 2023

Abstract
Little has been published on the factors influencing the safety and quality of milk derived from water buffalo in Bangladesh. This study aims to describe the milk hygiene parameters and milk chain characteristics of unpasteurized raw milk sold to consumers in order to improve milk hygiene. A quantitative study design evaluated somatic cell counts, total bacterial counts, and specific gram-negative (Enterobacteria) and gram-positive (staphylococci) pathogens in 377 aseptically collected milk samples. Samples were collected at multiple nodes along the buffalo milk value chain: 122 bulk tank milk samples were collected at the farm level, 109 milk samples at the middlemen level, and 111 milk samples at the milk collection centers. In addition, 35 samples were taken from various milk products at the retail level. It was found that progressively increasing somatic cell counts and bacterial counts, including potential pathogens, occurred along the milk chain. A seasonal increase in spring was found, varying based on the farming system (semi-intensive versus intensive). Other factors included water purity and cleanliness of containers, mixing buffalo and cow's milk, and the location of the water buffalo milk producer (coastal or river basin). This study demonstrated how improving udder health and milk hygiene along the water buffalo milk value chain would increase the safety and quality of water buffalo milk in the study area.

Keywords Milk hygiene · Unpasteurized raw milk · Milk value chain · Somatic cell count · Staphylococci · Enterobacteria

✉ Shuvo Singha
shuvo.singha@unimi.it

1 Department of Veterinary Medicine and Animal Sciences, Università degli Studi di Milano, Via dell'Università 6, 26900 Lodi, Italy

2 Department of Physiology, Faculty of Veterinary, Animal and Biomedical Sciences, Sylhet Agricultural University, Sylhet 3100, Bangladesh

3 Department of Medicine and Surgery, Faculty of Veterinary Medicine, Chattogram Veterinary and Animal Sciences University, Chattogram 4225, Bangladesh

4 Udder Health Bangladesh, Chattogram 4225, Bangladesh

5 Sustainable Enterprise Project, Sagarika Samaj Unnayan Sangstha, Subarnachar, Noakhali 3812, Bangladesh

6 Buffalo Research and Development Project, Bangladesh Livestock Research Institute, Savar, Dhaka 1341, Bangladesh

7 Department of Animal Health and Antimicrobial Strategies, National Veterinary Institute, 75189 Uppsala, Sweden

8 Department of Biomedical Sciences and Veterinary Public Health, The Swedish University of Agricultural Sciences, 75007 Uppsala, Sweden

Introduction

Milk provides an excellent nutrient source for many people worldwide, particularly in low- and middle-income countries (Adesogan and Dahl, 2020). Demand for milk products is projected to increase by 1 % in the next decade, forecasting an increase of 1.7 % in global milk production (OECD/FAO, 2019). Cow milk dominates global production (81 %), whereas water buffalo is the principal non-cow dairy production species, contributing 15 % of milk output (Minervino et al., 2020). About 97 % of the buffalo population resides in Asia, with water buffalo being the primary milk source in South Asia (Hegde, 2019).

In Bangladesh, small-holder farmers dominate the water buffalo farming sector by utilizing fallow land and feed resources and providing income-generation opportunities (Habib et al., 2017). Water buffalo are increasingly reared in a free-range system (locally known as "Bathan"), followed by household subsistence systems in the coastal areas, sugarcane belt, and marshland of Bangladesh (Hamid et al., 2016; Sultana, 2018).

Published online: 16 June 2023

Research in Veterinary Science 158 (2023) 17–25

Contents lists available at ScienceDirect

Research in Veterinary Science

journal homepage: www.elsevier.com/locate/rvsc

The prevalence and risk factors of subclinical mastitis in water buffalo (*Bubalis bubalis*) in Bangladesh

Shuvo Singha [a,b,c,d,1,*], Gerrit Koop [d,e], Fabrizio Ceciliani [a,d], Marjolein Derks [d,f], Md. Ahasanul Hoque [c,d], Mohammed Kawser Hossain [d,g], Md. Matiar Rahman Howlader [b], Md. Mizanur Rahman [c,d], Momena Khatun [d,h,i], Sofia Boqvist [d,j], Ylva Persson [d,k]

[a] Department of Veterinary Medicine and Animal Sciences, Università degli Studi di Milano, Via dell'Università 6, 26900 Lodi, Italy
[b] Department of Physiology, Veterinary, Animal and Biomedical Sciences, Sylhet Agricultural University, Sylhet 3100, Bangladesh
[c] Department of Medicine and Surgery, Chattogram Veterinary and Animal Sciences University, Chattogram 4225, Bangladesh
[d] Udder Health Bangladesh, Chattogram 4225, Bangladesh, www.uhb.org.bd
[e] Sustainable Ruminant Health, Department of Population Health Sciences, Faculty of Veterinary Medicine, Utrecht University, Yalelaan 7, 3584, CL, Utrecht, the Netherlands
[f] Farm Technology Group, Wageningen University and Research, Droevendaalsesteeg 1, 6708, PB, Wageningen, the Netherlands
[g] Department of Surgery and Theriogenology, Faculty of Veterinary, Animal and Biomedical Sciences, Sylhet Agricultural University, Sylhet 3100, Bangladesh
[h] Veterinary Physiology, Vetsuisse Faculty, University of Bern, Bremgartenstr. 109a, CH-3001 Bern, Switzerland
[i] Department of Surgery and Obstetrics, Faculty of Veterinary Science, Bangladesh Agricultural University, Mymensingh 2202, Bangladesh
[j] Department of Biomedical Sciences and Veterinary Public Health, Swedish University of Agricultural Sciences, 750 07 Uppsala, Sweden
[k] Department of Animal Health and Antimicrobial Strategies, National Veterinary Institute, 751 89 Uppsala, Sweden

ARTICLE INFO

Keywords:
Bulk milk somatic cell count
California mastitis test
Teat and udder shape
Hand milking
Udder health
Survey
Bangladesh farms

ABSTRACT

Subclinical mastitis (SCM) in water buffalo is responsible for reduced milk yield and quality. This cross-sectional study was carried out to a) estimate the prevalence of SCM, b) identify risk factors associated with SCM, and c) identify farm-level risk factors associated with bulk milk somatic cell count (BMSCC).

The buffalo farms included in this study represented five rearing systems: free-range, semi-free-range, household, semi-intensive, and intensive, providing a total of 3491 functional quarters of 880 lactating buffalo on 248 farms. The California mastitis test score was used to identify SCM. Bulk milk samples ($n = 242$) were used for farm-level BMSCC. Quarter and buffalo-level risk factors for SCM were measured using questionnaires and observations.

The overall SCM prevalence was high at 27.9% at the quarter-level (25th and 75th percentiles: 8.3% and 41.7%) and 51.5% at buffalo-level (25th and 75th percentiles: 33.3% and 66.7%). The geometric mean BMSCC was 217,000 cells/mL of milk (ranging from 36,000-1,213,000 cells/mL), which is low on average, but some farms could improve substantially. The buffalo rearing system, udder location (left versus right), teat shape, udder asymmetry, number of milkers, and having a quarantine facility were associated with buffalo udder health. Our findings suggest that mainly using free-range rearing systems may help decrease the prevalence of SCM primarily by employing buffalo breeding and better farm biosecurity, and udder health control strategies can be designed based on our findings.

1. Introduction

Subclinical mastitis (SCM) is the inflammation of mammary tissue in the absence of clinical signs. This disease reduces the milk yield in dairy animals, impairs animal welfare, and is associated with milk quality deterioration, making milk less suitable for consumption and processing

Abbreviations: SCM, Subclinical mastitis; IMI, Intramammary infection; CMT, California mastitis test; BMSCC, Bulk milk somatic cell count; NGO, Non-governmental organization; PKSF, Palli Karma-Sahayak Foundation; UVH, Upazilla veterinary hospital; LRT, Likelihood ratio test; LBMSCC, Log10-transformed BMSCC; VIF, Variance inflation factor; *Staphylococcus, Staph.*.

* Corresponding author at: Department of Veterinary Medicine and Animal Sciences, Università degli Studi di Milano, Via dell'Università 6, 26900 Lodi, Italy.
E-mail address: shuvo.singha@unimi.it (S. Singha).
[1] Present address: Department of Veterinary Medicine and Animal Sciences, Università degli Studi di Milano, Via dell'Università 6, Lodi, 26900, Italy.

https://doi.org/10.1016/j.rvsc.2023.03.004
Received 24 November 2022; Received in revised form 20 February 2023; Accepted 4 March 2023
Available online 8 March 2023

Journal of Dairy Research

cambridge.org/dar

Occurrence and aetiology of subclinical mastitis in water buffalo in Bangladesh

Shuvo Singha[1,2,3,4], Carl David Ericsson[5], Salma Chowdhury[3,4], Sanjib Chandra Nath[3,4], Ovirup Bhushan Paul[3,4], Md. Ahasanul Hoque[3,4], Sofia Boqvist[5,4], Ylva Persson[6,4] and Md. Mizanur Rahman[3,4]

[1]Department of Veterinary Medicine, University of Milan, Lodi, Italy; [2]Department of Physiology, Sylhet Agricultural University, Sylhet, Bangladesh; [3]Department of Medicine and Surgery, Chattogram Veterinary and Animal Sciences University, Chattogram, Bangladesh; [4]Participating Partner, Udder Health Bangladesh, Chattogram, Bangladesh; [5]Department of Biomedical Sciences and Veterinary Public Health, Swedish University of Agricultural Sciences, Uppsala, Sweden and [6]Department of Animal Health and Antimicrobial Strategies, National Veterinary Institute, Uppsala, Sweden

Research Article

Cite this article: Singha S, Ericsson CD, Chowdhury S, Nath SC, Paul OB, Hoque MdA, Boqvist S, Persson Y and Rahman MdM (2021). Occurrence and aetiology of subclinical mastitis in water buffalo in Bangladesh. *Journal of Dairy Research* **88**, 314–320. https://doi.org/10.1017/S0022029921000698

Received: 18 January 2021
Revised: 12 June 2021
Accepted: 21 June 2021
First published online: 20 August 2021

Keywords:
CMT; intramammary infection; MALDI-TOF; pathogen; penicillinase test; SCC

Author for correspondence:
Shuvo Singha, Email: shuvo.singha@unimi.it

Abstract

Subclinical mastitis (SCM) in water buffalo is a production disease associated with decreased milk yield and impaired milk quality and safety. Water buffalo is an important livestock species in Bangladesh, but information about the occurrence and aetiology of SCM in this species is scarce. A cross-sectional study was conducted as part of the Udder Health Bangladesh Programme to (i) determine the occurrence of SCM and bulk milk somatic cell count (SCC) in water buffalo in Bangladesh, (ii) identify pathogens causing SCM and (iii) evaluate penicillin resistance in isolated staphylococci strains. Sixteen buffalo farms in the Bagerhat and Noakhali regions of Bangladesh were selected for study and a bulk milk sample was collected from each farm. In addition, 299 udder quarter milk samples were collected from 76 animals. The bulk milk samples were assessed by direct SCC and the quarter milk samples by California mastitis test (CMT). The occurrence of SCM calculated at quarter and animal level was 42.5 and 81.6%, respectively. Milk samples from 108 CMT-positive quarters in 48 animals and 38 randomly selected CMT-negative quarters in 24 animals were investigated using bacteriological culture. Estimated mean bulk milk SCC was 195 000 cells/ml milk (range 47 000– 587 000 cells/ml milk). On culture, estimated quarter-level intramammary infection (IMI) was 40.4%. The identity of isolated bacteria was confirmed by MALDI-TOF mass spectrometry. Non-aureus staphylococci (NAS) were the most common pathogens (24.7%) and, among 36 NAS tested, 36.1% were resistant to penicillin. Thus there was high occurrence of SCM on the study farms, with relatively high penicillin resistance in NAS. Further studies are needed to identify underlying risk factors and develop an udder health control strategy for water buffalo in Bangladesh.

Water buffalo (*Bubalus bubalis*) farming in Asian countries has grown exponentially over the past half century, and contributes around 13% of global milk production (Siddiky and Faruque, 2018). Water buffalo farming is also becoming popular in Bangladesh due to increasing demand for buffalo milk and milk products, greater resistance to many diseases compared with cows and lower management efforts and feeding costs (Hamid *et al.*, 2016). A significant impediment to milk production by water buffalo herds is mastitis, which affects the quantity, quality, and safety of milk, causes heavy economic loss, leads to increased use of antibiotics, and impairs animal welfare (Salvador *et al.*, 2012). Clinical mastitis (CM) can be diagnosed by visible changes in the milk, udder, and systemic condition of animals, but subclinical mastitis (SCM) remains undetectable in most cases due to lack of clinical signs (Patel *et al.*, 2019). In water buffalo, SCM is around three-fold more common than CM (Ali *et al.*, 2014) and is responsible for declining milk production, deteriorating milk quality and reduced milk processability. Subclinical mastitis is also a milk safety concern because of the presence of pathogenic microorganisms (Sharma *et al.*, 2011). As the clinical signs remain unnoticed, affected animals may act as reservoirs that shed microorganisms continuously to the environment and affect their herd mates (Ali *et al.*, 2014). Persistent infection also limits the efficacy of antimicrobial treatment by creating a fibrous barrier between the organism and the antibiotic (Putz *et al.*, 2020).

Somatic cells, a combination of leucocytes and epithelial cells released during regeneration of udder secretory tissue, provide a second line of defense of the mammary gland, so their numbers increase in response to intramammary infection (IMI). These cells are present in high amounts in normal milk, but IMI or stress results in a significant increase in the quantity of somatic cells present in milk (Dang *et al.*, 2007; Alhussien and Dang, 2018). Therefore, milk

Traditional buffalo milk chain in Bangladesh

AUTHOR

Shuvo Singha[1,2,3], Ylva Persson[4], Pallab Kumar Dutta[5], Fabrizio Ceciliani[1], , Md. Mizanur Rahman[3]

[1]Department of Veterinary and Animal Sciences, University of Milan, Via Festa del Perdono, 7, 20122 Milano MI, Italy

[2]Department of Physiology, Veterinary, Animal and Biomedical Sciences, Sylhet Agricultural University, Sylhet-3100, Bangladesh

[3]Department of Medicine and Surgery, Chattogram Veterinary and Animal Sciences University, Chattogram 4225, Bangladesh

[4]National Veterinary Institute, 751 89 Uppsala, Sweden

[5]Deputy Director (HRD),Department of Livestock Services, Krishi Khamar Sharak, Dhaka, Bangladesh

Shuvo.singha@unimi.it

UN SDGs

Summary

Location: Bangladesh

IDF Welfare Action Area: Husbandry practice; Health management

INTRODUCTION

Buffalo milk production is becoming an interesting business in Bangladesh. Water buffaloes are one of the main sources of milk in south Asia, except in Bangladesh, where this species is sharing 1.5 million heads contributing only 4% of national milk production [1]. Traditional buffalo production in a mixed-crop based livestock system is becoming popular in Bangladesh because of lower management efforts and feeding costs needed [2]. Among different types of production systems, the bathan (local name in Bengali) or free-ranging system is solely dependent on grazing on fallow land in remote islands and they are shifted to other islands based on the availability of green roughages. The household rearing system allows 5-7 hours of grazing combining supply of the minimum amount of straw, grass, and concentrates. The milk production per animal ranges from 1 to 3 L [3]. Marketing approaches of milk in these two production systems are based on supply to household needs and the remaining larger portion is used in the preparation of buffalo milk products [4]. Buffalo milk products, especially curd prepared from raw milk has a consumer preference in southern parts of Bangladesh whereas curd prepared from boiled milk, cheese, and ghee are more popular in the northern parts of Bangladesh.

TRADITIONAL, FAMILY-RUN BUFFALO MILK CHAIN IN BANGLADESH RELIES ON HAND MILKING

Agro-climatic coastal and semi-costal districts in Bangladesh dominate the water buffalo population in this country. At present, Meghna-Ganga and Jamuna-Brahmaputra river flood plains are the buffalo pockets in Bangladesh but scattered throughout the country. Milking from buffalo is performed by hand milking traditionally because of being the feasible technique in terms of economic reason and less technical knowledge is required and then raw milk is carried in a special bamboo vessel or aluminium container from bathans, and household farms in the countryside. Milk is finally carried to the milk collection centre and sweet shops. The raw milk is carried by boat to cross riverine areas and then motorbike or by walk is chosen to reach the milk collection centre. Cool chain is not maintained during the transportation but in some places, middlemen mix ices or sink palm or banana leaves in the milk container during travel to keep the bulk milk cool in a traditional way. To evaluate the present status of the buffalo milk chain in Bangladesh, we are working on a Bangladesh-Sweden-Italy-Netherlands collaborated project entitled "Climate change mitigation by a sustainable water buffalo dairy chain in Bangladesh" to identify the risk factors and best practices for better milk quality and safety from udder to consumer, focusing on udder health, handling practices and milk production.

> "Buffalo milk production is rising in Bangladesh because of lower management efforts and feeding costs needed."
>
> Shuvo Singha

CONCLUSION

In this report, we briefly illustrated the traditional buffalo milk supply chain in Bangladesh. Despite a significant number of buffalo heads, buffalo milk production is certainly low compared to dairy cows in this country. There are only a few studies that have been reported in buffalo in Bangladesh and evidence about the hygienic quality of milk and milk products along the milk value chain is limited. Especially traditional curd made from raw buffalo milk might have health consequences with the possibility of contamination with zoonotic pathogens such as *FÊW¼ü¯®oðooÔ‰ WÔÉÔ, &›oüÉ/¢üdW‰ðð®* etc. Enhancement of buffalo milk and milk product hygiene can be regulated by Govt. and private company supported milk chilling centres in the buffalo pockets which has been planned recently. Milk pasteurization and final buffalo milk products can be prepared in central buffalo milk stations to supply nationwide. This can be an effective way to establish an ideal buffalo milk chain in Bangladesh. Motivating people in consuming safe buffalo milk and milk products should be a priority.

REFERENCES

1. Hamid, M.; Ahmed, S.; Rahman, M.; Hossain, K. Status of buffalo production in Bangladesh compared to SAARC countries. Asian J. Anim. Sci. 2016, 10, 313-329, doi:10.3923/ajas.2016.313.329.
2. Habib, M.R.; Haque, M.N.; Rahman, A.; Aftabuzzaman, M.; Ali, M.M.; Shahjahan, M. Dairy buffalo production scenario in Bangladesh: a review. Asian J. Med. Biol. Res. 2017, 3, 305-316.
3. Samad, M. A systematic review of research

Protist 172 (2021) 125804
http://www.elsevier.de/protis
Published online date 26 March 2021

Protist

ORIGINAL PAPER

Molecular Epidemiology and Characterization of *Theileria* in Goats

Md. Faridul Islam [a], Pran Gopal Rudra [a], Shuvo Singha [a], Tridip Das [a], Hagos Gebrekidan [b], Md Bashir Uddin [c,1], and Mohammed Yousuf Elahi Chowdhury [d,1]

[a] Chattogram Veterinary and Animal Sciences University, Khulsi, 4225, Bangladesh
[b] Faculty of Veterinary and Agricultural Sciences, The University of Melbourne, Werribee, Victoria, Australia
[c] Department of Medicine, Sylhet Agricultural University, Sylhet 3100, Bangladesh
[d] Department of Medicine and Surgery, Chattogram Veterinary and Animal Sciences University, Khulsi, 4225, Bangladesh

Submitted October 31, 2020; Accepted March 6, 2021
Monitoring Editor: C. Graham Clark

The study was carried out to determine the prevalence and associated risk factors of theileriosis in goats of Chattogram district, Bangladesh. Molecular characterization of circulating *Theileria* in this area was also undertaken. A total of 400 samples were collected from goats of different breeds, ages and sex with relevant information of rearing and management. The prevalence of theileriosis was 8.50% (34/400) by polymerase chain reaction though all of those samples were test-negative by microscopic examination. Among different risk factors season, breed and tick infestation were found to be significantly ($p \leq 0.05$) associated with the prevalence of theileriosis in goats. Serous nasal discharge and swollen lymph nodes were determined to be significant clinical signs. The *Theileria* spp. detected in the present study closely resemble isolates which were previously detected in Myanmar and China. Further large scale epidemiological studies are required to identify the circulating species and responsible vectors, which would facilitate control measures for this disease in Bangladesh.

Key words: Prevalence; risk factors; PCR, *Theileria* spp.; goat.

Introduction

Goat is the most important food animal species in the tropics having a unique ability to adapt to harsh condition with different economically important characteristics like shorter generation intervals, higher rates of prolificacy and good market acceptance. Around 26.1 million goats are reared in Bangladesh (Livestock Economy at a Glance 2017-18) where it is known as “poor man’s cow”, helping to boost up the income of landless marginal people, particularly poor women having small capital (Nath et al. 2014). However, different types of diseases are contributing to huge economic losses by increasing mortality,

[1] Corresponding authors;
e-mails bashir.vetmed@sau.ac.bd (M.B. Uddin), yousuf_elahi@cvasu.ac.bd (M.Y.E. Chowdhury).

https://doi.org/10.1016/j.protis.2021.125804

animals

MDPI

Article

Incidence, Etiology, and Risk Factors of Clinical Mastitis in Dairy Cows under Semi-Tropical Circumstances in Chattogram, Bangladesh

Shuvo Singha [1,2,*], Gerrit Koop [2,3], Ylva Persson [2,4], Delower Hossain [2,5], Lauren Scanlon [2,6], Marjolein Derks [2,7], Md. Ahasanul Hoque [1,2] and Md. Mizanur Rahman [1,2]

1 Department of Medicine and Surgery, Chattogram Veterinary and Animal Sciences University, Chattogram 4225, Bangladesh; md.hoque@my.jcu.edu.au (M.A.H.); mizanuhcp@cvasu.ac.bd (M.M.R.)
2 Udder Health Bangladesh, Chattogram Veterinary and Animal Sciences University, Chattogram 4225, Bangladesh; g.koop@uu.nl (G.K.); ylva.persson@sva.se (Y.P.); delowervet@sau.edu.bd (D.H.); Lauren.Scanlon@tufts.edu (L.S.); marjolein.derks@wur.nl (M.D.)
3 Department of Population Health Sciences, Faculty of Veterinary Medicine, Utrecht University, Yalelaan 7, 3584 CL Utrecht, The Netherlands
4 National Veterinary Institute, 751 89 Uppsala, Sweden
5 Department of Medicine and Public Health, Sher-e-Bangla Agricultural University, Sher-e-Bangla Nagar, Dhaka 1207, Bangladesh
6 Cummings School of Veterinary Medicine, Tufts University, 200 Westboro Road, North Grafton, MA 01536, USA
7 Farm Technology Group, Wageningen University and Research, Droevendaalsesteeg 1, 6708 PB Wageningen, The Netherlands
* Correspondence: shuvosingha@cvasu.ac.bd; Tel.: +880-1767-040023

Citation: Singha, S.; Koop, G.; Persson, Y.; Hossain, D.; Scanlon, L.; Derks, M.; Hoque, M.A.; Rahman, M.M. Incidence, Etiology, and Risk Factors of Clinical Mastitis in Dairy Cows under Semi-Tropical Circumstances in Chattogram, Bangladesh. *Animals* **2021**, *11*, 2255. https://doi.org/10.3390/ani11082255

Academic Editor: Richard Laven

Received: 29 April 2021
Accepted: 27 July 2021
Published: 30 July 2021

Simple Summary: Bovine clinical mastitis is an inflammatory disease of the mammary gland associated with visual changes in the milk and/or the udder. We show that the incidence of clinical mastitis in commercial dairy farms in Bangladesh is high but with large variation between farms. Streptococci and non-aureus Staphylococci were the most frequently isolated bacteria from quarter milk samples. *Staphylococcus aureus* and non-aureus Staphylococci were often resistant against penicillin and oxacillin. This work suggests an urgent need for improved udder health management and specifically a more prudent use of antimicrobial agents following a treatment protocol.

Abstract: Clinical mastitis (CM) is an important production disease in dairy cows, but much of the knowledge required to effectively control CM is lacking, specifically in low-income countries where most farms are small and have specific dairy management, such as regular udder cleaning and practicing hand milking. Therefore, we conducted a 6-month-long cohort study to (a) estimate the incidence rate of clinical mastitis (IRCM) at the cow and quarter level, (b) identify risk factors for the occurrence of CM, (c) describe the etiology of CM, and (d) quantify antimicrobial susceptibility (AMS) against commonly used antimicrobial agents in *S. aureus* and non-aureus *Staphylococcus* spp. (NAS) in dairy farms in the Chattogram region of Bangladesh. On 24 farms, all cows were monitored for CM during a 6-month period. Cases of CM were identified by trained farmers and milk samples were collected aseptically before administering any antimicrobial therapy. In total, 1383 lactating cows were enrolled, which totaled 446 cow-years at risk. During the study period, 196 new cases of CM occurred, resulting in an estimated crude IRCM of 43.9 cases per 100 cow-years, though this varied substantially between farms. Among the tested CM quarter samples, Streptococci (22.9%) followed by non-aureus staphylococci (20.3%) were the most frequently isolated pathogens and resistance of *S. aureus* and NAS against penicillin (2 out of 3 and 27 out of 39 isolates, respectively) and oxacillin (2 out of 3 and 38 out of 39 isolates, respectively) was common. The IRCM was associated with a high milk yield, 28 to 90 days in milk, and a higher body condition score. Our results show that there is substantial room for udder health improvement on most farms.

Veterinary Research Communications
https://doi.org/10.1007/s11259-022-09948-x

REVIEW ARTICLE

Subclinical mastitis in dairy cows in south-Asian countries: a review of risk factors and etiology to prioritize control measures

Md Saiful Bari[1,2] · Md. Mizanur Rahman[2,3] · Ylva Persson[2,4] · Marjolein Derks[2,5] · Md. Abu Sayeed[2,6] · Delower Hossain[2,7] · Shuvo Singha[2,3] · Md. Ahasanul Hoque[2,3] · Subramnian Sivaraman[8] · Palika Fernando[9] · Ijaz Ahmad[10] · Abdul Samad[11] · Gerrit Koop[2,12]

Received: 9 January 2022 / Accepted: 30 May 2022

Abstract

Mastitis is a major production disease, causing significant economic losses for dairy farmers in South-Asian countries, as well as other parts of the world. Udder health control programs (UHCP) have been established in developed countries as an effective strategy for mastitis control but have not yet been introduced in South-Asian low-income countries like Bangladesh, India, Pakistan, and Sri Lanka. To launch UHCP successfully in dairy herds in South-Asia, it is important to know the current prevalence and risk factors for subclinical mastitis (SCM). Therefore, a narrative literature review was conducted with the aim to describe the dairy sector, the prevalence of SCM and its causal agents, risk factors for mastitis occurrence and the control measures suggested by different studies conducted in the selected countries. The literature revealed that India had the highest cattle population. Milking was mainly done by hand in all of the studied countries. Stall feeding was done in Bangladesh and Sri Lanka and limited access to grazing was also reported in some farms in India and Pakistan. There was substantial variation in the prevalence of SCM between studies in all 4 countries, ranging from about 20% to about 80%, but the average prevalence across all studies was high (50%). The most common causal agents for SCM were non-aureus staphylococci (NAS), *Staphylococcus (S.) aureus*, *Streptococcus* spp. and *Escherichia (E.) coli*. The management related risk factors reported for SCM were stall feeding of cows, a higher stock density, cracked floors, open drains, the presence of flies, poor drainage, peri-parturient diseases, infrequent dung removal and earth floors. The control measures suggested in these studies were to improve the hygiene and sanitation of cows, to improve the cleanliness of farms and milker's hands, to apply dry cow therapy, supplementing micronutrients and routine screening for SCM combined with taking intervention measures like isolation of cows or milking infected cows last, and proper treatment. Also, full hand milking, complete milking, machine milking, and providing feed and water immediately after milking have been recommended. Finally, we show that current literature often studies the same set of (non-manageable) risk factors, so more research is needed to obtain a comprehensive picture of the determinants of SCM. Randomized controlled trials are needed to truly quantify the effect of intervention under field conditions. Altogether, our work gives an overview of the udder health situation in South-Asia and provides the basis for the design of UHCP in this region.

Keywords Intramammary infection · Udder health · Prevalence · Incidence · Causal agent

Introduction

Throughout the world, mastitis is one of the major production diseases affecting the health, welfare, and productivity of dairy cows (Kee 2012; Zigo et al. 2021). Because of this, mastitis has been the subject of a vast amount of research for many years. However, much of this research has only focused on mastitis in developed countries, where cows have a high milk yield, are kept in large herds and are machine milked (Kumar et al. 2013). Substantially less research effort has been invested in mastitis in low-income countries, where herd sizes are generally small and hand milking is much more common. However, the effect of mastitis in these settings can be much more devastating (Islam et al. 2019) as smallholder farmers' livelihoods are directly dependent on

✉ Md Saiful Bari
saifulbari@cvasu.ac.bd

Extended author information available on the last page of the article

Published online: 14 June 2022

MDR-2023-0026-ver9-Nath_1P
Type: research-article

MICROBIAL DRUG RESISTANCE
Volume 00, Number 00, 2023

DOI: 10.1089/mdr.2023.0026

ORIGINAL ARTICLE

Open camera or QR reader and scan code to access this article and other resources online.

Colistin Resistance in Multidrug-Resistant *Escherichia coli* Isolated from Retail Broiler Meat in Bangladesh

Chandan Nath,[1] Tridip Das,[2] Md Sirazul Islam,[3] F.M. Yasir Hasib,[3,4] Shuvo Singha,[5] Avijit Dutta,[1] Himel Barua,[1] and Md Zohorul Islam[1,6,7]

The emergence of colistin resistance in *Escherichia coli* is a global public health concern. Contaminated food can accelerate the spread of colistin-resistant *E. coli* to humans. This study aimed to detect and characterize colistin-resistant *E. coli* from broiler meat in Bangladesh. We analyzed 136 pooled broiler meat samples from 240 carcasses collected from 40 live bird markets in urban and rural areas and 8 metropolitan supermarkets. The mean count of *E. coli* in broiler meat samples collected from rural retail shops, metropolitan supermarkets, and urban retail shops was 5.3 ± 1.1, 4.1 ± 1.4, and 3.9 ± 0.8 $\log_{10}$ colony-forming unit per gram, respectively. Colistin-resistant *E. coli* (minimum inhibitory concentration >2 mg/L) was found in 78% (95% confidence interval 70.2–84.1%) of the samples. All colistin-resistant isolates harbored the *mcr*-1 gene, while the rest of the *mcr* genes (*mcr-2* to *mcr-9*) were not detected. Most colistin-resistant *E. coli* isolates (98%) showed coresistance to tetracycline, sulfamethoxazole/trimethoprim followed by ciprofloxacin (95%). Alarmingly, all of the colistin-resistant isolates were found to be multidrug resistant. Phylogenetic analysis showed close similarities of the *mcr*-1 gene sequences of this study with many strains of Enterobacterales isolated from humans, animals, and the environment. This study detected colistin-resistant *E. coli* contamination in broiler meat, which can pose a serious public health threat.

Keywords: broiler meat, colistin-resistant *E. coli*, MDR, *mcr-1*, public health

Introduction

Escherichia coli is a gut commensal of warm-blooded animals comprising both pathogenic and nonpathogenic strains; however, the pathogenic strains may cause various acute and invasive infections in animals and humans.[1,2] The emergence of multidrug resistance in *E. coli* poses a significant public health risk. Due to the lack of strict enforcement of regulations and easy availability of colistin sulfate, this antimicrobial is commonly used in poultry production in Bangladesh.[3,4] As colistin sulfate is considered a last-resort antimicrobial to treat infections caused by multidrug resistant (MDR) Gram-negative bacteria, the emergence and dissemination of antimicrobial resistance (AMR) limit the treatment options for MDR bacterial infections in humans. Indiscriminate antimicrobial usage might have played a role in the emergence of resistance against colistin. Although the shared mechanism of colistin resistance is associated with the chromosomal mutation of pmrAB, phoQ, and mgrB genes,[5] a plasmid-mediated colistin resistance gene (*mcr-1*) conferring resistance to colistin in *E. coli* was reported in China for the first time in 2016.[6] Later, nine *mcr* genes and their variants were identified in various species and geographical areas.[7–9] Colistin-

[1]Department of Microbiology and Veterinary Public Health, Chattogram Veterinary and Animal Sciences University, Chattogram, Bangladesh.
[2]School of Agricultural, Environmental and Veterinary Sciences, Faculty of Sciences and Health, Charles Sturt University, Wagga Wagga, New South Wales, Australia.
[3]Department of Pathology and Parasitology, Chattogram Veterinary and Animal Sciences University, Chattogram, Bangladesh.
[4]Department of Infectious Diseases and Public Health, City University of Hong Kong, Hong Kong SAR, China.
[5]Department of Veterinary and Animal Sciences, Università degli Studi di Milano, Lodi, Italy.
[6]Department of Veterinary and Animal Sciences, University of Copenhagen, Copenhagen, Denmark.
[7]CSIRO Health & Biosecurity, Australian Centre for Disease Preparedness, Geelong, Victoria, Australia.

Thank you very much!