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Characterization of Staphylococci and Streptococci Isolated from Bovine Mastitis: Genotypes, Virulence Profiles and Antimicrobial Resistance Patterns of *Staphylococcus aureus* Strains and *Streptococcus uberis* Strains

VET/05 – Infectious Diseases of Domestic Animals

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List of Abbreviations

AMR – Antimicrobial Resistance

CC – Clonal Complex

CDT – Cytolethal Distending Toxin

CL - Cluster

CLSI – Clinical and Laboratory Standards Institute

CM – Clinical Mastitis

DCT – Dry-Cow Therapy

ddPCR – droplet digital PCR

dPCR – digital PCR

ELISA –Enzyme Linked Immunosorbent Assay

GBS - Group B Streptococci

HPCIA – Highest-Priority Critically Important Antibiotic

ID – Identification

IEC – Immune Evasion Cluster

IMI – Intramammary Infection

ITS – Internal Transcribed Spacer

LOD – Limit of Detection

MALDI-TOF MS – Matrix-Assisted Laser Desorption/Ionization Time-Of-Flight Mass Spectrometry

MDR – Multi Drug Resistance

MGE – Mobile Genetic Element

MIC – Minimum Inhibitory Concentration

MIC_{50} – The MIC inhibiting the growth of 50% of the isolates

MIC_{90} – The MIC inhibiting the growth of 90% of the isolates

MLST - Multi-Locus Sequence Typing

mPCR – multiplex PCR

MRSA – Methicillin-Resistant *Staphylococcus aureus*

MSSA – Methicillin-Sensitive *Staphylococcus aureus*

NAS - Non-aureus Staphylococci

PBP – Penicillin Binding Protein

PCR - Polymerase Chain Reaction

PCR-ELISA – PCR-Enzyme Linked Immunosorbent Assay

PFGE – Pulsed-Field Gel Electrophoresis

PVL – Panton-Valentine Leucocidin

qPCR – quantitative PCR

RAPD-PCR – Random Amplified Polymorphic DNA-PCR

RFLP - Restriction Fragment Length Polymorphism

RS-PCR – Ribosomal Spacer-PCR

RT-PCR – Reverse-Transcriptase PCR

SAg – Superantigen

SCC - Somatic Cell Count

SCC*mec* - Staphylococcal Cassette Chromosome *mec*

SCM - Subclinical Mastitis

SE – Staphylococcal Enterotoxin

SFP – Staphylococcal Food Poisoning

STEC – Shiga Toxin-producing *Escherichia coli*

VTEC - Vero Toxin-producing *Escherichia. coli*

WGS - Whole Genome Sequencing

Abstract

Mastitis is the most expensive disease in dairy production. Among mastitis causative agents, *Staphylococcus aureus* and *Streptococcus uberis* are recognized as the major pathogens responsible for contagious and environmental IMIs, respectively. Both *S. aureus* and *Strep. uberis* strains can acquire antimicrobial resistance and can express a multitude of factors strongly linked to the outcomes of the disease, but only few genetic lineages, characterized by specific resistance phenotypes and molecular characteristics, are responsible for the spread of infections within bovine population.

The principal aims of this project were to investigate the genotypic variability, the virulence and the antimicrobial resistance in *S. aureus* and *Strep. uberis* isolates collected from bovine mastitis cases.

The study on *S. aureus* determined the phenotypic antimicrobial susceptibility, the occurrence of selected antimicrobial resistance genes and other virulence genes in 93 isolates from clinical mastitis collected in different countries and previously genotyped by RS-PCR. The results revealed the low prevalence of the IEC genes and of the multidrug resistance in *S. aureus*, with the detection of only two MRSA strains. The occurrence of AMR genes did not always correspond with their actual expression.

The studies on *Strep. uberis* firstly developed 2 low-cost and fast multiplex PCRs to detect simultaneously 10 housekeeping and virulence *Strep. uberis* genes. These molecular assays were subsequently used for a 4-month epidemiological investigation of a *Strep. uberis* clinical mastitis outbreak on an Italian dairy farm. We explored the genotypic (RAPD) patterns, the virulence and the phenotypic antimicrobial resistance profiles of 71 *Strep. uberis* isolates. The same combination of virulence-associated genes was present in all the strains analyzed and a conserved RAPD pattern was detected within the herd, confirming the genetic similarity of *Strep. uberis* strains and suggesting their contagious transmission. *Strep. uberis* strains belonging to the same genotypic cluster differed in their resistance phenotypes. Most of them were resistant to at least one of the drugs tested and 25% were

not inhibited by three or more antimicrobial classes, but the majority were susceptible to β-lactams, including cephalosporins.

The last aim of this thesis was to provide an overview of the pathogens involved not only in the development of bovine IMI but also in foodborne human diseases, presenting further information on their characteristics. Different identification and detection techniques were reported and could be prospectively used to analyze other mastitis causative agents than *S. aureus* and *Strep. uberis.*

The relevance of this research would consist in filling the gap between the phenotypic and genetic traits of bovine mastitis pathogens and could help to set up control measures and treatment strategies based on their specific epidemiological and pathogenic properties.

Riassunto

Nel panorama mondiale dell'allevamento della bovina da latte, la mastite costituisce una delle principali cause di perdita economica. Tra gli agenti eziologici della mastite, *Staphylococcus aureus* rappresenta uno dei principali patogeni contagiosi, mentre *Streptococcus uberis* è riconosciuto come uno dei più comuni batteri ambientali. I ceppi di *S. aureus* e *Strep. uberis* possono sviluppare la resistenza a molteplici antimicrobici e possono esprimere diversi geni fortemente correlati alla patogenesi della mastite, ma solo pochi genotipi, caratterizzati da specifici profili di antibiotico-resistenza e da particolari caratteristiche molecolari, sono da considerarsi responsabili della diffusione e della persistenza delle infezioni intramammarie all'interno della mandria.

Gli obiettivi principali di questa tesi sono stati l'analisi dei cluster genotipici, dei fattori di virulenza e di antibiotico resistenza nei ceppi di *S. aureus* e *Strep. uberis* isolati da casi di mastite bovina.

Lo studio su *S. aureus* si è focalizzato sul determinare la suscettibilità antimicrobica, la presenza di geni di resistenza ed altri geni di virulenza in 93 isolati da campioni di latte mastitico, provenienti da diversi Paesi del mondo e precedentemente genotipizzati tramite RS-PCR. Questo lavoro ha rivelato la bassa diffusione nei ceppi di *S. aureus* sia dei geni IEC sia di quelli responsabili della resistenza a diversi tipi di antibiotici; in particolare, sono stati trovati solo due ceppi di MRSA. Tuttavia, è stata riscontrata una discrepanza tra i risultati genotipici relativi alla presenza dei geni di resistenza e quelli fenotipici corrispondenti alla loro effettiva espressione.

Gli studi su *Strep. uberis* si sono concentrati sullo sviluppo di due multiplex PCR per rilevare la presenza simultanea di 10 diversi geni housekeeping e di virulenza nei ceppi di *Strep. uberis.* La messa a punto di questa metodica rapida ed economica ha permesso di condurre un'indagine epidemiologica della durata complessiva di quattro mesi, durante un focolaio di mastite clinica da *Strep. uberis* in un allevamento bovino italiano. Un totale di 71 isolati di *Strep. uberis* sono stati caratterizzati, determinando i loro cluster genotipici mediante RAPD – PCR, i loro pattern di geni di virulenza e i loro fenotipi di resistenza. La stessa combinazione di geni di virulenza era presente in

tutti i ceppi analizzati e un profilo RAPD predominante è stato rilevato all'interno della mandria, confermando l'alta similarità dei ceppi di *Strep. uberis* e suggerendone la trasmissione di carattere contagioso. Tuttavia, i ceppi di *Strep. uberis* appartenenti allo stesso cluster genotipico hanno mostrato differenti profili di suscettibilità fenotipica. La maggior parte di essi era resistente ad almeno uno degli antibiotici testati ed il 25% non era inibito da tre o più classi antimicrobiche, ma la maggioranza era suscettibile ai β-lattamici, comprese le cefalosporine.

Il fine ultimo di questa tesi è stato di fornire una panoramica generale dei patogeni coinvolti non solo nello sviluppo delle infezioni intramammarie bovine ma anche delle intossicazioni alimentari nell'uomo, presentando ulteriori informazioni sulle loro caratteristiche. I differenti metodi di identificazione descritti potrebbero rivelarsi utili all'analisi di altri agenti eziologici della mastite, e al confronto di questi ultimi con *S. aureus* e *Strep. uberis*.

I risultati di questa ricerca potrebbero contribuire ad indagare ulteriormente l'associazione tra i tratti fenotipici e genetici dei microrganismi isolati dalla mastite bovina, e potrebbero aiutare a formulare strategie di controllo e di trattamento basate sulle loro specifiche proprietà epidemiologiche e patogene.

Introduction

Bovine Mastitis

Mastitis, whatever has an infectious or non-infectious etiology, is defined as the inflammation of the mammary gland (Bradley, 2002), including not only intramammary tissues but also related anatomical structures (Contreras and Rodríguez, 2011). In fact, the mammary gland is composed of teat cistern and gland cistern, milk ducts and glandular tissue, which contains millions of alveoli, lined with milk-producing epithelial cells and surrounded by muscle cells (Figure 1; Schroeder, 2012).

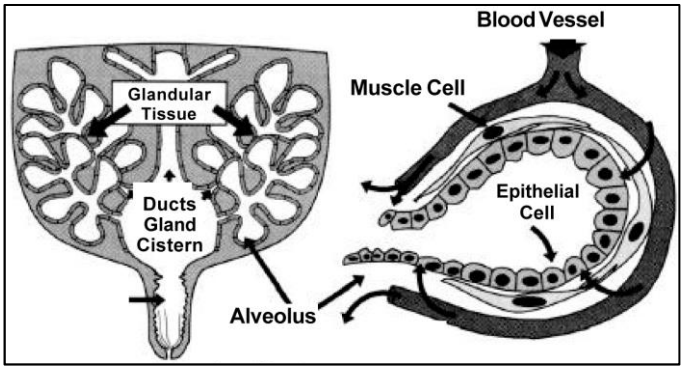

Figure 1. Anatomy of the bovine mammary gland.
Adapted from Schroeder, 2012.

In veterinary medicine, mastitis is usually referred to an intramammary inflammatory reaction caused by a bacterial agent, whose penetration through the teat duct into the teat cistern usually occurs when the teat orifice is open (Bradley, 2002; Schroeder, 2012). In fact, the teat apex and the teat canal represent an effective barrier against the entry of microorganisms into the udder, but certain conditions, such as the pre-partum loss of the keratin lining of the teat canal or the post-milking dilation of the teat sphincter, can compromise this first-line defense mechanism of the mammary gland (Derakhshani et al., 2018).

The disease development is based on a process consisting of three different phases: the invasion of the udder by a pathogen, followed by the infection, with the establishment of the microorganism in

the mammary gland, and the inflammation caused by the expression of several virulence determinants in the bacteria (Ruegg, 2017). The outcomes can be classified depending on the lactation stage, clinical manifestations, or their course (Contreras and Rodríguez, 2011).

Mastitis can be detected in both lactating and nonlactating cows, including in animals during dry-off period as well as in pre-partum heifers (De Vliegher et al., 2012), in which IMI was recognized for the first time in 1995 (Nickerson et al., 1995). However, the probability of detecting a mastitis case is consistently higher during the early lactation (Ruegg, 2011), as the result of the infections acquired during the dry period and the early fresh period (Bradley and Green, 2004). Therefore, periparturient diseases can have a great impact on animal performance, negatively affecting the health of the udder and its ability to produce milk through-out lactation (Rollin et al., 2015).

According to only changes in some parameters of the milk or also visible signs of infection, mastitis can be classified as subclinical or clinical, respectively (Schroeder, 2012). In dairy cows, the SCC, quantified as the number of cells per ml of milk, above 200,000 cells/mL defined the diagnosis of SCM (Bradley, 2002). Milk somatic cells are normally secreted in healthy milk and are influenced by host-associated factors, including milk productivity, cow health, parity, lactation stage and breed, but also by environmental factors (Alhussien and Dang, 2018). In normal milk, the majority is constituted by epithelial cells, derived from the desquamation of the mammary epithelium of the alveoli and the ducts, and by leucocytes (Alhussien and Dang, 2018). Among leucocytes, macrophages represent the second line defense of the udder (Derakhshani et al., 2018; Bronzo et al., 2020). As they play an essential role in the host immune response, a decreased macrophage number could be linked to a high incidence of mastitis in quarters with particularly low SCC (Schukken et al., 1999). In case of IMI, macrophages are responsible for the recruitment of neutrophils, phagocytic leucocytes which predominate in infected glands (Bronzo et al., 2020). As their percentage increases dramatically under disease conditions, milk differential leukocyte counts could help to detected SCM. In addition to the presence of elevated SCC of the milk, clinical mastitis can be detected by direct

observation of abnormalities not only in milk but also in the udder or in the animal (Ruegg, 2011). Clinical infection results in several negative outcomes for the cow, including hyperemia, pain, decreased production and increased gland size; culling or death may also occur. Based on the symptoms, cases of CM can be classified as non-severe, if they present only mild (changes of organoleptic characteristics, flakes, or clots in the milk) or moderate and local (abnormal secretion and hot, swollen quarter or udder) signs. Severe CM includes also systemic signs, such as anorexia, fever, and depression (Oliveira et al., 2013; Oliveira and Ruegg, 2014).

The severity of the disease is strongly related to many pathogen-specific, host-associated and environmental factors (Figure 2). Among them, the outcome of IMI depends on the udder defense efficiency that is influenced by genetics, nutritional and immune status, age, parity, and stage of lactation of the animal (Bronzo et al., 2020). As previously reported, the intense genetic selection of cows, and the high levels of milk production in the postpartum and early lactation were associated with physiological dysfunctions, including immunosuppression. The reduction of the immune competence, linked to negative energy balance, oxidative stress and hypocalcemia, increases the risk for mastitis and for severe systemic inflammatory response (Curone et al., 2018).

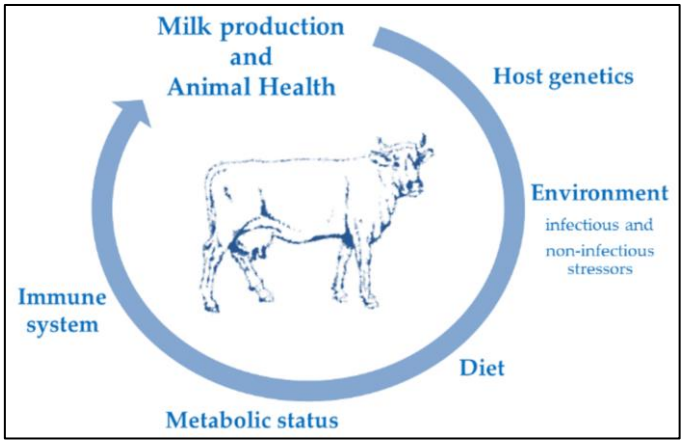

Figure 2. Factors involved in host immune response and in bovine mastitis. Reproduced from Bronzo et al., 2020.

Economic Impact of Bovine Mastitis

In dairy herds, bovine mastitis has existed since at least 3100 BC (Ruegg, 2017), when cows started to be milked. Despite the efforts involved in control and treatment, it continues to cause serious economic consequences for the milk production worldwide (Huijps et al., 2010). Although the calculations of the economic impact of mastitis on dairy industry vary among countries, IMI is still globally recognized as the most expensive disease in the dairy sector (Halasa et al., 2007). In the United States, the cost of a mastitis case was estimated between $325.75 for first lactation heifers and $426.50 for multiparous cows (Liang et al., 2017). In Europe, the losses varied from €149 to €570 per case (Sørensen et al., 2010; van Soest et al., 2016) with €458 as average cost of CM (Heikkilä et al., 2012).

Economic losses can be divided into direct costs associated with treatment (increased use of drugs and veterinary service) and mortality (high culling rate but low sale value of affected cows), and indirect costs due to negative effects on milk yield and quality (Halasa et al., 2007). In both clinical and subclinical mastitis, decreased milk production was the most expensive cost component, followed by culling and preventive measures, including the labor required for their implementation (van Soest et al., 2016). In North America and Europe, subclinical cases are more prevalent than CM, whose incidence ranges from 7 to 30 cases per 100 cows per year (Olde Riekerink et al., 2008; Contreras and Rodríguez, 2011), and most of the mastitis costs (48%) are attributed to SCM (Aghamohammadi et al., 2018). Therefore, the most cost-effective strategies are intended to prevent and control subclinical IMI (Gussmann et al., 2019a). At herd level, multiparous cows in late lactation are considered as the major contributors to production loss caused by SCM (Hagnestam-Nielsen et al., 2009). In addition to cow-specific treatment and culling decisions, farm-level measures should be taken for the efficient management of mastitis (Halasa et al., 2007; Gussmann et al., 2019a).

Epidemiology of Bovine Mastitis

There are more than one hundred pathogens involved in IMI, together with the cow and the environment. The microorganism can influence the host immune response, and the progression of disease depends on the numbers and types of bacteria that are present in the environment and, thus, the cow's udder is exposed to (Schroeder, 2012).

Among several mastitis causative agents, the gram-positive staphylococci and streptococci, the gram-negative *Enterobacteriaceae* are frequently isolated from milk samples of cows with IMI (Bradley et al., 2007; Keane et al., 2013). Although their prevalence varies from region to region, all of them are responsible for expensive diseases in dairy industry worldwide (Zadoks and Fitzpatrick, 2009). They are generally classified in contagious or environmental species, based on their epidemiological behavior in dairy herds (Schukken et al., 2012). *Staphylococcus aureus* remains one of the main pathogens responsible for contagious infection, while the other ones are *Streptococcus agalactiae* and *Mycoplasma* spp. (Bradley, 2002). Bovine udder represents their primary reservoir, that results in their transmission from cow to cow or between quarters of the same animal, especially during the milking process via the milking machine or the farmer's hands; consequently, these bacteria are spread within the herd (Zadoks et al., 2002). Among environmental species, *Streptococcus* spp. other than *Strep. agalactiae*, especially *Streptococcus uberis*, and NAS and coliforms, including *Escherichia coli* and *Klebsiella* spp., are commonly found in the dairy farms. They are usually detected in extramammary locations, including cow's skin and body sites, manure and bedding materials (Zadoks et al., 2005; Ericsson et al., 2009). Although strategies for control of environmental pathogens are focused on reducing the risk of infection by improving hygienic milking and housing measures (Krömker et al., 2014), some strains may show a contagious transmission under specific environment or host conditions (Wald et al., 2020).

Changing Trends in Bovine Mastitis

For decades, *Strep. agalactiae* and *S. aureus* were considered as the most important mastitis causative agents (Ruegg, 2017). *Strep. agalactiae* was responsible for about 90% of IMI by 1937 (Williams, 1937). In the 1940s-1960s, the incidence of mastitis attributed to *S. aureus* kept getting higher (Bradley, 2002). Between the 1960s and the 1980s, the prevalence of *Strep. agalactiae* drastically decreased as a result of the application of several infection control strategies. In particular, the National Mastitis Council (NMC)'s Five-Point Mastitis Control Plan included (1) effective post-milking teat dipping, (2) use of antibiotic dry cow therapy in every quarter at the end of each lactation, (3) rapid identification and appropriate treatment of clinical cases within lactation, (4) culling of chronically affected cows, and (5) maintenance of milk equipment to ensure stable teat end vacuum (Hillerton and Booth, 2018). The implementation of its later extension to 10-point plan made significant progress in the management of contagious mastitis; also *S. aureus* infections declined markedly between 1994 and 2001 (Makovec and Ruegg, 2003), but success in their prevention has been variable and a *S. aureus* challenge still exists (Ruegg, 2017). Although *S. aureus* is mainly considered as a contagious pathogen, whose proliferation is strongly related to colonization and invasion of mammary gland, this bacterium can survive in the farm and several isolates were previously detected in extramammary sites (da Costa et al., 2014). In addition to the ability of *S. aureus* to act as an environmental pathogen, the characteristic to irregularly shed in milk and the low cure rate after antibiotic therapy (Sol et al., 1997) may contribute to difficulties in its control (Sommerhauser et al., 2003; Klaas and Zadoks, 2018).

At the same time there has been an emergence of environmental pathogens, also found as responsible for persistent infection (Oliveira et al., 2013). Among them, *E. coli* and *Strep. uberis* have become the predominant bacteria isolated from mastitis cases (Bradley et al., 2007).

E. coli IMIs are commonly transient infections of short duration, characterized by high bacteria levels and associated with a severe inflammatory response (Schukken et al., 2011), taking place in the

alveoli and generally resulting in non-permanent damages to the mammary tissue (Zhao and Lacasse, 2008); however, the death of the host can occur. Recurrent cases due to repeated episodes of infection and cure, as well as persistent cases with alternating clinical and subclinical episodes, can also be observed and they are associated with large, long-lasting milk losses (Hertl et al., 2014).

Strep. uberis is strictly an animal pathogen and primarily an opportunistic environmental mastitis agent, but within-cow or cow-to-cow transmission may also occur (Zadoks et al., 2011), as previously demonstrated by the identification of clonal strains in different cows in the same herd (Davies et al., 2016; Tomazi et al., 2019). *Strep. uberis* IMIs may be transient or persistent with high levels of bacteria and with high levels of SCC in milk (Schukken et al., 2011), resulting in repeated isolation of the same strain over time (Zadoks et al., 2011). The clinical or subclinical outcome of these infections, as well as their duration and the host immune response to them, depend on the infecting strain (Zadoks et al., 2003; Tassi et al., 2013).

Corynebacterium and non-aureus staphylococci species are also frequently detected in bovine mammary quarters (Bradley et al., 2007), but they are referred to as ‘minor pathogens’ with limited impact on udder health and productivity (Schukken et al., 2009; Taponen and Pyörälä, 2009). They can be isolated from mastitis cases, characterized by mild inflammation of the mammary gland, milk leukocytosis, generally without clinical signs (Benites et al., 2002). Furthermore, they can also be incidentally found in healthy quarters, where they may have a protective role against major pathogens, arising from `competitive exclusion'. It has been demonstrated that quarters infected with *Corynebacterium* spp. or NAS at drying off or in the prepartum period have less probabilities of developing new IMI with a severe pathogen in early lactation (Bradley, 2002; De Vliegher et al., 2003).

Mastitis Diagnosis

Clinical mastitis is often diagnosed directly by visible signs of infection, while the high presence of subclinical forms in dairy cattle worldwide is difficult to be detected and requires the use of specific tests (NMC, 2016). Different diagnostic tools have been developed to rapidly identify quarters with IMI, and their sensitivities have substantial effects on the cost-efficiency of control and management strategies (Gussmann et al., 2019b); among them, SCC testing is considered the standard method (Ruegg, 2017). However, only microbiological analysis of milk allows for the identification of causative agents (Contreras and Rodríguez 2011). As bacterial culture is needed for routine etiological diagnosis of mastitis, the collection of not contaminated milk samples, by using the standard protocols proposed by NMC (National Mastitis Council) or IDF (International Dairy Federation), is mandatory to obtain accurate results (Goodridge et al., 2004; NMC, 2017).

During the recent years, MALDI-TOF MS, reliable in detecting specific peaks in bacterial mass spectra, has become the reference method for the instant identification of microbial species during the routine practices (Barreiro et al., 2010; Cordovana et al., 2019). Moreover, the application of the molecular techniques, including the best well-known PCR, has significantly reduced the processing time required for bacterial identification, increasing the sensitivity and the specificity of the routine procedures (Moroni et al., 2011).

Pathogen Detection and Characterization

Strain typing of *E. coli*, *Strep. uberis*, and *S. aureus* isolates represents an additional mean to study these pathogens and provides insights into their molecular epidemiology. Two common subtyping methods include MLST, based on sequencing of seven housekeeping genes, and PFGE, that compares banding patterns after restriction digestion of bacterial genomic DNA (Keane, 2019).

In recent years, omics technologies, such as metagenomic and transcriptomic, have been introduced to investigate pathogenic bacteria, and shed further light on their infection characteristics (Capra et al., 2017). Next generation sequencing increases discriminatory power, thereby allowing to distinguish closely related strains and to understand their dissemination in dairy herds. High-throughput whole-genome sequencing enables to identify the most prevalent IMI strains and to examine the association between their gene patterns and mastitis pathogenesis (Keane, 2019). Furthermore, omics-related approaches for analyzing milk samples have changed the landscape of bovine mastitis; microbial identification, based on sequencing of the 16S ribosomal RNA gene, may provide further knowledge about the role of microbiota to maintain the health of the mammary gland (Curone et al., 2018; Bronzo et al., 2020).

Mastitis Treatment

Mastitis, responsible for morbidity and mortality, is still the most common and costly problem in dairy cows worldwide. Antimicrobial treatment, indispensable for prevention and cure of mastitis, plays a decisive role in keeping bovine udder health and animal welfare. In dairy cattle, antibiotics are usually used to treat CM within lactation (27%) and to cure existing infections or prevent new IMI at drying off (73%) (Krömker and Leimbach, 2017).

However, the wide and sometimes inappropriate use of drugs have come in a decrease of their efficacy and, thus, of the profitability for farmers (Gussmann et al., 2019b). This phenomenon is strictly associated with the emergence of antimicrobial resistant mastitis causative agents, resulting in several therapeutic problems at cow- and herd-levels (Cheng et al., 2019).

Antimicrobial Resistance of Mastitis Pathogens

Antimicrobial resistance occurs because microorganisms, changing over time, may be able to overcome the effects of drugs used in the past, and no longer respond to therapy; as a consequence, infections become increasingly difficult to treat, resulting in severe illness and death. In this century, AMR represents the most serious threat to public health in Europe (ECDC et al., 2015) as well as around the world (WHO, 2014; 2017). Currently, AMR is responsible for about 700,000 human deaths each year, and it has been estimated that infections caused by resistant bacteria will result in the death of approximately 10 million people in 2050 (Krömker and Leimbach, 2017). In food-producing animals, the large use of antimicrobials to prevent disease's spreading may have contributed to the overall problem of resistance (Marshall and Levy, 2011). Over several decades, bacteria causing infections in food animals have developed resistance to essential drugs also in human medicine, with important implications for both animal and human health (Davies and Davies 2010).

Recently, interest in methicillin-resistant *S. aureus* has increased, because of the diffusion of the *mecA* and the *mecC* (Sawant et al., 2009; Paterson et al., 2014). These genes are carried by a mobile staphylococcal cassette chromosome (*SCCmec*), and are responsible for synthesis of PBP2a, characterized by low affinity for β-lactams, but also for other antibiotic classes (Figure 3; Foster, 2004; 2017); MRSA isolates are defined as multidrug-resistant (Amoako et al., 2019).

The evolution of MDR in food animals is also due to the increasing prevalence of genes associated with the production of extended-spectrum beta-lactamase (ESBL) in *Enterobacteriaceae*, especially *E. coli* (Ajiboye et al., 2009; Ali et al., 2017).

The dissemination of resistance genes via vertical transmission or horizontal gene transfer (Peton and Le Loir, 2014) results in their spread within an animal population, but also between animal and human populations. For this reason, they are involved in the emergence of AMR and MDR, together with the selective pressure by the antibiotic use (Pol and Ruegg, 2007).

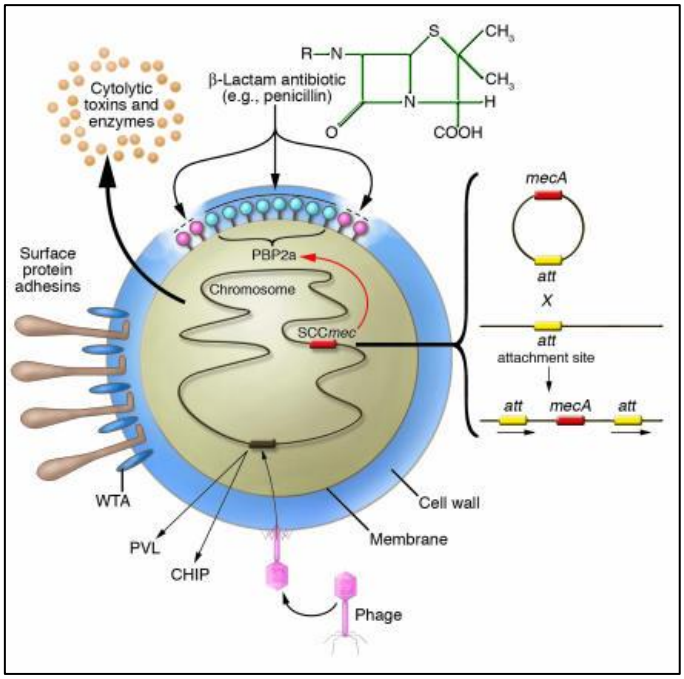

Figure 3. Mechanism of methicillin resistance in *S. aureus*. Reproduced from Foster, 2004.

Facing with this reality, immediate actions should be taken to preserve the effectiveness of critically important antibiotics for human health, by restricting their use in animal production (Krömker and Leimbach, 2017).

The World Health Organization (WHO), the American Food and Drug Administration (FDA), and the European Medicines Agency (EMA) have divided the available active substances into different groups, based on their importance for treating human illnesses (Krömker and Leimbach, 2017). In particular, EMA have classified drugs in four categories, from A to D. Category A must be avoided in veterinary medicine, while category B comprises polymyxins, quinolones, 3rd- and 4th-generation cephalosporins, whose application in animal production exhibited high risk for human health and whose use should be limited (EMA, 2020). In the European Union, their administration should be restricted to cows with IMI caused by multidrug-resistant pathogens. In the United States, ceftiofur (a 3rd-generation cephalosporin) is a broad-spectrum drug, listed as a HPCIA, but used to treat dairy cattle (Ruegg, 2018); particularly, it is considered as the primary mastitis therapy for cows with systemic symptoms (Rajala-Schultz et al., 2004). Antibiotics of category C are admitted in veterinary

as well as human medicine, but the old-established substances belonging to category D should be employed as first choice in dairy production. They are mainly narrow-spectrum drugs, usually active against either gram-positive or gram-negative bacteria (Krömker and Leimbach, 2017; Ruegg, 2018). As any antimicrobial use is associated with the chance of inducing resistance among bacteria (WHO, 2015), the identification of the most appropriate mastitis therapy can be helpful to avoid the misuse of active substances and the consequent risk of MDR spreading. According to the commission notice (2015/C 299/04), the choice of treatment should be based on knowledge of the antimicrobial susceptibility of mastitis causative agents, measured by in vitro sensitivity tests (European Commission, 2015). Disk diffusion method and MIC assay assess the ability of a range of selected antimicrobials to inhibit the bacterial growth at a concentration below the clinical cut-off values (Walker, 2006). However, specific breakpoints for mastitis pathogens are often lacking and they are adopted from other animal species, other groups of bacteria, or human medicine standards (McDougall et al., 2014; Entorf et al., 2016; Kaczorek et al., 2017).

Antimicrobial Treatment of Clinical Mastitis

In lactating cows, the use of antibiotics for SCM should be avoided because of the reduced bacteriological cure in lactation. The decision for CM, on the other hand, should be based on the clinical grade of infection, as well as the identification of the mastitis causative agent (Krömker and Leimbach, 2017). In severe cases, a parenteral therapy is required, while a local treatment is recommended for CM with mild or moderate signs (Oliveira and Ruegg, 2014), depending on the results of on-farm culture, conventional bacteriological culture, MALDI-TOF MS or PCR (Duarte et al., 2015). Previous studies demonstrated that infections caused by gram-negative bacteria, such as *E. coli*, should not be treated, as they are quickly and successfully cleared by the host immune system, thus spontaneously resolve without antibiotic administration (Lago et al., 2011a; Suojala et al., 2013). Similarly, high self-cure rates were observed in mild and moderate cases of CM caused by

opportunistic pathogens, especially NAS (Lago et al., 2011a). The spontaneous cure rates of environmental streptococci vary among species, but the antibiotic use improves the clinical cure, because *Streptococcus* spp. respond well to intramammary therapy (Ruegg, 2018). Local treatment is recommended also for *S. aureus* IMI, although the antimicrobial efficacy is lower against *S. aureus* than against the other gram-positive bacteria (Barkema et al., 2006); culling of cows infected with *S. aureus* can be the first choice in chronic cases (Ruegg, 2017).

The selective treatment should be restricted to CM cases that can benefit from the antimicrobial usage. Recently, no-growth cases have become the most common clinical outcome of culturing milk samples and do not require antimicrobial therapy (Lago et al., 2011a; Ruegg, 2018). Selective treatment protocols should also consider the cow's medical history and the characteristics related to the host immune response. Antibiotics should only be used when cows may be responsive to antibiotic therapy and when bacterial infections are likely to be sensitive to approved intramammary antibiotics (Ruegg, 2018). The knowledge on etiology of CM cases represents an opportunity to decrease by about half the antibiotic use on dairy farms, without affecting the short-term clinical and bacteriological cures, but also the long-term health and production potential of the animals (Lago et al., 2011a; Lago et al., 2011b).

Dry-Cow Antimicrobial Therapy

Dry-cow therapy is an effective way to eliminate existing infections at the end of lactation and prevent new IMIs that frequently occur during the first weeks of the dry off and result in CM cases in the early next lactation (Bradley and Green, 2004). Blanket DCT was introduced many years ago (Neave et al., 1950; 1969) and have become an important component of mastitis control in the majority of dairy herds since 1996 (USDA et al., 1996). Long-acting antimicrobials, usually consisting of beta-lactams used alone or in combination with other agents such as streptomycin or novobiocin, are still routinely administered to all quarters of all cows at the end of lactation by dairy farmers worldwide.

However, public health concerns have been raised regarding the overuse of antimicrobials and the indiscriminate treatment of uninfected cows and quarters, that could provide a strong selective pressure for the emergence of AMR (Rajala-Schultz et al., 2004). Although dry period can be particularly difficult to manage in the absence of antibiotic therapy, governmental regulations encourage the reduced use of antimicrobials for a prophylactic purpose; the comprehensive DCT should be avoided, according to the commission notice (2015/C 299/04; European Commission, 2015). Facing with the biggest challenge of the modern dairy industry, a potential mean of reducing antimicrobial usage can be selective DCT, reserved for cows or quarters suspected of having IMI. The accurate identification of the infected cows can be based on the monthly recorded cow SCC (Bradley and Green, 2004), the California mastitis test at dry-off or the CM history of the cow (Torres et al., 2008). In addition, external and internal teat sealants can be administered to protect quarters/cows, in order to improve the overall udder health during dry-off period (Timms, 2001). Their use, alone to healthy quarters or in the presence of selective DCT, has been shown to be effective in preventing new IMIs at drying off and calving, and CM cases in early lactation (Rabiee and Lean, 2013; Dufour et al., 2019; Kabera et al., 2021). This approach could reduce the antimicrobial usage on dairy farms, without negative effects at cow- and herd-levels.

Vaccination

An effective vaccination strategy against the major mastitis pathogens could be an option to increase the host immunocompetence, as well as to reduce the incidence of IMIs and their pathologic consequences in dairy herds, and the overall antimicrobial usage on dairy farms (Sordillo, 2018). Until now, the developed vaccines against the most common mastitis agents have provided limited protection, depending on several factors also associated with the host and the environment (Gomes and Henriques, 2016). Their efficacy to prevent new infections and to reduce the severity of diseases is highly controversial (Ismail, 2017).

One of the vaccine products against *E. coli* is based on the mutant bacterin of strain J5 (Enviracor™ J-5, Zoetis, Parsippany, New Jersey), and is efficacious in reducing clinical signs of coliform infections, and consequent milk loss (Wilson et al., 2009; Herry et al., 2017). Other gram-negative vaccines are a broad bacterin-toxoid from the *E. coli* mutant strain (J-VAC®, Merial Ltd., Duluth, Georgia) and a bacterin-toxoid formulated from a Re-17 mutant of *Salmonella typhimurium* (ENDOVAC-Dairy®, Immvac Inc., Columbia, Missouri; Sordillo, 2018). Furthermore, the commercially available *Klebsiella pneumoniae* siderophore receptor protein vaccine (Vaxxon® SRP® Klebsiella, Epitopix, Willmar, MN) have successfully been used for protection against *Klebsiella* mastitis and for cross-protection against all coliforms (Gorden et al., 2018).

Recently, a high priority has been given to the development of an effective vaccine against *S. aureus* in both human and veterinary medicine, because the large use of drugs in staphylococcal infections have increased the risk of MRSA spreading (Keane, 2019). The use of potential antigens such as *S. aureus* virulence factors and surface proteins (SpA, FnBPA, FnBPB and ClfA) can limit the transmission of the contagious pathogen, even if it cannot provide a complete protection against *S. aureus* (Pankey et al., 1985; Shkreta et al., 2004). In the U.S. dairy herds, the administration of the only marketed *S. aureus* mastitis bacterin (Lysigin®, Boehringer Ingelheim Vetmedica Inc., St. Joseph, Missouri) has reported to increase the spontaneous cure rate and decrease SCC in cows, and to reduce the incidence of IMIs in heifers (Nickerson and Ryman, 2019). The polyvalent vaccine, including a bacterin based on *S. aureus* SP140 in addition to inactivated *E. coli* J5, is available in Europe and Canada (Startvac®, Hipra, Spain; Prenafeta et al., 2010).

Currently, a *Mycoplasma bovis* bacterin (Mycomune®AgriLabs, St. Joseph, Missouri) is labeled for bovine mastitis, and has shown to prevent new infections and reduce positive bulk tank cultures caused by *Mycoplasma bovis* (Sordillo, 2018; Nickerson and Ryman, 2019).

Autogenous *Mycoplasma* and *S. aureus* vaccines have also been developed with specific strains of *Mycoplasma* and *S. aureus*, respectively, isolated from individual cows with IMI within a single dairy

farm. There is some evidence that their use can protect other animals of the same herd against further udder infection (Nickerson and Ryman, 2019).

In the field of *Strep. uberis* mastitis vaccination, subunit vaccines against the plasminogen activator pauA, the cell surface associated protein GapC, and the adhesion molecule SUAM may only provide a strain-specific protection (Leigh et al., 1999; Prado et al., 2011; Song et al., 2017). The commercial inactivated *Strep. uberis* vaccine is based on lipoteichoic acid from biofilm adhesion component of strain 5616 (UBAC, Laboratorios Hipra S.A., Amer, Spain; Collado et al., 2018).

As immune stimulation cannot be sufficient, high hygiene conditions, satisfactory housing and feeding conditions during both the dry and the lactation periods should be kept to reduce the IMI risk and the antimicrobial usage on dairy farms (Curone et al., 2018).

Complementary and Alternative Therapeutic Approaches

In the last decades, the rising public awareness regarding the antimicrobial usage in food production and the consequence emergence of AMR worldwide made it necessary to find alternative treatments for bovine mastitis (Ruegg, 2003). As the environmental and animal conditions take part in the development of IMIs, the improvement of dairy cattle management can ensure cow welfare that is a key issue for the host immune response (Pyörälä, 2002). Among the factors involved in immunocompetence and disease resistance, appropriate nutrition appears crucial to achieve a positive balance in the gastrointestinal microbiota of cattle but also in the mammary gland (Barkema et al., 2015; Sommer et al.,2017). Pre- and probiotics help to establish and restore microbiota health (Bronzo et al., 2020), while the proper use of immunomodulators, such as lactoferrin, and the supplementation of selenium, copper, zinc, vitamin A and E can have positive effects on udder health and immune defense (Trevisi et al., 2014; Sordillo, 2018).

References mentioned in the Introduction are reported in the Bibliography.

Aims

A unified view of the epidemiology of bovine mastitis could be needed to develop a unique approach to controlling and treating the disease in dairy cattle.

The present project was focused on the antimicrobial susceptibility and the molecular characterization of the most common pathogens detected in milk. The phenotypic and genotypic results could be useful to establishing the prevalence and distribution of antimicrobial-resistant strains within bovine populations and to associate their resistance patterns with their genotypic clusters and virulence profiles. The importance of this research consisted of improving our knowledge about the genetic diversity within bacterial species involved in IMI and present-day antibiotic resistance trends.

The principal aims of this project were to determine the genotypic variability, the virulence and the antimicrobial resistance in *S. aureus* and *Strep. uberis* isolates collected from bovine mastitis.

I. A collaboration among 6 countries contributed *S. aureus* isolates. The strains, previously genotyped by RS – PCR, were analyzed for selected virulence and antimicrobial resistance genes by PCR and for phenotypic antimicrobial susceptibility by MIC assay.

II. The epidemiology of *Strep. uberis* was investigated along with the pathogenic properties. Two mPCR assays were developed for the simultaneous detection of 10 *Strep. uberis* genes and were subsequently used to identify and quickly characterize *Strep. uberis* isolates. We subtyped the *Strep. uberis* strains by RAPD – PCR to understand their genetic heterogeneity and their transmission mode within a dairy herd. We also explored the distribution of the antimicrobial resistance across different RAPD patterns by MIC assay.

III. The last aim of this project was to give a general overview of the main pathogens involved in the development of IMI and the relationship to foodborne diseases. From a practical point of view, the last part of this thesis provided further knowledge of IMI characteristics and their impact on both

animal and human health. From a wider perspective, the information on different techniques for their identification and detection could be useful in bridging the gap between the phenotypic and genetic traits of mastitis causative agents other than *S. aureus* and *Strep. uberis*.

Part I: *Staphylococcus aureus*

Staphylococcus is a genus comprising 51 species and 27 subspecies of gram-positive bacteria belonging to the family of *Staphylococcaceae* whose members are ubiquitous and highly versatile (Founou et al., 2018). In dairy cattle, *Staphylococcus aureus* is the best known among coagulase-positive staphylococci and is predominantly classified as a contagious pathogen (Schroeder, 2012). This microorganism is characterized by low recovery rates despite the efforts in controlling its presence and diffusion in dairy herds (Barkema et al., 2006).

Molecular genotyping of the isolates is an important tool in epidemiological studies of staphylococcal infections and contributes to understand *S. aureus* dissemination among animal populations. Typing of *S. aureus* isolates by DNA sequencing of the variable spacer region of the staphylococcal *spa* gene (Harmsen et al., 2003) may give insight into the epidemiology of *S. aureus* strains and into their likely origin. Multilocus sequence typing is an alternative method to discriminate *S. aureus* strains of different clonal complexes (Enright et al., 2000). Among them, the CC8, well documented in human infections, has been frequently found in bovine IMIs due to the recent bovine adaption of this cluster as a consequence of a new human-to-cow host jump (Cremonesi et al., 2015). Because of its high variability, another technique for *S. aureus* genotyping is the PCR amplification of the 16S-23S rRNA intergenic spacer region (Jensen et al., 1993). The RS-PCR is a reliable but expensive means for the rapid characterization of *S. aureus* strains and the results were similar to those obtained from more costly and time consuming methods (Cremonesi et al., 2015). Recent studies have used RS-PCR to demonstrate that a *S. aureus* genotype predominates on each farm, due to its contagious nature, and only a few genotypes are linked to the high within-cow and within-herd presence of IMIs (Fournier et al., 2008; Graber et al., 2009). In European dairy herds, the major bovine *S. aureus* genotypes, combined with their variants into genotypic clusters, comprised of: CLB, CLC, CLR, CLF and CLI (Cosandey et al., 2016). Cluster B, consisting mainly of *S. aureus* CC8 strains, is strongly associated with the bovine mammary gland and is frequently detected in milk samples from bovine mastitis, as

it is characterized by high contagiousness and pathogenicity (van den Borne et al., 2017). Cluster C, considered as "dairy cattle specific" together with CLR, is usually involved in individual cow diseases and infects single quarters (Cosandey et al., 2016). The others are primarily observed on bovine surfaces (e.g., teat skin) and in the environment but are not related to the on-farm presence of IMI (Leuenberger et al., 2019).

The wide spread of some genotypic clusters could be due to their particular variable genome, consisting of accessory genes encoding different virulence factors. The combination of these factors, involved in binding to host cells and damaging host tissues and evading host immune defenses, plays a decisive role in the onset and progression of the infection (Grumann et al., 2014; Peton and Le Loir, 2014). Among them, the surface adhesins mediate adherence to different substrates of the host (Clarke and Foster, 2006; Speziale et al., 2009). The successive invasion of host tissues involves the production of the haemolysins (*hla, hlb, hld* and *hlg*) which destroys many different types of cells, including epithelial and endothelial cells. The *hlg* gene cluster gives rise to γ-haemolysin, a bi-component leukotoxin (Grumann et al., 2014). *S. aureus* strains frequently produce other leukotoxins, among which LukAB/GH, LukED and LukS/F-PV (also known as PVL) can kill neutrophils, macrophages and dendritic cells (Ventura et al., 2010; Dumont et al., 2011; Alonzo et al., 2012; Vandenesch et al., 2012). The PVL is mainly found in strains of human origin and is weakly active on bovine neutrophils (Schlotter et al., 2012) but the recent detection in isolates from bovine mastitis has suggested that a *S. aureus* transmission between farmers and cows may also occur (Mekonnen et al., 2018; Antók, et al., 2019). Similarly, the IEC genes are more prevalent among human colonizing *S. aureus* strains than in isolates of ruminant origin (Verkaik et al., 2011), although they also appear to be involved in invading animal tissues (Cuny et al., 2015). The SAgs, comprising toxic shock syndrome toxin–1 (*tsst-1*) and SEs, are highly potent immunostimulatory molecules, implicated not only in the pathogenesis of bovine mastitis (Magro et al., 2017), but also in human illness from foodborne diseases (Figure 4; Foster, 2005), as described in detail in the Part III. The function of each

extracellular protein in the development of IMI has yet to be defined and it is still uncertain which of these play a decisive role as virulence factors in cattle.

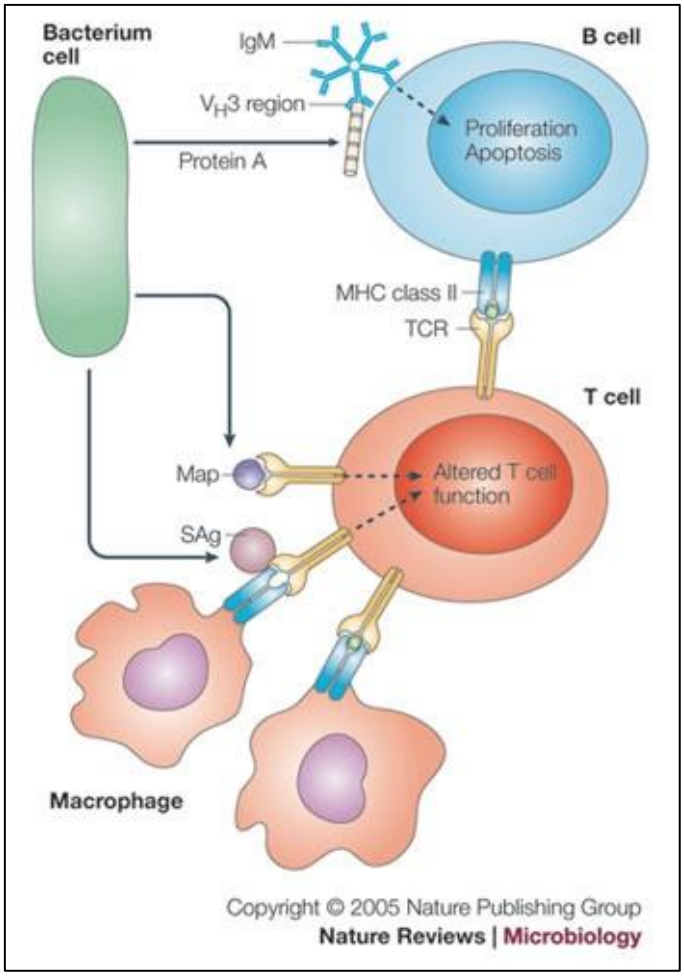

Figure 4. Host defense mechanisms of *Staphylococcus aureus*. Reproduced from Foster, 2005.

The accessory genes encode not only virulence but also AMR genes. The feature of *S. aureus* acquiring resistance quickly and successfully to the most common drugs is strongly linked to the stability and evolution of this pathogen (McCallum et al., 2010; Gao et al., 2012). The *mecA* and *mecC* genes are responsible for resistance to methicillin and are also associated with decreased affinity to other β-lactams, cephalosporins, aminoglycosides, and macrolides (Bloemendaal et al., 2010; Bitrus et al., 2017; Foster, 2017). Interest in MRSA has risen since the first appearance in 1961 (Jevons et al., 1963), followed by the increasing diffusion of Hospital-Acquired (HA-MRSA) and

Community-Acquired (CA-MRSA) infections (Basset et al. 2011). Recently a high priority has been given to the emergence of Livestock-Associated MRSA (LA-MRSA) strains (Graveland et al., 2011; Paterson et al., 2014). LA-MRSA CC398, first described as a pig-adapted lineage in 2005 (Voss et al., 2005), has rapidly spread among other animals, including ruminants, poultry, horses and pets, but also in humans, suggesting the risk of its zoonotic transmission (McCarthy et al., 2012). On dairy farms, the evolution of MRSA has depended on the large use of β-lactams to prevent and cure mastitis cases and represents the main reason for treatment failure (Mekonnen et al., 2005). The use of penicillin and ampicillin for extended periods of time has led to the development of *S. aureus* resistance against these active substances (Mekonnen et al., 2018; Antók, et al., 2019; Ndahetuye et al., 2019). In particular, penicillin has been the drug of choice for several decades and has resulted in the wide spread of *blaZ*, another gene encoding β-lactamase (Olsen et al., 2006) since 1944 (Kirby, 1944). By the late 1960's, the number of penicillin-resistant *S. aureus* strains had significantly increased (Olsen et al., 2006). Recently the prevalence of *S. aureus* penicillin resistance has started to decrease (Ruegg et al., 2015) simultaneously with an increase in resistance to methicillin (Bitrus et al., 2017) and this may vary considerably among countries (Barkema et al., 2006). Besides the extensively used β-lactams, another class of effective antibiotics for staphylococcal mastitis consists of the macrolides, including erythromycin (Barkema et al., 2006). The most important resistance mechanism for this drug is regulated by *erm* genes (Gatermann et al., 2007). The emergence of tetracycline-resistant *S. aureus* strains is strongly related to the carriage of *tetM* and *tetO* genes, expressing ribosomal protection by elongation factor-like proteins (Connell et al., 2003), or *tetK* and *tetL* genes, encoding active efflux (Figure 5; Levy et al., 1999).

S. aureus responds poorly to treatment with many different drugs, but antimicrobial therapy still plays a significant role in prevention and cure of bovine staphylococcal mastitis. Since the selection of increasingly antibiotic-resistant *S. aureus* strains can cause several therapeutic problems, the greatest challenge to treat *S. aureus* infections is the identification of the most appropriate antibiotic agents

(Pol and Ruegg, 2007; USDA et al., 2007). The choice of treatment should be based on knowledge of the antimicrobial resistance of the infecting strain. The selection of antibiotics based on susceptibility tests cannot guarantee success of mastitis therapy as the use of human and different animal species breakpoints (Entorf et al., 2016; Kaczorek et al., 2017) do not take into account the pharmacokinetic aspects of ruminants and mammary gland. The phenotypic methods can be combined with molecular analysis to investigate the presence of antibiotic resistance genes (Cockerill, 1999; Walker, 2006). As previously described (Ruegg et al., 2015), the discrepancy between genotypic and phenotypic results may demonstrate that the detection of genes does not necessarily implicate their expression or else *S. aureus* resistance to a specific antimicrobial might occur via mechanisms associated with different resistance genes. Further research is necessary to test a broader selection of antibiotic resistance genes and to consider multiple loci related to these genes in order to validate resistance breakpoints. These results can be meaningful for practical management.

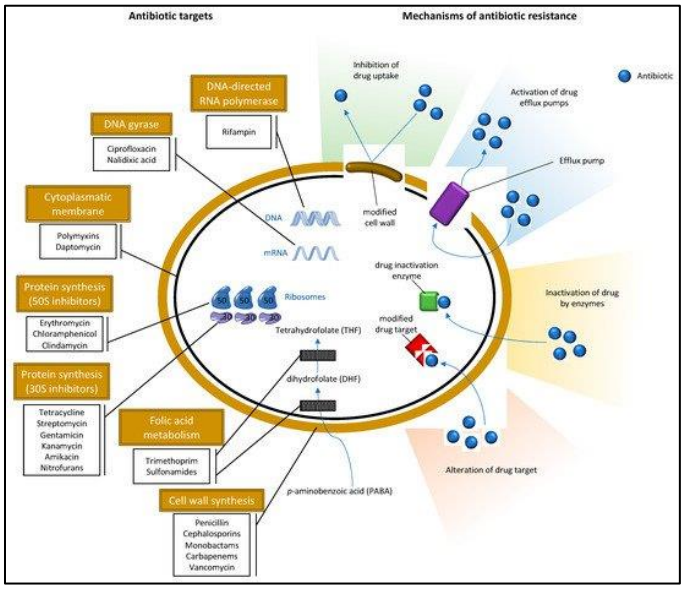

Figure 5. Mechanisms of antimicrobial resistance in *S. aureus*. Reproduced from Silva et al., 2020.

Staphylococcus aureus Isolates from Bovine Mastitis in Eight Countries: Genotypes, Detection of Genes Encoding Different Toxins and Other Virulence Genes

During the internship period in 2017, I had the opportunity to participate in an interesting collaborative research project between University of Milan and Institute of Agricultural Biology and Biotechnology – Italian National Research Council (IBBA-CNR), with the involvement of a great number of countries contributing *S. aureus* isolates. A comprehensive data collection was undertaken to give an overview of *S. aureus* molecular characteristics. A large collection of *S. aureus* isolates was analyzed and their genotypes were associated with their virulence profiles. The confirmation of a worldwide association between genotypic clusters and specific genes could be of great relevance from the epidemiological point of view and help with the identification of the harmful strains involved in bovine mastitis, requiring separate control measures and treatment strategies.

A total of 120 *S. aureus* isolates from bovine CM and high SCC samples were collected from 8 different countries: Argentina, Brazil, Colombia, Germany, Italy, the United States, South Africa, and Tunisia (Figure 6).

They were transported to the microbiology laboratory of the Department of Veterinary Medicine at the University of Milan during 2017 and were selected for a worldwide study on *S. aureus* (Monistero et al., 2018). All the 120 *S. aureus* isolates were genotyped by RS-PCR to increase knowledge of the circulating genetic lineages among the cow populations with mastitis and were analyzed by PCR for a total of 26 virulence-associated genes (Monistero et al., 2018).

We determined genotypes of bovine *S. aureus* strains and a specific genotypic cluster was found for each country (Figure 7; Monistero et al., 2018).

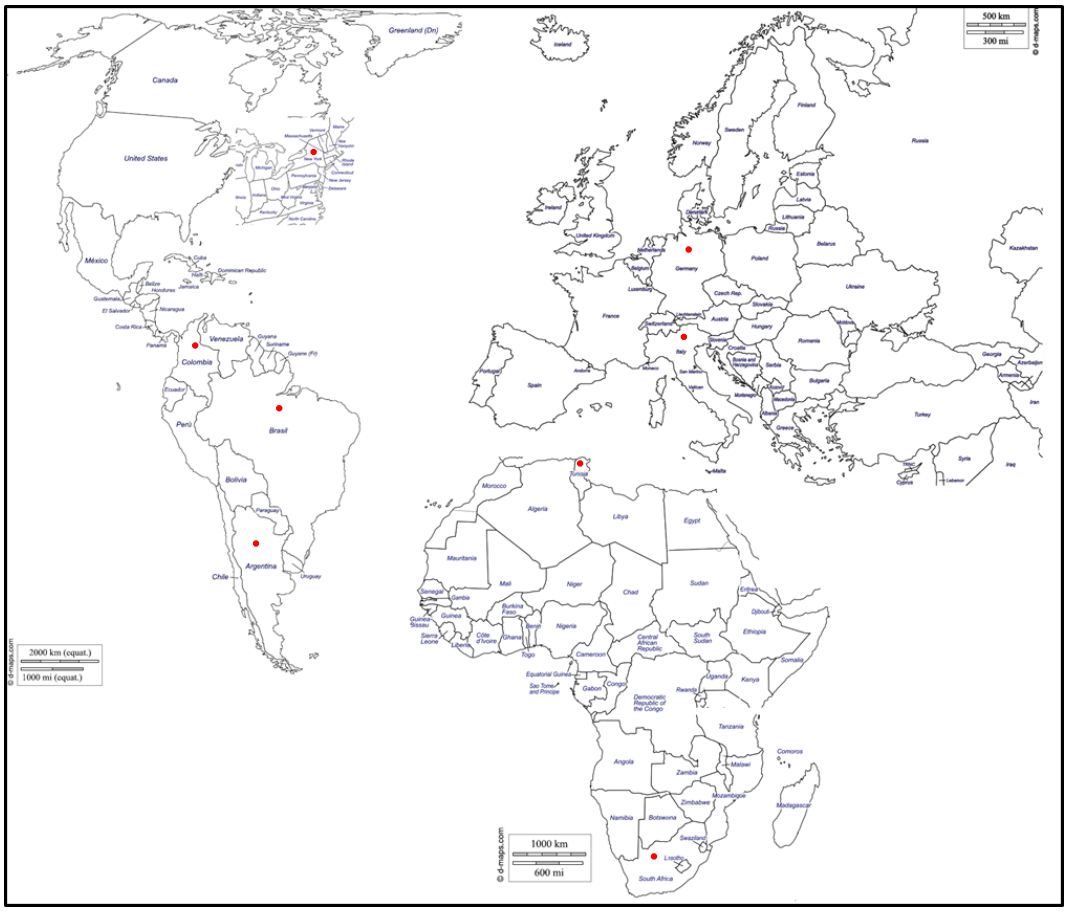

Figure 6. World survey on bovine *S. aureus* isolates: participating countries are marked by red dots. Adapted from http://www.d-maps.com.

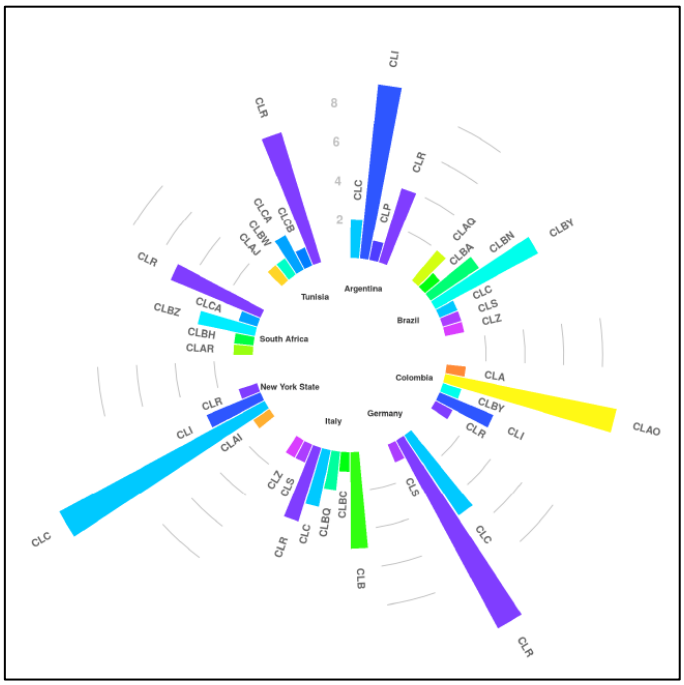

Figure 7. Representation of the major genotypes with their variants combined into genotypic clusters. Reproduced from Monistero et al., 2018.

This study showed that *S. aureus* differed among countries with each having a particular association of genotypic clusters and virulence profiles. As the genetic characteristics of *S. aureus* strains could be related to their origin, considering their geographical isolation could help to monitor the staphylococcal intramammary infection spread. Few genotypic clusters were disseminated worldwide. The CLC and CLR, in association with some virulence factors involved in binding to host cell and tissues, were found with high frequency. These combinations could be related to the bacterial ability to colonize and invade the udder and their screening in *S. aureus* isolates could be useful to identify harmful strains and predict clinical outcomes (Monistero et al., 2018).

This collaborative research added to my understanding of *S. aureus*. To understand further, I involved researchers from IBBA-CNR in the work below (Monistero et al., 2020). We used 93 of the 120 strains previously analyzed selecting only isolates recovered from CM cases. As staphylococcal genotypes are highly associated with virulence but also AMR profiles, we carried out the phenotypic and genotypic analysis of antimicrobial resistance in the *S. aureus* strains.

References mentioned in the Part I are reported in the Bibliography.

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Different Distribution of Antimicrobial Resistance Genes and Virulence Profiles of *Staphylococcus aureus* Strains Isolated from Clinical Mastitis in Six Countries

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Abstract

Staphylococcus aureus is recognized worldwide as one of the main contagious mastitis agents in cattle and can express a set of antimicrobial resistance genes and virulence-associated genes that explain the wide range of outcomes of intramammary infections. *Staphylococcus aureus* strains are heterogeneous: their different resistance and virulence patterns, associated with the host-level factors and treatment factors, are related to the severity of infection. The aim of this study was to determine phenotypic antibiotic susceptibility, occurrence of selected antimicrobial resistance genes and other virulence genes in 93 *S. aureus* strains isolated from clinical mastitis in 6 different countries: Argentina, Brazil, Germany, Italy, the United States (New York State), and South Africa. These isolates were tested against a total of 16 drugs (amoxicillin-clavulanate, ampicillin, cefazolin, cefoperazone, cefquinome, enrofloxacin, erythromycin, gentamicin, kanamycin, lincomycin, oxacillin, penicillin, rifampin, spiramycin, sulfamethoxazole/trimethoprim, tylosin) by minimum inhibitory concentration (MIC) assay, and examined for the presence of 6 antibiotic-resistance genes (*blaZ, mecA, mecC, ermA, ermB, ermC*) and 6 virulence-associated genes (*scn, chp, sak, hla, hlb, sea*) via PCR analysis. The phenotypic results of this study revealed the presence of 19.4% penicillin-resistant strains, whereas 22.6% of the strains were classified as having resistance (5.4%) or

intermediate resistance (17.2%) to erythromycin. Most (96.8%) of the isolates were inhibited by cephalosporins, and all were susceptible to amoxicillin-clavulanate. Two strains (1 from Germany, 1 from Italy) were resistant to oxacillin and were positive for *mecA.* Among the other antimicrobial resistance genes, the most frequently detected was *blaZ* (46.2%), and 32.3% of the isolates were positive for *erm* genes: *ermC* (21.5%) and *ermB* (10.8%). The most prevalent virulence gene was *hla* (100%), followed by *hlb* (84.9%) and *sea* (65.6%). These results show a low prevalence of antibiotic multidrug resistance in *S. aureus* isolates, even if the detection of selected antimicrobial resistance genes did not always correspond with the occurrence of phenotypic antibiotic resistance; the immune evasion cluster gene prevalence was quite low in the samples analyzed.

Keywords: dairy cow, mastitis, *Staphylococcus aureus*, virulence gene, antimicrobial resistance gene, MIC

Introduction

Mastitis is a common disease of dairy cows and a major concern for the dairy industry because of economic losses due to the decreased animal health and increased antibiotics usage (Heikkilä et al., 2018; Gussmann et al., 2019). *Staphylococcus aureus* is one of the major agents of contagious mastitis, responsible for mainly subclinical but also clinical infections in cattle worldwide (Barkema et al., 2006). This pathogen, in combination with both the bovine host and environmental factors, is characterized by low cure rates compared to other mastitis pathogens because of its capability to acquire antibiotic resistance and produce a wide array of virulence factors (Malinowski et al., 2002; Moroni et al., 2006; Sakwinska et al., 2011; Gao et al., 2012). Higher parity is associated with a lower probability of cure, which is lower also in older cows with high SCC and in cows infected in hindquarters during early and mid-lactation (Sol et al. 1997). Although *S. aureus* responds poorly to treatment with many different antimicrobial agents, antibiotic therapy still plays a significant role in the prevention and cure of bovine staphylococcal mastitis. The infection of cows with increasingly

antibiotic-resistant strains can cause several therapeutic problems and is one of the main reasons for monitoring drug resistance (Pol and Ruegg, 2007; USDA, 2007; Saini et al., 2012a). The measurement of *S. aureus* antimicrobial resistance using phenotypic susceptibility tests, such as disk diffusion or MIC assay, is essential in order to select the most appropriate and efficient therapy (Walker, 2006). These methods can be combined with molecular analysis, as phenotypic *S. aureus* resistance to the most commonly used antimicrobials is related to the expression of antibiotic-resistance genes (Cockerill, 1999). The genes associated with resistance to β-lactams are often detected in *S. aureus* isolates from bovine milk samples, because β-lactams have been widely used to prevent and treat mastitis cases for several decades (Saini et al., 2012a; Saini et al., 2012b). Among the genes encoding β-lactamase, *blaZ* is responsible for resistance to penicillin (Olsen et al., 2006), whereas the *mecA* (Sawant et al., 2009) and *mecC* (Paterson et. al., 2014) genes confer resistance to methicillin, a semisynthetic penicillinase-resistant penicillin. Another concern is the emergence of erythromycin resistance regulated by the *ermA, ermB,* and *ermC* genes encoding different ribosomal methylases (Gatermann et al., 2007). In addition to antibiotic-resistance genes, *S. aureus* strains can harbour virulence genes in different combinations, thereby expressing factors used to attach, colonize, invade, and infect the host, which contribute largely to the establishment and severity of bovine mastitis (Jarraud et al., 2002). Many *S. aureus* virulence factors can be described as toxins (Otto, 2014). Among them, the hemolysins are cytolytic toxins able to lyse different types of cells. *Staphylococcus aureus* isolates from bovine mastitis, in particular, show a high level of expression of α-toxin (*hla*), exhibiting dermonecrotic and neurotoxic effects on a wide range of mammalian cells (Berube and Bubeck Wardenburg, 2013). β-toxin (*hlb)* is a sphingomyelinase that damages cell membranes rich in this lipid; it is produced abundantly by isolates of animal origin (Clarke and Foster, 2006), as it increases the adherence of *S. aureus* to bovine mammary epithelial cells (Magro et al., 2017). *Staphylococcus aureus* can also produce a wide array of enterotoxins (from SEA to SEQ): SEA, in particular, is able to promote the ability of *S. aureus* to evade host immune defenses by negatively affecting the activity of neutrophils (Xu et al., 2014). The gene for enterotoxin A (*sea*)

belongs to the immune evasion cluster (**IEC**), which also includes *scn, chp, sak* and other enterotoxin genes (*sep*, *sek*, or *seq*; Cuny et al., 2015). This cluster can interfere with host immunity and is common in methicillin-resistant *S. aureus* (**MRSA**) strains isolated from humans (McCarthy and Lindsay, 2013); in animals, it was previously detected in MRSA isolates from horses (Cuny et al., 2015).

Because *S. aureus* virulence and antimicrobial resistance profiles are associated with specific genotypes (Fournier et al., 2008), a greater understanding of the epidemiology of *S. aureus* genotypes in dairy herds may help monitor the emergence of antimicrobial-resistant strains associated with their virulence characteristics. The aim of this study was to determine (1) the phenotypic antimicrobial susceptibility and (2) the prevalence of selected antimicrobial resistance genes and other virulence genes in 93 *S. aureus* isolates from clinical mastitis milk samples collected in 6 different countries; all these isolates were previously genotyped by RS-PCR (Monistero et al., 2018).

Materials and Methods

Herd and Isolate Enrollment Criteria

Ninety-three *S. aureus* isolates from single-quarter (Q) and composite (C) milk samples of cows with clinical mastitis were collected between 2012 and 2017 from 76 farms in 6 countries: Argentina, Brazil, Germany, Italy, the United States (New York State), and South Africa (Table 1). Farms enrolled in the present and prior study (Monistero et al., 2018) were required to have a minimum of 120 lactating cows, to participate in monthly DHI testing or to use monthly California Mastitis Test for all lactating animals, to use a milking routine including fore-stripping of quarters for detection of mastitis, and to have a farm survey once a year by sending quarter or composite milk samples to the reference laboratory. The isolates were selected based on a non-probability convenience sample, and

only isolates from clinical mastitis were selected for this study. Considering a within-herd mastitis prevalence of 20%, of which 10 to 15% were clinical cases, this yielded 2 to 4 isolates per farm.

Table 1. Source and type of *Staphylococcus aureus* isolates identified in this study

Country	Total isolates per country	Number of farms	Type of sample[1]	Date of isolation
Argentina	16	10	C	April 2015 to June 2017
Brazil	15	12	Q	July 2014 to May 2015
Germany	17	17	Q	May 2012 to August 2016
Italy	17	15	Q	September 2012 to December 2016
United States (New York State)	17	13	Q	January 2017 to April 2017
South Africa	11	9	Q	August 2016 to February 2017

[1]C = composite milk sample; Q = quarter milk sample.

Sample Collection

Milk samples were collected by farm personnel trained to detect mastitis cases. After disinfection of teat ends and discarding the first streams of foremilk, milk was collected in 10-mL sterile vials, labeled with cow number and quarter. Clinical mastitis was defined as visibly abnormal milk from a mammary quarter (Wenz el al., 2001; Ruegg, 2011).

Milk samples were stored at 4°C and shipped to their respective laboratories. Ten microliters of each sample were plated on blood agar plates, and bacterial cultures were evaluated after 24 h of aerobic incubation at 37°C. *Staphylococcus aureus* colonies were round, smooth, substantial, opaque, characterized by hemolysis, and were positive in the tube coagulase test (Cookson, 1997). One colony of each *S. aureus* isolate was subcultured and stored at -20°C.

The isolates were shipped frozen on either dry ice or wet ice, depending on the distance, to the Department of Veterinary Medicine (University of Milan, Italy). Upon arrival, isolates were stored at -20°C.

Antimicrobial Susceptibility Testing

The MIC of 16 antimicrobials were determined for 93 *S. aureus* isolates, using the broth dilution test according to the procedure described in Clinical and Laboratory Standards Institute (CLSI) guidelines VET01-A4 (CLSI, 2013). The MIC were performed with a customized commercial microdilution MIC system (Micronaut-S MIC Mastitis, Merlin Diagnostika, GmbH, Bornheim, Germany) used for routine laboratory testing of mastitis isolates. Results were interpreted using available CLSI resistance breakpoints according to VET01-S2 guidelines (CLSI, 2013) or other breakpoints reported in the literature if CLSI standards were not established. If breakpoints were differentiated for host species, cattle breakpoints were selected. The CLSI breakpoints were used for the following antimicrobials: amoxicillin-clavulanate, ampicillin, cefazolin, enrofloxacin, erythromycin, gentamicin, oxacillin, penicillin, rifampin, and sulfamethoxazole/trimethoprim. The breakpoints standardized by the Société française de microbiologie (2018) were used for lincomycin, kanamycin, and spiramycin; literature references were used for cefoperazone (Feβler et al., 2012), cefquinome (Lang et al., 2002), and tylosin (Simjee et al, 2011). Furthermore, the MIC inhibiting the growth of 90% of the isolates (MIC_{90}) was calculated for each antimicrobial. The MIC plates reading was performed manually, and the last concentration of antimicrobial that did not show turbidity or a deposit of cells at the bottom of the well was recorded. The MIC value of each isolate, expressed as micrograms per milliliter, was defined as the lowest concentration of the antimicrobial agent that completely inhibited the growth after the incubation period. *Staphylococcus aureus* ATCC 29213 was used as a quality-control strain in each MIC batch, and a double negative control was used for each plate. The antimicrobials used on the plate were selected based on their activity against mastitis pathogens and on their registrations for dairy cattle. Ceftiofur was not included in the plate, because this drug is not approved for mastitis treatment in Europe, as opposed to the United States and Canada. Considering that third-generation cephalosporins are generally not advised for *S. aureus* treatment, specific testing for this antimicrobial was not performed in the present study.

β-Lactamase Detection

Phenotypic β-lactamase activity was determined using the nitrocefin-based test (nitrocefin disks, Sigma-Aldrich, St. Louis, MO), performed according to the manufacturer's instructions and to VET08 guidelines (CLSI, 2018). *Staphylococcus aureus* ATCC 29213 and *S. aureus* ATCC 25923 were used as positive and negative controls, respectively.

Molecular Analysis

DNA Extraction

DNA was extracted from the isolates following the protocol described by Cremonesi and coworkers (2006). After the measurement of its amount and quality using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE), DNA was stored at -20°C.

Molecular Characterization

The DNA extracted from the 93 isolates was amplified via specific PCR analysis to determine the occurrence of 6 antibiotic-resistance genes (*blaZ, mecA, mecC, ermA, ermB, ermC*), the hemolysins (*hla* and *hlb*) and the IEC genes (*chp, sak, scn,* and *sea*). All these genes were investigated using primers and protocols described in literature (Table 2). Each PCR reaction contained a total of 12.5 µL of Phusion High-Fidelity Master Mix 2× (ThermoFisher Scientific, Waltham, MA) for detection of *blaZ, ermA,* and *hla* or 12.5 µL of PCR Master Mix 2× (Thermo Fisher Scientific) to investigate the other genes considered; 0.2 µL of each primer (100 µ*M*) were added to 2 µL of genomic DNA (5 ng/µL).

Table 2. Primer sequences, melting temperature (T_m) values and sizes of PCR product for the amplification of 93 *Staphylococcus aureus* isolates analyzed

Target gene	Primer sequence	T_m (°C)	Amplification size (bp)	Reference
blaZ	5'-AAGAGATTTGCCTATGCTTC-3' 3'-GCTTGACCACTTTTATCAGC-5'	50	517	Sawant et al., 2009
mecA	5'-GTAGAAATGACTGAACGTCCGATAA-3' 3'-CCAATTCCACATTGTTTCGGTCTAA-5'	56	310	McClure et al., 2006
mecC	5'-CATTAAAATCAGAGCGAGGC-3' 3'-CATTAAAATCAGAGCGAGGC-5'	52	188	Paterson et al., 2012
ermA	5'-TCTAAAAAGCATGTAAAAGAA-3' 3'-CTTCGATAGTTTATTAATATTAG-5'	52	645	Sutcliffe et al., 1996
ermB	5'-CATTTAACGACGAAACTGGC-3' 3'-GGAACATCTGTGGTATGGCG-5'	55	424	Jensen et al., 1999
ermC	5'-ATCTTTGAAATCGGCTCAGG-3' 3'-CAAACCCGTATTCCACGATT-5'	55	294	Jensen et al., 1999
chp	5'-TTTTTAACGGCAGGAATCAGTA-3' 3'-TGCATATTCATTAGTTTTTCCAGG-5'	55	404	Sung et al., 2008
sak	5'-TGAGGTAAGTGCATCAAGTTCA-3' 3'-CCTTTGTAATTAAGTTGAATCCAGG-5'	55	403	Sung et al., 2008
scn	5'-ATACTTGCGGGAACTTTAGCAA-3' 3'-TTTTAGTGCTTCGTCAATTTCG-5'	55	320	Sung et al., 2008
hla	5'-GGTTTAGCCTGGCCTTC-3' 3'- CATCACGAACTCGTTCG-5'	53	534	Salasia et al., 2004
hlb	5'-GCCAAAGCCGAATCTAAG-3' 3'-CGCATATACATCCCATGGC-5'	50	833	Salasia et al., 2004
sea	5'-TAAGGAGGTGGTGCCTATGG-3' 3'-CATCGAAACCAGCCAAAGTT-5'	56	180	Cremonesi et al., 2005

Table 3. *Staphylococcus aureus* reference strains used as positive controls in PCR reactions for detection of selected genes investigated in this study

Reference strains	Target genes
ATCC[1] 19040	*chp, hlb*
ATCC[1] 19041	*sea, hla*
ATCC[1] 19048	*blaZ, nuc, sak, scn*
ATCC[1] 700699	*mecC, ermA*
IZSLER[2] 182828/321	*ermB*
IZSLER[2] 194588/52A	*ermC*
IZSLER[2] STAU26	*mecA*

[1] Reference strains with known genotype.
[2] Isolates from the collection of IZSLER (Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna).

As positive controls, *S. aureus* reference strains (ATCC 19040, ATCC 19041, ATCC 19048, ATCC 700699, or *S. aureus* isolates from the collection of IZSLER, previously analyzed by molecular tests) were used in each PCR assay (Table 3). All amplified PCR fragments were visualized on 2% agarose

gel electrophoresis (GellyPhor, Euroclone, Milan, Italy), stained with ethidium bromide (0.05 mg/mL; Sigma-Aldrich), and visualized under UV transilluminator (BioView Ltd., Nes Ziona, Israel). A 100-bp DNA ladder (Finnzymes, Espoo, Finland) was included in each gel.

Results

Antimicrobial Profiling and Virulence Profiling

The 93 *S. aureus* strains analyzed in this study were identified with the same identification (**ID**) numbers used in the previous study (Monistero et al., 2018). All of them were positive for the gene for α-haemolysis (*hla*) but negative for a gene involved in host cell invasion (*chp*) and 2 antimicrobial resistance genes, 1 responsible for resistance to methicillin (*mecC*) and 1 conferring resistance to erythromycin (*ermA*). The MIC assay demonstrated 100% phenotypical susceptibility to tylosin and amoxicillin-clavulanate.

Argentina

All of the 16 isolates from Argentina showed phenotypic intermediate or complete resistance to spiramycin, except 1 (ID 5); 7 (43.8%) isolates were not inhibited by erythromycin, and 5 (31.3%) were also not sensitive to lincomycin. Only 1 isolate (ID 12) was phenotypically resistant to ampicillin and penicillin. The nitrocefin-based method detected 2 (12.5%) β-lactamase-positive isolates (Table 4).

The molecular analysis revealed that all strains carried *ermC*, except 1 (ID 2), which was negative for this gene. The *ermC* gene was the only erythromycin-resistance gene found in Argentina, although *blaZ* was detected with a frequency of 18.8% (Table 4).

The *hlb* gene was detected in 93.8% of the Argentinian strains. Among the IEC genes, the most prevalent was *sea* (56.3%), whereas *sak* was carried by 5 (31.3%) isolates, and none harboured *scn* (Table 4).

Table 4. Phenotypic antimicrobial resistance and molecular characteristic of *Staphylococcus aureus* strains isolated in Argentina

Argentinian isolates[1]	Phenotypic antimicrobial intermediate(I) or complete resistance(R)[2]	β-Lactamase detection	*blaZ*	*mecA*	*ermB*	*ermC*	*hlb*	*sea*	*scn*	*sak*
1	SPM(R)	-	-	-	-	+	+	+	-	-
2	SPM(I)	-	-	-	-	-	+	-	-	+
3	ERY(I), SPM(I)	-	-	-	-	+	+	-	-	-
4	ERY(R), SPM(R), LIN(R)	-	-	-	-	+	+	+	-	-
5	-[3]	-	-	-	-	+	+	-	-	-
6	SPM(I)	-	-	-	-	+	+	+	-	+
7	SPM(R)	-	-	-	-	+	+	+	-	+
8	ERY(I), SPM(I)	-	-	-	-	+	-	+	-	+
9	ERY(R), SPM(R), LIN(R)	-	-	-	-	+	+	-	-	-
10	ERY(R), SPM(R), LIN(R)	-	-	-	-	+	+	+	-	+
11	ERY(I), SPM(R), LIN(R)	-	+	-	-	+	+	+	-	-
12	AMP(R), PEN(R), SPM(I)	+	+	-	-	+	+	-	-	-
13	SPM(I)	-	-	-	-	+	+	-	-	-
14	SPM(I)	-	-	-	-	+	+	+	-	-
15	ERY(R), SPM(R), LIN(R)	+	-	-	-	+	+	+	-	-
16	SPM(I)	-	+	-	-	+	+	-	-	-

[1]Isolate identification numbers correspond to those in Monistero et al. (2018).
[2]SPM = spiramycin; ERY = erythromycin; LIN = lincomycin; AMP = ampicillin; PEN = penicillin.
[3]Isolate 5 demonstrated phenotypical susceptibility to all 16 antimicrobials tested by the minimum inhibitory concentration assay.

Brazil

All 15 isolates collected in Brazil showed phenotypic intermediate or complete resistance to spiramycin, except 1 (ID 29). Among the Brazilian isolates, 46.7 % were not susceptible to lincomycin, and 13.3% were not inhibited by the range of concentration tested for erythromycin. Out

of the 15 isolates analyzed, 3 (20%) were found to be β-lactamase-positive by the nitrocefin-based method, also demonstrating in vitro resistance to ampicillin and penicillin, and 3 (20%) showed resistance to the combination of trimethoprim and sulfamethoxazole (Table 5).

The genotypic results showed that 46.7% of the Brazilian strains were positive for *blaZ*, and all were negative for both *mecA* and *erm* genes (Table 5).

As reported in Table 5, the gene for β-haemolysin (*hlb*) was present in 100% of the Brazilian strains. The majority (53.3%) of them carried *sea*, but none harbored the other IEC genes investigated, *scn* and *sak*.

Table 5. Phenotypic antimicrobial resistance and molecular characteristics of *Staphylococcus aureus* strains isolated in Brazil

Brazilian isolates[1]	Phenotypic antimicrobial intermediate(I) or complete resistance(R)[2]	β-Lactamase detection	*blaZ*	*mecA*	*ermB*	*ermC*	*hlb*	*sea*	*scn*	*sak*
17	SPM(R), SX-T(R), LIN(R)	-	-	-	-	-	+	+	-	-
18	SPM(R), LIN(R)	-	-	-	-	-	+	+	-	-
19	ERY(I), SPM(R)	-	-	-	-	-	+	-	-	-
20	AMP(R), PEN(R), SPM(I), LIN(R)	+	+	-	-	-	+	-	-	-
21	SPM(I)	-	+	-	-	-	+	-	-	-
22	AMP(R), ERY(I), PEN(R), SPM(I), LIN(R)	+	+	-	-	-	+	-	-	-
23	AMP(R), PEN(R), SPM(I), LIN(R)	+	+	-	-	-	+	-	-	-
24	SPM(I)	-	-	-	-	-	+	+	-	-
25	SPM(R), SX-T(R), LIN(R)	-	-	-	-	-	+	+	-	-
26	SPM(R)	-	+	-	-	-	+	+	-	-
27	RF(I), SPM(R), SX-T(R)	-	+	-	-	-	+	+	-	-
28	SPM(R), LIN(I)	-	-	-	-	-	+	-	-	-
29	-[3]	-	+	-	-	-	+	-	-	-
30	SPM(R)	-	-	-	-	-	+	+	-	-
31	SPM(R)	-	-	-	-	-	+	+	-	-

[1]Isolate identification numbers correspond to those in Monistero et al. (2018).
[2]SPM = spiramycin; SX-T = sulfamethoxazole/trimethoprim; LIN = lincomycin; ERY = erythromycin; AMP = ampicillin; PEN = penicillin; RF = rifampin.
[3]Isolate 29 demonstrated phenotypical susceptibility to all 16 antimicrobials tested by the minimum inhibitory concentration assay.

Germany

Table 6. Phenotypic antimicrobial resistance and molecular characteristics of *Staphylococcus aureus* strains isolated in Germany

German isolates[1]	Phenotypic antimicrobial intermediate(I) or complete resistance(R)[2]	β-Lactamase detection	*blaZ*	*mecA*	*ermB*	*ermC*	*hlb*	*sea*	*scn*	*sak*
47	ERY(I), SPM(R), SX-T(R), LIN(R)	-	-	-	-	-	+	+	-	-
48	SPM(R), SX-T(R), LIN(R)	-	-	-	-	-	+	+	-	-
49	SPM(R)	-	-	-	-	-	+	+	-	-
50	ERY(I), SPM(I), LIN(R)	-	-	-	-	-	-	+	-	-
51	SPM(R)	-	-	-	-	-	+	+	-	-
52	-[3]	-	-	-	+	-	+	-	-	-
53	AMP(R), CEZ(I), CPZ(R), CEQ(I), ERY(R), OXA(R), PEN(R), SPM(R), LIN(R)	+	+	+	+	-	+	-	-	-
54	ERY(I), SPM(R)	-	+	-	+	-	+	+	-	-
55	SPM(R)	-	+	-	+	-	+	+	-	-
56	SPM(I)	-	+	-	+	-	+	+	-	-
57	SPM(R)	-	-	-	+	-	+	+	-	-
58	SPM(I)	-	-	-	-	-	-	+	-	-
59	SPM(R)	-	+	-	-	-	+	+	-	-
60	SPM(I)	-	-	-	-	-	-	+	-	-
61	CPZ(I), SPM(I)	-	+	-	-	-	-	+	-	-
62	ERY(I), SPM(R), LIN(R)	-	+	-	-	-	-	+	-	-
63	ERY(I), SPM(R)	-	+	-	-	-	-	+	-	-

[1]Isolate identification numbers correspond to those in Monistero et al. (2018).
[2]ERY = erythromycin; SPM = spiramycin; SX-T = sulfamethoxazole/trimethoprim; LIN = lincomycin; AMP = ampicillin; CEZ = cefazolin; CPZ = cefoperazone; CEQ = cefquinome; OXA: oxacillin; PEN = penicillin.
[3]Isolate 52 demonstrated phenotypical susceptibility to all 16 antimicrobials tested by the minimum inhibitory concentration assay.

In the MIC assay, 94.1% of German isolates were phenotypically resistant to spiramycin. Out of 17 strains analyzed, 6 (35.3%) showed in vitro resistance to erythromycin and 5 (29.4%) to lincomycin. One isolate (ID 53) was also resistant to ampicillin, oxacillin, penicillin, and the 3 cephalosporins tested (cefazolin, cefoperazone, and cefquinome); this isolate was the only one detected by the nitrocefin-based method. Another single German isolate (5.9%) was classified as having intermediate

resistance to cefoperazone, and another 2 (11.8%) were classified as resistant to sulfamethoxazole/trimethoprim (Table 6).

As shown in Table 6, 47.1% of the German strains were potentially resistant to penicillin, harboring *blaZ*, whereas the *erm* genes were less prevalent, with 35.3% of the strains positive for *ermB* but none for *ermC*. The single strain phenotypically resistant to 9 different antimicrobials, including methicillin, was the only one (6.9%) positive for *mecA*.

Most (64.7%) of the strains isolated from Germany were positive for *hlb*. The result related to the presence of the IEC genes indicated that 15 (88.2%) strains carried *sea*, but none possessed the virulence factors associated with suppressing innate immunity (*scn* and *sak;* Table 6).

Italy

The results of antimicrobial susceptibility testing (Table 7) showed that more than a half (58.8%) of the isolates collected in Italy were not inhibited by spiramycin or penicillin or both. Out of 10 penicillin-resistant isolates, 9 (52.9%) also showed resistance to ampicillin, and 9 revealed phenotypic β-lactamase activity. One other isolate was detected by the nitrocefin-based method, for a total of 10 (58.8%) β-lactamase positive isolates with this test.

Besides penicillin and ampicillin, 1 isolate (ID 77) was not susceptible to the other 5 drugs (spiramycin, cefoperazone, enrofloxacin, oxacillin, and lincomycin). Two (11.8%) Italian isolates were considered resistant to gentamycin, and 1 (5.9%) also showed in vitro resistance to kanamycin. Only 1 isolate (ID 79) was classified as having intermediate resistance to erythromycin.

Of 17 Italian strains, 14 (82.4%) were potentially penicillin-resistant, carrying *blaZ*, and 5 strains (29.4%) also harbored *ermC*. A single strain (5.9%) was positive for *mecA*: this was phenotypically resistant to 7 different antimicrobials, including methicillin, but negative for the *erm* genes (Table 7).

Table 7 shows that 94.1% of the strains isolated from Italy carried *hlb;* the *sea* gene was detected in 58.8% of the strains, but only 1 (ID 78) was also positive for both *scn* and *sak* genes.

Table 7. Phenotypic antimicrobial resistance and molecular characteristics of *Staphyloccus aureus* strains isolated in Italy

Italian isolates[1]	Phenotypic antimicrobial intermediate(I) or complete resistance(R)[2]	β-Lactamase detection	*blaZ*	*mecA*	*ermB*	*ermC*	*hlb*	*sea*	*scn*	*sak*
64	AMP(R), PEN(R), SPM(R)	+	+	-	-	-	+	-	-	-
65	AMP(R), GEN(R), PEN(R)	+	+	-	-	+	+	-	-	-
66	AMP(R), PEN(R), SPM(R)	+	+	-	-	-	+	-	-	-
67	AMP(R), PEN(R), SPM(R)	+	+	-	-	+	+	-	-	-
68	AMP(R), PEN(R), SPM(R)	+	+	-	-	+	+	-	-	-
69	GEN(R), KAN(R), SPM(I)	-	+	-	-	-	+	-	-	-
70	SPM(I)	-	-	-	-	-	-	-	-	-
71	SPM(I)	+	+	-	-	+	+	+	-	-
72	SPM(I)	-	-	-	-	-	+	+	-	-
73	AMP(R), PEN(R)	+	+	-	-	+	+	+	-	-
74	AMP(R), PEN(R)	-	+	-	-	-	+	+	-	-
75	-[3]	-	+	-	-	-	+	+	-	-
76	-[3]	-	-	-	-	-	+	+	-	-
77	AMP(R), CPZ(R), ENRO(I), OXA(R), PEN(R), SPM(R), LIN(R)	+	+	+	-	-	+	+	-	-
78	AMP(R), PEN(R)	+	+	-	-	-	+	+	+	+
79	ERY(I), PEN(R)	+	+	-	-	-	+	+	-	-
80	SPM(I)	-	+	-	-	-	+	+	-	-

[1]Isolate identification numbers correspond to those in Monistero et al. (2018).
[2]AMP = ampicillin; PEN = penicillin; SPM = spiramycin; GEN = gentamicin; KAN = kanamycin; CPZ = cefoperazone; ENRO = enrofloxacin; OXA = oxacillin; LIN = lincomycin; ERY = erythromycin.
[3]Isolate 75 and 76 demonstrated phenotypical susceptibility to all 16 antimicrobials tested by the minimum inhibitory concentration assay.

United States (New York State)

All American isolates, with 1 exception (ID 97), exhibited resistance to spiramycin. From these spiramycin-resistant isolates, 1 (ID 90) was classified as having intermediate resistance to erythromycin, 1 (ID 82) as having intermediate resistance to rifampicin, and 1 (ID 88) as resistant to lincomycin (Table 8).

Among the antimicrobial resistance genes investigated, *blaZ* had a prevalence of 41.2%, and *erm* genes were not identified (Table 8). By contrast, the *hlb* gene was found to be quite diffused (88.2%); *sea* was carried by 52.9%, and *scn* and *sak* were not found (Table 8).

Table 8. Phenotypic antimicrobial resistance and molecular characteristics of *Staphylococcus aureus* strains isolated in the United States (New York State)

US isolates[1]	Phenotypic antimicrobial intermediate(I) or complete resistance(R)[2]	β-Lactamase detection	*blaZ*	*mecA*	*ermB*	*ermC*	*hlb*	*sea*	*scn*	*sak*
81	SPM(R)	-	-	-	-	-	+	+	-	-
82	RF(I), SPM(I)	-	+	-	-	-	+	+	-	-
83	SPM(R)	-	-	-	-	-	+	-	-	-
84	SPM(I)	-	-	-	-	-	+	-	-	-
85	SPM(R)	-	-	-	-	-	+	-	-	-
86	SPM(R)	-	+	-	-	-	+	-	-	-
87	SPM(I)	-	-	-	-	-	+	+	-	-
88	SPM(I), LIN(R)	-	+	-	-	-	+	+	-	-
89	SPM(R)	-	+	-	-	-	+	-	-	-
90	ERY(I), SPM(R)	-	-	-	-	-	+	+	-	-
91	SPM(R)	-	-	-	-	-	-	+	-	-
92	SPM(R)	-	-	-	-	-	+	-	-	-
93	SPM(R)	-	+	-	-	-	+	+	-	-
94	SPM(R)	-	+	-	-	-	+	+	-	-
95	SPM(R)	-	-	-	-	-	-	+	-	-
96	SPM(R)	-	+	-	-	-	+	-	-	-
97	-[3]	-	-	-	-	-	+	-	-	-

[1]Isolate identification numbers correspond to those in Monistero et al. (2018).
[2]SPM = spiramycin; RF = rifampin; LIN = lincomycin; ERY = erythromycin.
[3]Isolate 97 demonstrated phenotypical susceptibility to all 16 antimicrobials tested by the minimum inhibitory concentration assay.

South Africa

Among South African isolates, the highest rate of intermediate or complete resistance was found for spiramycin (100%), followed by erythromycin (36.4%). Of 11 isolates analyzed, 3 (27.3%) were

phenotypically resistant to penicillin and ampicillin, but only 1 (ID 103) showed phenotypic β-lactamase activity; a second isolate (ID 108) was detected by the nitrocefin-based method, for a total of 2 (18.2%) β-lactamase-positive isolates. Only 1 isolate (ID 100) was classified as having intermediate resistance to lincomycin (Table 9).

Of the 6 antimicrobial-resistance genes tested, *blaZ* and *ermB* were detected in the African strains with the same frequency (36.4%; Table 9). Of 11 South African strains analyzed, results (Table 9) showed that 7 (63.6%) carried the gene for β-haemolysin (*hlb*). Among the IEC genes, *sak* and *sea* were detected in 100% and 90.9% of the strains, respectively; 1 (ID 103) of them was also positive for *scn*.

Table 9. Phenotypic antimicrobial resistance and molecular characteristics of *Staphyloccus aureus* strains isolated in South Africa

South African isolates[1]	Phenotypic antimicrobial intermediate(I) or complete resistance(R)[2]	β-Lactamase detection	*blaZ*	*mecA*	*ermB*	*ermC*	*hlb*	*sea*	*scn*	*sak*
98	SPM(I)	-	-	-	-	-	+	+	-	+
99	ERY(I), SPM(R)	-	-	-	+	-	-	+	-	+
100	SPM(R), LIN(I)	-	-	-	+	-	-	+	-	+
101	AMP(R), ERY(I), PEN(R), SPM(R)	-	+	-	-	-	+	+	-	+
102	SPM(R)	-	-	-	+	-	+	+	-	+
103	SPM(R)	+	+	-	-	-	+	+	+	+
104	AMP(R), PEN(R), SPM(I)	-	+	-	-	-	-	+	-	+
105	SPM(R)	-	-	-	-	-	-	+	-	+
106	SPM(I)	-	-	-	-	-	+	+	-	+
107	ERY(I), SPM(I)	-	-	-	+	-	+	+	-	+
108	AMP(R), ERY(I), PEN(R), SPM(R)	+	+	-	-	-	+	-	-	+

[1]Isolate identification numbers correspond to those in Monistero et al. (2018).
[2]SPM = spiramycin; ERY = erythromycin; LIN = lincomycin; AMP = ampicillin; PEN = penicillin.

Association Between Phenotypic Resistance and Resistance Genes

The phenotypic results showed that most (93.6%) of the isolates had intermediate resistance or complete resistance to at least 1 of the 16 antimicrobial agents tested. Analyzing the resistance to multiple class of antimicrobials, 57.0% of isolates were resistant or intermediate to 1 class of antimicrobials, 25.8% to 2 different classes, 8.6% to 3 different classes, and 2.2% (the 2 MRSA isolates) to more than 3. Table 10 reports all the raw MIC values and the MIC_{90} of the isolates for each antimicrobial tested. The MIC_{90} of all antimicrobials tested was lower than the resistance breakpoint, except for penicillin, ampicillin, spyramicin, and tylosin. The MIC assay (Table 10) revealed that 50 (53.8%) isolates were not inhibited by the range of concentrations tested for spiramycin, which was the antimicrobial with the highest rate of resistance. Of 93 isolates, 21 (22.6%) were classified as having intermediate resistance or resistance to erythromycin, 20 (21.5%) to lincomycin, 18 (19.4%) to penicillin, and 17 (18.3%) to ampicillin. The nitrocefin-based method detected a total of 18 (19.4%) isolates producing β-lactamase: 15 of these were also phenotypically resistant to penicillin, and 3 showed susceptibility to this drug.

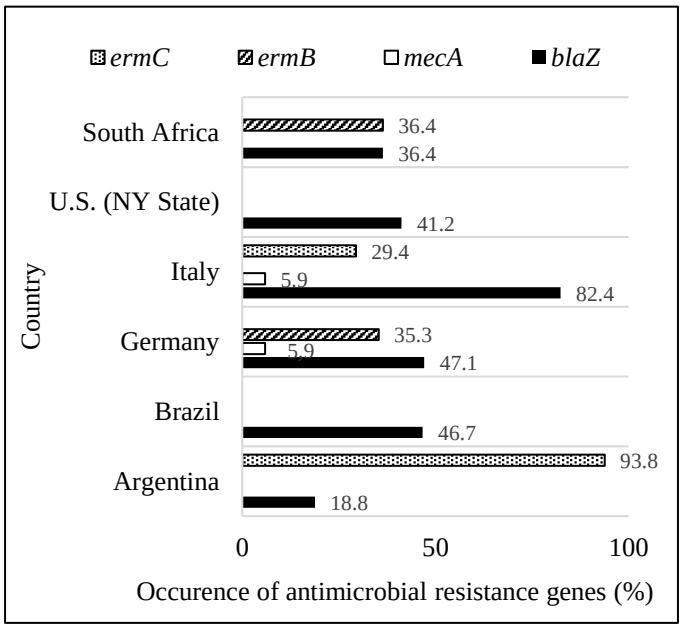

Figure 1. Occurrence of antimicrobial resistance genes in 93 *Staphylococcus aureus* isolates from different countries.

In estimating the occurrence of genes responsible for antibiotic resistance using PCR analysis, *blaZ* had the highest frequency (46.2%). Additionally, we investigated the presence of *erm* genes and *mec* genes that can confer resistance to erythromycin and methicillin, respectively. The genotypic results (Figure 1) showed that the resistance rate to erythromycin was 32.3%, and the most frequently detected erythromycin-resistance gene was *ermC* (21.5%), followed by *ermB* (10.8%). The prevalence of methicillin-resistant *S. aureus* strains was low among the isolates analyzed, as only 2 (2.2%) strains harbored *mecA*.

Figure 2 shows the association between the occurrence of genes conferring antibiotic resistance (x-axis) and laboratory-tested phenotypic resistance to antimicrobials (y-axis). The association was calculated as the sum of co-occurrences of genetic and phenotypic resistance to antibiotics, normalized over sample size (Buzydlowski, 2015). The molecular detection of the antibiotic-resistance genes was not always directly proportional to the phenotypic expression of these genes (Figure 2). The gene responsible for resistance to penicillin (*blaZ*) was the most prevalent (46.2%), but only 19.4% of the strains analyzed demonstrated phenotypic resistance to this drug; the same percentage (19.4%) of isolates were reported to be resistant due to a positive nitrocefin test result. All isolates that demonstrated phenotypic resistance to penicillin or β-lactamase activity carried the *blaZ* gene. In addition, 32.3% of the isolates were positive for *erm* genes, but the phenotypic results showed that 21 (22.6%) of the 93 strains analyzed were resistant (5.4%) or had intermediate resistance (17.2%) to erythromycin; 10 (10.8%) of the intermediate erythromycin-resistant strains were negative for *ermB* or *ermC*. The 2 phenotypically oxacillin-resistant strains were the only ones that harbored *mecA*.

Figure 3 shows the comparison of penicillin MIC distribution and frequency of *blaZ*-positive and *blaZ*-negative isolates. The distribution of *blaZ*-positive MIC is scattered along the dilution range, without bimodal distribution and with most of the isolates (23) having the lower MIC. The *blaZ*-negative isolates are gathered in the last 2 MIC dilutions with a clear unimodal distribution. Figure 3 also displays the comparison of erythromycin MIC distribution and frequency of *erm*-positive and

erm-negative isolates. In this case, *erm*-positive isolates show a bimodal distribution, having as cutoff the resistance breakpoint, whereas *erm*-negative isolates are distributed only behind the resistance cutoff, with the major frequency at 0.5 µg/mL.

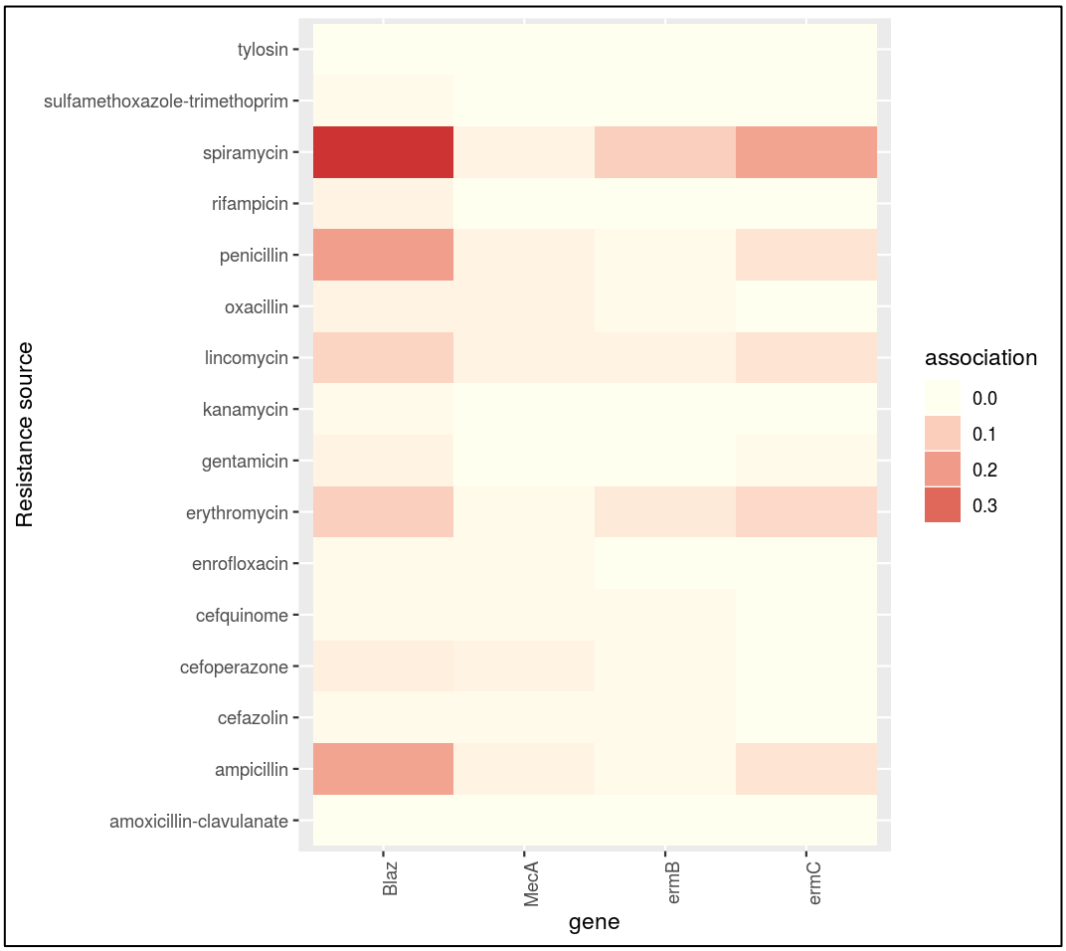

Figure 2. Comparison of phenotypic and genotypic antimicrobial resistance for selected genes in 93 *Staphylococcus aureus* isolates from different countries.

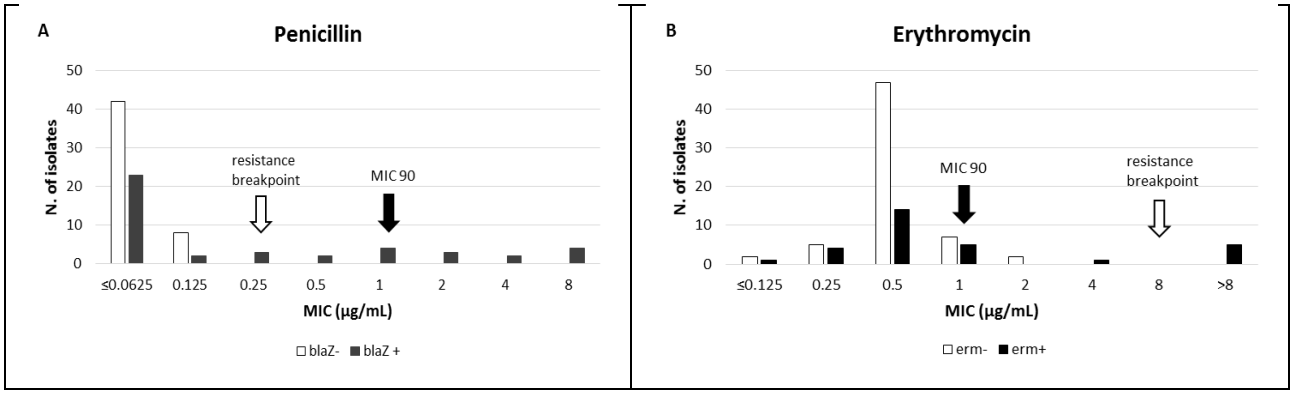

Figure 3. Comparison of (A) penicillin minimum inhibitory concentration (MIC) distribution and frequency of *blaZ*-positive and *blaZ*-negative isolates, and (B) erythromycin MIC distribution and frequency of *erm*-positive and *erm*-negative isolates. On the x-axis are displayed MIC values and on y-axis, the number of isolates. White arrows indicate the resistance breakpoint; black arrows, the MIC value inhibiting the growth of 90% of the isolates (MIC_{90}).

Table 10. Minimum inhibitory concentrations (MIC) and the MIC inhibiting the growth of 90% of the isolates (MIC_{90}) of the 16 antimicrobial agents tested for the 93 *Staphylococcus aureus* isolates

	Number of *S. aureus* isolates with MIC (µg/mL)											
Antimicrobials[1]	0.0625	0.125	0.25	0.5	1	2	4	8	16	32	MIC_{90}	Breakpoint reference
Amoxicillin-clavulanic acid[2]			82	7	2	1	1				0.5	CLSI vet01-S2 (2013)
Ampicillin		66	10	3	7	1	3	2		1	1	CLSI vet01-S2 (CLSI, 2013)
Cefazolin		16	22	48	5	1	1				0.5	CLSI vet01-S2 (CLSI,2013)
Cefoperazone			2	16	23	49	1	2			2	Feβler et al., 2012
Cefquinome		2	22	35	30	3	1				1	Lang et al., 2002
Enrofloxacin		88	3	1	1						0.125	CLSI vet01-s2 (CLSI, 2013)
Erythromycin		3	9	60	13	2	1		5		1	CLSI vet01-s2 (CLSI, 2013)
Gentamicin					91	1				1	1	CLSI vet01-s2 (CLSI, 2013)
Kanamycin						76	14	2		1	4	Société française de microbiologie, 2018
Lincomycin					60	13	1	1	18		>8	Société française de microbiologie, 2018
Oxacillin		38	37	15	1			2			0.5	CLSI vet01-s2 (CLSI, 2013)
Penicillin	65	10	3	2	1	3	2	4			1	CLSI vet01-s2 (CLSI, 2013)
Rifampin					91	2					1	CLSI vet01-S2 (CLSI, 2013)
Spiramycin					11	32		50			>4	Société française de microbiologie, 2018
Sulfamethoxazole/Trimethoprim[3]			87		1			5			0.25	CLSI vet01-s2 (CLSI, 2013)
Tylosin			2	4	28	59					2	Simjee et al, 2011

[1]The dilution ranges tested for each antibiotic are those within the shaded area. Values situated above the highest concentration tested indicate the number of isolates with a MIC greater than the highest dilution; values situated at the lower dilution tested indicate the number of isolates with a MIC lower than or equal to last dilution of antimicrobial. Resistance breakpoints are indicated with a double vertical line to the right of the breakpoint value; intermediate breakpoints are indicated with a single vertical line to the right of the breakpoint value.

[2]In the shaded area is reported the concentration of amoxicillin. Amoxicillin-clavulanic acid concentration ratio is 2:1.

[3]In the shaded area is reported the concentration of trimethoprim. Sulfamethoxazole/trimethoprim- concentration ration is 1:19.

Discussion

Of the 93 *S. aureus* strains analyzed, only 2 European isolates harbored *mecA*. None were positive for *mecC*, a recently identified *mecA* homolog detected in humans and in a wide range of domestic and wild animals from different European countries (Schlotter et al., 2014). Our results confirmed the low prevalence of MRSA among *S. aureus* strains collected from bovine mastitis samples (Hendriksen et al., 2008; Silva et al., 2013; da Costa Krewer et al., 2015; Luini et al., 2015). The β-lactam antibiotics have been largely used to treat *S. aureus* mastitis for several decades, but their efficiency is reduced by bacterial β-lactamases. The *blaZ* gene, which encodes the β-lactamase and confers resistance to penicillin (Olsen et al., 2006), was the most frequently detected resistance gene, found in 43 strains. Of these, 21, including the 2 MRSA isolates, were reported to be phenotypically resistant to penicillin based on MIC or nitrocefin-based test results. Therefore, the remaining 50% of the *blaZ*-positive isolates was phenotypically susceptible to penicillin, in agreement with previous results reported by Ruegg and collaborators (2015). Haveri et al. (2005) suggested that the occurrence of isolates with phenotypic resistance to a certain antibiotic might not always be proportional to the presence of the corresponding resistance gene. Considering the isolates susceptible to penicillin but positive for *blaZ* as potentially resistant (Haveri et al., 2005), our results confirmed that resistance to penicillin was the most frequently observed resistance mechanism, although with a lower prevalence than the over 60% reported by Malinowski and collaborators (2002, 2008). On the other hand, looking at the comparison of penicillin MIC distribution and the frequency of *blaZ*-positive and *blaZ*-negative isolates, most of the isolates had the lowest MIC. This discrepancy between phenotypic and genotypic results may demonstrate that the detection of genes does not necessarily implicate their expression; indeed, the percentage of isolates phenotypically resistant to penicillin was in agreement with previous results (Ruegg et al., 2015), reporting that the resistance rate to this drug has declined (Makovec and Ruegg, 2003), even with differences among geographical areas. Previous studies demonstrated that the occurrence of phenotypically penicillin-resistant *S. aureus* strains was higher

in Argentina (40%; Gentilini et al., 2000) than in Germany (17%; Tenhagen et al., 2006) or in the United States (10%; Anderson et al., 2006). Accordingly, we found higher resistance rates in Argentina and Germany compared with New York State, but the rates were overall lower (12.5% in Argentina, 5.9% in Germany, and 0% in New York State).

Of 18 phenotypically penicillin-resistant isolates, 17 were not inhibited even by the highest concentration of ampicillin tested, in accordance with previous studies (El Behiry et al., 2012; Jagielski et al., 2014). This outcome could be explained by the presence of *blaZ* in all these strains, because penicillin, as well as ampicillin, is inactivated by the β-lactamase encoded by *blaZ.* Among them, 3 were negative for the β-lactamase test; this discrepancy could be due to the lower sensitivity of the nitrocefin test compared with MIC assay and PCR analysis for the *blaZ* gene (Ferreira et al., 2017). All 93 isolates analyzed in this study were susceptible to the association of amoxicillin and clavulanate, with a very low MIC_{90} (0.5 μg/mL). Considering the uncertainties connected to laboratory methods for detection of β-lactamase-producing *S. aureus* strains, the use of amoxicillin and clavulanate could be recommended when only phenotypic methods are available to test resistance to penicillin, given also its demonstrated efficiency in mastitis therapy (Güler et al., 2005).

In addition to the genes responsible for resistance to β-lactams, we investigated the presence of the genes encoding resistance to erythromycin. We detected *erm* genes in 30 of the 93 isolates analyzed and found that *ermC* was the most prevalent gene, similar to the results of Aarestrup and Schwarz (2006) and Sawant et al. (2009). The phenotypic results showed that the MRSA strain collected in Germany was positive for *ermB* and was also classified as phenotypically erythromycin-resistant, whereas the other MRSA isolate from Italy was negative for both *erm* genes tested and was susceptible to erythromycin. Of 30 isolates positive for *erm* genes, 5 were classified as having complete resistance and 6 as having intermediate resistance to erythromycin. The susceptibility of the remaining 19 isolates could be due to lack of expression of methylases encoded by *erm* genes, in agreement with previous studies (Fluit et al., 2001). The other 10 isolates negative for *erm* genes

showed intermediate resistance to erythromycin, but they were very close to the CLSI breakpoints; therefore, we cannot discard the possibility that some of them could be considered susceptible.

Surprisingly, among the macrolides tested, we found a high number of isolates resistant to spiramycin but susceptible to erythromycin, when the genes responsible for resistance to erythromycin usually also confer resistance to other macrolides. The possibility of an uncorrected resistance breakpoint should be taken into account, and the MIC distribution could be helpful to analyze the data. Spiramycin MIC results (Table 10) show a bimodal distribution, with an epidemiological cutoff at 4 µg/100 mL, which seems to split the isolates into 2 different phenotypical populations and which corresponds to the resistance breakpoint. A greater number of isolates and dilution points in the area of resistance would be needed to assess the accuracy of the breakpoint. Therefore, possible bias due to an incorrect resistance breakpoint cannot be excluded, even if the epidemiological cutoff is consistent with the breakpoint for this set of data. Another possible explanation of this phenomenon could be the presence among the isolates of other genes encoding resistance to macrolides that have not been tested in this study. The emergence of macrolide-resistance genes conferring resistance to spiramycin but not to erythromycin has been described in *Streptococcus uberis* (Achard et al., 2008).

The cephalosporins, usually classed into different generations based on their antimicrobial spectrum, are often used to treat mastitis in dairy ruminants (Moroni et al., 2005). Globally, there are intramammary formulations of first-generation cephalosporins (cefazolin, cephalexin, cephalotin, cephalonium, and cephapirin), second-generation (cefuroxime), third-generation (cephoperazone and ceftiofur), and fourth-generation (cefquinome; Moroni et al., 2005; Oliveira and Ruegg, 2014; Ruegg et al., 2015). We tested cefazolin as a first-generation cephalosporin, and cephoperazone and cefquinome as third- and fourth-generations, respectively. These last 2 antimicrobials, classified by the World Health Organization (WHO, 2019) as highest-priority critically important antibiotics (HPCIA), were tested in this study because they were included in the MIC plates used for routine testing. It is important to highlight that the use of third- and fourth-generation cephalosporins should be limited only to gram negative bacteria that show resistance to antibiotics different from HPCIA

(WHO, 2017). The use of other drugs, such as first-generation cephalosporins or amoxicillin-clavulanate, should be preferred to these cephalosporins for the treatment of staphylococcal mastitis. Results showed that the 2 MRSA isolates both displayed resistance to cefoperazone (MIC 8 µg/mL). Moreover, the MRSA strain isolated in Germany had a MIC of 4 µg/mL, classified as intermediate, to cefazolin and cefquinome, whereas that isolated in Italy had a MIC of 2 µg/mL, classified as susceptible, to both drugs. The 2 MRSA isolates were resistant to oxacillin with a MIC value >4 µg/mL, outside of the dilution range. These data highlight that only oxacillin or cefoxitin should be used to phenotypically assess the presence of MRSA, confirming the detection of the *mecA* gene, as advised by CLSI (2013). However, bovine mastitis caused by *S. aureus* strains positive for the *mecA* gene and treated by administration of cephalosporins show clinical outcomes with low probability of cure (Pol and Ruegg, 2007; Oliveira and Ruegg. 2014; Krömker and Leimbach, 2017). For this reason, antibiotic treatment decisions should be based not only on the diagnosis of the mastitis causative agents obtained through microbiological and sensitivity test results but also on the identification of animals with high healing prospects (Krömker and Leimbach., 2017). The MIC_{90} was lower than the resistance breakpoint for the majority of the antimicrobials tested. This outcome was expected, in agreement with previous study (Gentilini et al., 2000; Ruegg et al., 2015). Therefore, for many antimicrobials, we selected a dilution range wider in the lower side and narrow in the upper part, to possibly detect the presence of bimodal distribution also in the susceptibility dilution range. This reduced the possibility of investigating the extent of the resistance level for some antimicrobials, such as lincomycin, spiramycin, and tylosin.

Analysis of the virulence profiles of the 93 *S. aureus* strains revealed that the 2 adhesion factors Hla and Hlb, also involved in host invasion (Moroni et al., 2011), were the most frequently detected. In fact, the genes for α- and β-haemolysins were found to be widely distributed in all the 6 countries analyzed, in agreement with Aarestrup et al. (1999). All strains were positive for *hla* (100%), and 79 (84.9%) also carried *hlb*. The gene for α-toxin is present in essentially all *S. aureus* strains (Monecke et al., 2014), including strains isolated from humans, whereas the β-toxin gene, whose activity may

be important in the pathogenesis of mastitis, is more frequent in bovine rather than human isolates (Larsen et al., 2002).

We also investigated the occurrence of the IEC genes that play an important role in human medicine (Baptistão et al., 2016), especially in the infections caused by MRSA (McCarthy and Lindsay, 2013). This cluster comprises the staphylococcal complement inhibitor gene (*scn*) and the chemotaxis inhibitory protein (*chp*), which are located on an 8-kb region at the conserved 3′ end of β-hemolysin (*hlb*)-converting bacteriophages (βC-φs). The region at the conserved 3′ end encodes the genes *sak*, *sea*, or *sep* (van Wamel et al., 2006). Seven different IEC types (A to G) were previously identified, based on the occurrence of *sea*, *sep*, *sak*, *chp*, and *scn* genes, and type B (*sak-chp-scn*) was the most prevalent (van Wamel et al., 2006). The presence of this cluster in large animals was previously investigated in MRSA and methicillin-sensitive *S. aureus* (MSSA) isolates from different kinds of infections in pigs and horses as well as in humans with occupational exposure to pigs and horses (Cuny et al., 2015). Cuny and collaborators (2015) detected the IEC genes only in MRSA from horse clinics and the colonization of veterinary personal, probably for a re-adaptation to humans. Acquisition of the IEC is probably one of the first steps in the process of adaptation to animals and including loss or acquisition of genetic elements (Schijffelen et al., 2010). In this context, we investigated the occurrence of the IEC genes in *S. aureus* isolates from bovine intramammary infection. Our results showed that neither strain of MRSA found in our study carried these genes: the one from Germany was negative for all of them, whereas that from Italy carried only the gene encoding for enterotoxin A. However, the IEC type D was detected in 1 Italian and 1 South African MSSA, carrying *scn*, *sak*, and *sea*. This cluster type has been described as quite common in human MRSA (van Wamel et al., 2006). The gene *chp* was overall absent, but *sak* was present in 31.3% of Argentinian and 100% of South African strains. Our findings are in accordance with a recent paper (Magro et al., 2017) reporting that only bovine isolates were devoid of such prophage, probably because the untruncated *hlb* is necessary in ungulates for the different structure of erythrocyte membranes. In contrast to our results, the IEC genes were reported to be quite frequent in a recent

Tunisian study on *S. aureus* strains collected from cow and ewe milk: IEC type B was predominant (Khemiri et al., 2019). The *sea* gene was carried, on average, by half of the isolates from each country, with the exception of Germany and South Africa, where the prevalence of this gene was 88.2% and 90.1%, respectively.

Conclusions

Although it is not straightforward to generalize to the global dairy population, given the limited study size and the non-probability convenience sampling scheme of this study, our results strengthen the knowledge of the virulence and antibiotic-resistance patterns of *S. aureus* strains in dairy cows. Few specific genes were frequently detected in the strains analyzed, suggesting that they could be related to the ability of *S. aureus* to colonize the host. The *blaZ* gene was identified in most of the isolates analyzed, even though the detection of this gene, as well as of *erm* genes, did not correspond with the relative occurrence of phenotypic resistance; further research will be necessary to validate phenotypic susceptibility testing and genotypic testing. Notwithstanding the ongoing alert on methicillin-resistant *S. aureus* strains, only 2 MRSA isolates were identified in this study; all other isolates were susceptible to oxacillin, and the majority were also susceptible to most antimicrobials tested. Therefore, the presence of highly multidrug-resistant isolates was low, and the emergence of widespread *S. aureus* multidrug resistance is limited to MRSA, in agreement with the previously mentioned works. The results of the present work show that the prevalence of antimicrobial-resistant *S. aureus* strains vary depending on country and herd, but collection of more comprehensive data through collaboration with a greater number of countries can provide further information on the spreading of antibiotic resistance; these findings could be used for further studies or meta-analysis on combined datasets. To date, the results suggest that it is necessary to maintain the described antimicrobial resistance trends, making antibiotic treatment decisions based on rapid diagnostic and

resistance tests, and to keep an adequate level of surveillance on the presence of MRSA in dairy cattle, to avoid the spreading of these strains in dairy cattle populations and beyond.

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References

Aarestrup, E. M., H. D. Larsen, N. H. R. Eriksen, C. S. Elsberg, and N. E. Jensen. 1999. Frequency of alpha- and beta-haemolysin in *Staphylococcus aureus* of bovine and human origin. A comparison between pheno- and genotype and variation in phenotypic expression. APMIS. 107(4):425-30.

Aarestrup, F. M., and S. Schwarz. 2006. Antimicrobial resistance in staphylococci and streptococci of animal origin. Pages 187–212 in Antimicrobial Resistance in Bacteria of Animal Origin. F. M. Aarestrup, ed. ASM Press, Washington, DC.

Achard, A., V. Guérin-Faublée, V. Pichereau, C. Villers, and R. Leclercq. 2008. Emergence of macrolide resistance gene *mph(B)* in *Streptococcus uberis* and cooperative effects with *rdmC*-like gene. Antimicrob Agents Chemother. 52:2767–2770. https://doi.org/10.1128/AAC.00481-08.

Anderson, K. L., R. L. Lyman, S. M. Bodeis-Jones, and D. G. White. 2006. Genetic diversity and antimicrobial susceptibility profiles among mastitis-causing *Staphylococcus aureus* isolated from bovine milk samples. Am. J. Vet. Res. 67:1185–1191. https://doi.org/10.2460/ajvr.67.7.1185.

Baptistão, L. G., N. C. C. Silva, E. C. R. Bonsaglia, B. F. Rossi, I. G. Castilho, A. Fernandes Junior, and V. L. M. Rall. 2016. Presence of immune evasion cluster and molecular typing of methicillin-susceptible *Staphylococcus aureus* isolated from food handlers. Journal of Food Protection. J Food Prot. 79(4):682-6. https://doi.org/10.4315/0362-028X.JFP-15-401.

Barkema, H.W., Y. H. Schukken, and R. N. Zadoks. 2006. Invited review: the role of cow, pathogen, and treatment regimen in the therapeutic success of bovine *Staphylococcus aureus* mastitis. J. Dairy Sci. 89:1877–1895. https://doi.org/10.3168/jds.S0022-0302(06)72256-1.

Berube, B. J., and J. Bubeck Wardenburg. 2013. *Staphylococcus aureus* α-toxin: nearly a century of intrigue. Toxins (Basel). 5(6):1140-66. https://doi.org/10.3390/toxins5061140.

Buzydlowski, Jan W. 2015. Co-occurrence analysis as a framework for data mining. Journal of Technology Research. Volume 6.

Clarke, S.R., and S. J. Foster. 2006. Surface adhesins of *Staphylococcus aureus*. Adv Microb Physiol. 51:51. https://doi.org/10.1016/S0065-2911(06)51004-5.

CLSI. 2013. Performance standards for antimicrobial disk and dilution susceptibility tests for bacteria isolated from animals; approved standard - Fourth Edition. CLSI document Vet 01-A4. Clinical and Laboratory Standards Institute, Wayne, PA.

CLSI. 2018. Performance standards for antimicrobial disk and dilution susceptibility tests for bacteria isolated from animals. 4th ed. CLSI supplement VET 08. Clinical and Laboratory Standards Institute, Wayne, PA.

Cockerill, F.R. 1999. Genetic methods for assessing antimicrobial resistance. Antimicrob. Agents Chemother. 43:199–212.

Cookson, B. 1997. *Staphylococcus aureus*. Page 109 in Principles of Clinical Bacteriology. M. Emmerson, C. Kibbler, and P. Hawkey, John Wiley, Oxford.

Cremonesi, P., M. Luzzana, M. Brasca, S. Morandi, R. Lodi, C. Vimercati, D. Agnellini, G. Caramenti., P. Moroni, and B. Castiglioni. 2005. Development of a multiplex PCR assay for

the identification of *Staphylococcus aureus* enterotoxigenic strains isolated from milk and dairy products. Mol Cell Probes. 19(5):299-305. https://doi.org/10.1016/j.mcp.2005.03.002.

Cremonesi, P., B. Castiglioni, G. Malferrari, I. Biunno, C. Vimercati, P. Moroni, S. Morandi, and M. Luzzana. 2006. Technical note: Improved method for rapid DNA extraction of mastitis pathogens directly from milk. Journal of Dairy Science. 89:163–169. https://doi.org/10 .3168/jds.S0022-0302(06)72080-X.

Cuny, C., M. Abdelbary, F. Layer, G. Werner, and W. Witte. 2015. Prevalence of the immune evasion gene cluster in *Staphylococcus aureus* CC398. Veterinary Microbiology. 177:219–223. https://doi.org/10.1016/j.vetmic.2015.02.031.

da Costa Krewer C, E. Santos Amanso, G. Veneroni Gouveia, R. de Lima Souza, M. M. da Costa, and R. Aparecido Mota. 2015. Resistance to antimicrobials and biofilm formation in *Staphylococcus* spp. isolated from bovine mastitis in the Northeast of Brazil. Trop Anim Health Prod. 47:511–518. https://doi.org/10.1007/s11250-014-0752-9.

El Behiry, A. E., G. Schlenker, I. Szabo, and U. Roesler. 2012. In vitro susceptibility of *Staphylococcus aureus* strains isolated from cows with subclinical mastitis to different antimicrobial agents. J. Vet. Sci. 13:153–161. https://doi.org/10.4142/jvs.2012.13.2.153.

Ferreira A. M., Martins K. B., Silva V. R., Mondelli A. L., and Cunha M. L. 2017. Correlation of phenotypic tests with the presence of the *blaZ* gene for detection of beta-lactamase. Braz J Microbiol. 48:159-166. https://doi.org/10.1016/j.bjm.2016.10.011.

Feßler A. T., H. Kaspar, C. J. Lindeman, M. R. Stegemann, T. Peters, J. Mankertz, J. L. Watts, and S. Schwarz. 2012. A proposal of interpretive criteria for cefoperazone applicable to bovine mastitis pathogens. Vet. Microbiol. 157:226-231. https://doi.org/10.1016/j.vetmic.2011.11 .032.

Fluit A. C., M. R. Visser, and F. J. Schmitz. 2001. Molecular detection of antimicrobial resistance. Clinical Microbiology Reviews. 14:836–871. https://doi.org/10.1128/CMR.14.4.836-871.2001.

Fournier, C., P. Kuhnert, J. Frey, R. Miserez, M. Kirchhofer, T. Kaufmann, A. Steiner, and H. U. Graber. 2008. Bovine *Staphylococcus aureus*: association of virulence genes, genotypes and clinical outcome. Res. Vet. Sci. 85:439–448. https://doi.org/10.1016/j.rvsc.2008.01.010.

Gao, J., M. Ferreri, F. Yu, X. Liu, L. Chen, J. Su, and B. Han. 2012. Molecular types and antibiotic resistance of *Staphylococcus aureus* isolates from bovine mastitis in a single herd in China. Vet. J. 192:550–552. https://doi.org/10.1016/j.tvjl.2011.08.030.

Gatermann, S. G., T. Koschinski, and S. Friedrich. 2007. Distribution and expression of macrolide resistance genes in coagulase-negative staphylococci. Clinical Microbiology and Infection. 13:777–781. https://doi.org/10.1111/j.1469-0691.2007.01749.x.

Gentilini, E., G. Denamiel, P. Llorente, S. Godaly, M. Rebuelto, and O. DeGregorio. 2000. Antimicrobial susceptibility of *Staphylococcus aureus* isolated from bovine mastitis in Argentina. J. Dairy Sci. 83:1224–1227. https://doi.org/10.3168/jds.S0022-0302(00)74988-5.

Güler, L., U. Ok, K. Gündüz, Y. Gülcü, and H. H. Hadimli. 2005. Antimicrobial susceptibility and coagulase gene typing of *Staphylococcus aureus* isolated from bovine clinical mastitis cases in Turkey. J Dairy Sci. 88(9):3149-54. https://doi.org/10.3168/jds.S0022-0302(05)72998-2.

Gussmann, M., W. Steeneveld. C. Kirkeby, H. Hogeveen, M. Farre, and T. Halasa. 2019. Economic and epidemiological impact of different intervention strategies for subclinical and clinical mastitis. Prev Vet Med. 166:78-85. https://doi.org/10.1016/j.prevetmed.2019.03.001.

Haveri, M., S. Suominen, L. Rantala, T. Honkanen-Buzalski, and S. Pyorala. 2005. Comparison of phenotypic and genotypic detection of penicillin G resistance of *Staphylococcus aureus* isolated from bovine intramammary infection. Vet. Microbiol. 106:97–102. https://doi.org/10.1016/j.vetmic.2004.12.015.

Heikkilä, A. M., E. Liski, S. Pyörälä, and S. Taponen. 2018. Pathogen-specific production losses in bovine mastitis. J Dairy Sci. 101(10):9493-9504. https://doi.org/10.3168/jds.2018-14824.

Hendriksen, R. S., D. J. Mevius, A. Schroeter, C. Teale, D. Meunier, P. Butaye, A. Franco, A. Utinane, A. Amado, M. Moreno, C. Greko, K. Stärk, C. Berghold, A. L. Myllyniemi, D.

Wasyl, M. Sunde, and F. M. Aarestrup. 2008. Prevalence of antimicrobial resistance among bacterial pathogens isolated from cattle in different European countries: 2002–2004. Acta Vet Scand. 50:28. https://doi.org/10.1186/1751-0147-50-28.

Jagielski, T., E. Puacz, A. Lisowski, P. Siedlecki, W. Dudziak, J. Międzobrodzki, and H. Krukowski. 2014. Antimicrobial susceptibility profiling and genotyping of *Staphylococcus aureus* isolates from bovine mastitis in Poland. J. Dairy Sci. 97:6122–6128. https://doi.org/10.3168/jds.2014-8321.

Jarraud, S., C. Mougel, J. Thioulouse, G. Lina, H. Meugnier, F. Forey, X. Nesme, J. Etienne, and F. Vandenesch. 2002. Relationships between *Staphylococcus aureus* genetic background, virulence factors, *agr* groups (alleles), and human disease. Infect Immun. 70:631-641. https://doi.org/10.1128/IAI.70.2.631-641.2002.

Jensen, L. B., N. Frimodt-Moller, and F. M. Aarestrup. 1999. Presence of *erm* gene classes in 271 gram-positive bacteria of animal and human origin in Denmark. FEMS Microbiol. Lett. 170:151–158. https://doi.org/10.1111/j.1574-6968.1999.tb13368.x.

Khemiri, M., M.S. Abbassi, H. Elghaieb, M. Zouari, R. Dhahri, C. Pomba, and S. Hammami. 2019. High occurrence of enterotoxigenic isolates and low antibiotic resistance rates of *Staphylococcus aureus* isolated from raw milk from cows and ewes. Letters in Applied Microbiology. 68:573-579. https://doi.org/10.1111/lam.13152.

Krömker, V., and S. Leimbach. 2017. Mastitis treatment-reduction in antibiotic usage in dairy cows. Reprod Domest Anim. 3:21-29. https://doi.org/10.1111/rda.13032.

Lang I., M. Rose, E. Thomas, and E. Zschiesche. 2002. A field study of cefquinome for the treatment of pigs with respiratory disease. Revue Méd. Vét. 153:575-580.

Larsen, H. D., F. M. Aarestrup, and N. E. Jensen. 2002. Geographical variation in the presence of genes encoding superantigenic exotoxins and β-hemolysin among *Staphylococcus aureus* isolated from bovine mastitis in Europe and USA. Vet Microbiol. 85(1):61-67. https://doi.org/10.1016/S0378-1135(01)00478-3.

Luini, M., P. Cremonesi, G. Magro, V. Bianchini, G. Minozzi. B. Castiglioni, and R. Piccinini. 2015. Methicillin-resistant *Staphylococcus aureus* (MRSA) is associated with low within-herd prevalence of intra-mammary infections in dairy cows: genotyping of isolates. Vet Microbiol. 178(3-4):270-274. https://doi.org/10.1016/j.vetmic.2015.05.010.

Magro, G., S. Biffani, G. Minozzi, R. Ehricht, S. Monecke, M. Luini, and R. Piccinini. 2017. Virulence genes of *S. aureus* from dairy cow mastitis and contagiousness risk. Toxins. 9:195. https://doi.org/10.3390/toxins9060195.

Makovec, J. A., and P. L. Ruegg. 2003. Antimicrobial resistance of bacteria isolated from dairy cow milk samples submitted for bacterial culture: 8,905 samples (1994–2001). J. Am. Vet. Med. Assoc. 222:1582–1589. https://doi.org/10.3168/jds.S0022-0302(03)73951-4.

Malinowski, E., A. Kłossowska, M. Kaczmarowski, H. Lassa, and K. Kuźma. 2002. Antimicrobial susceptibility of staphylococci isolated from affected with mastitis cows. Bull. Vet. Inst. Pulawy. 46:289–294.

Malinowski, E., H. Lassa, S. Smulski, A. Kłossowska, and M. Kaczmarowski. 2008. Antimicrobial susceptibility of bacteria with mastitis in 2006–2007. Bull. Vet. Inst. Pulawy. 52:565–572.

McCarthy, A.J., and J.A. Lindsay. 2013. *Staphylococcus aureus* innate immune evasion is lineage-specific: a bioinformatics study. Infect. Genet. Evol. 19:7–14. https://doi.org/10 .1016/j.meegid .2013 .06.012.

McClure, J. A., J. M. Conly, V. Lau, S. Elsayed, T. Louie, W. Hutchins, and K. Zhang. 2006. Novel multiplex PCR assay for detection of the staphylococcal virulence marker Panton-Valentine leukocidin genes and simultaneous discrimination of methicillin-susceptible from -resistant staphylococci. J. Clin. Microbiol. 44:1141–1144. https://doi.org/10.1128/JCM.44.3.1141-1144.2006.

Monecke, S., E. Müller, J. Büchler, B. Stieber, and R. Ehricht. 2014. *Staphylococcus aureus* in vitro secretion of alpha toxin (*hla*) correlates with the affiliation to the clonal complexes. PLoS One. 9(6): e100427. https://doi.org/10.1371/journal.pone.0100427.

Monistero, V., H. U. Graber, C. Pollera, P. Cremonesi, B. Castiglioni, E. Bottini, A. Ceballos-Marquez, L. Lasso-Rojas, V. Kroemker, N. Wente, I.M. Petzer, C. Santisteban, J. Runyan, M. Veiga Dos Santos, B.G. Alves, R. Piccinini, V. Bronzo, M.S. Abbassi, M.B. Said, and P. Moroni. 2018. *Staphylococcus aureus* isolates from bovine mastitis in eight countries: genotypes, detection of genes encoding different toxins and other virulence genes. Toxins. 10(6):247. https://doi.org/10.3390/toxins10060247.

Moroni, P., G. Pisoni, C. Vimercati, M. Rinaldi, B. Castiglioni, P. Cremonesi, and P. Boettcher. 2005. Characterization of *Staphylococcus aureus* isolated from chronically infected dairy goats. J. Dairy Sci. 88:3500–3509. https://doi.org/10.3168/jds.S0022-0302(05)73035-6.

Moroni P., G. Pisoni. M. Antonini, R. Villa, P. Boettcher, and S. Carli S. 2006. Short communication: Antimicrobial drug susceptibility of *Staphylococcus aureus* from subclinical bovine mastitis in Italy. J. Dairy Sci. 89:2973–2976. https://doi.org/10.3168/jds.S0022-0302(06)72569-3.

Moroni, P., G. Pisoni, P. Cremonesi, B. Castiglioni. 2011. *Staphylococcus*. Pages 307-317 in Molecular Detection of Human Bacterial Pathogens. Dongyou Liu, CRC Press, Boca Raton.

Oliveira, L., and P. L. Ruegg. 2014. Treatments of clinical mastitis occurring in cows on 51 large dairy herds in Wisconsin. J. Dairy Sci. 97:5426–5436. https://doi.org/10.3168/jds.2013-7756.

Olsen, J.E., H. Christensen, and F. M. Aarestrup. 2006. Diversity and evolution of *blaZ* from *Staphylococcus aureus* and coagulase-negative staphylococci. Journal of Antimicrobial Chemotherapy. 57:450–460. https://doi.org/10.1093/jac/dki492.

Otto, M. 2014. *Staphylococcus aureus* toxins. Current Opinion in Microbiology. 17:32-37. https://doi.org/10.1016/j.mib.2013.11.004.

Paterson, G. K., A. R. Larsen, A. Robb, G. E. Edwards, T. W. Pennycott, G. Foster, D. Mot, K. Hermans, K. Baert, S. J. Peacock, J. Parkhill, R. N. Zadoks, and M. A. Holmes. 2012. The newly described *mecA* homologue, mecALGA251, is present in methicillin-resistant *Staphylococcus aureus* isolates from a diverse range of host species. J. Antimicrob. Chemother. 67:2809–2813. https://doi.org/10.1093/jac/dks329.

Paterson, G. K., E. M. Harrison, and M. A. Holmes. 2014. The emergence of *mecC* methicillin-resistant *Staphylococcus aureus*. Trends Microbiol. 22:42–47. https://doi.org/10.1016/j.tim .2013.11.003.

Pol, M., and P. L. Ruegg. 2007. Relationship between antimicrobial drug usage and antimicrobial susceptibility of gram-positive mastitis pathogens. J. Dairy Sci. 90:262–273. https://doi.org/10.3168/jds.S0022-0302(07)72627-9.

Ruegg, P.L. 2011. Managing mastitis and producing quality milk. Pages 207-232 in Dairy Production Medicine. C.A. Risco, and P.R. Melendez, editors, West Sussex (Shire) John Wi-ley & Sons.

Ruegg, P. L., L. Oliveira, W. Jin, and O. Okwumabua. 2015. Phenotypic antimicrobial susceptibility and occurrence of selected resistance genes in gram-positive mastitis pathogens isolated from Wisconsin dairy cows. J. Dairy Sci. 98:4521–4534. https://doi.org/10.3168/jds.2014-9137.

Saini, V., J. T. McClure, D. Leger, S. Dufour, A. G. Sheldon, D. T. Scholl, and H. W. Barkema. 2012a. Antimicrobial use on Canadian dairy farms. J. Dairy Sci. 95:1209–1221. https://doi.org/10.3168/jds.2011-4527.

Saini, V., J. T. McClure, D. Leger, S. Dufour, A. G. Sheldon, D. T. Scholl, and H. W. Barkema. 2012b. Antimicrobial use on Canadian dairy farms. J. Dairy Sci. 95:1209–1221. https://doi.org/10.3168/jds.2011-4527.

Sakwinska O., D. Morisset, J-Y Madec, A. Waldvogel, P. Moreillon, and M. Haenni. 2011. Link between genotype and antimicrobial resistance in bovine mastitis-related *Staphylococcus aureus* strains, determined by comparing Swiss and French isolates from the Rhone Valley. Appl. Environ. Microbiol. 77:3428–3432. https://doi.org/10.1128/AEM.02468-10.

Salasia, S. I., Z. Khusnan, C. Lammler, and M. Zschock. 2004. Comparative studies on pheno- and genotypic properties of *Staphylococcus aureus* isolated from bovine subclinical mastitis in central Java in Indonesia and Hesse in Germany. J. Vet. Sci. 5:103–109.

Sawant, A.A., B. E. Gillespie, and S. P. Oliver. 2009. Antimicrobial susceptibility of coagulase-negative *Staphylococcus* species isolated from bovine milk. Vet. Microbiol. 134:73–81. https://doi.org/10.1016/j.vetmic.2008.09.006.

Schijffelen, M.J., C. H. Boel, J. A. van Strijp, and A. C. Fluit. 2010. Whole genome analysis of a livestock-associated methicillin-resistant *Staphylococcus aureus* ST398 isolate from a case of human endocarditis. BMC Geno-mics. 11:376. https://doi.org/10.1186/1471-2164-11-376.

Schlotter, K., R. Huber-Schlenstedt, A. Gangl, H. Hotzel, S. Monecke, E. Müller, A. Reißig, S. Proft, and R. Ehricht. 2014. Multiple cases of methicillin-resistant CC130 *Staphylococcus aureus* harboring *mecC* in milk and swab samples from a Bavarian dairy herd. J. Dairy Sci. 97:2782–2788. https://doi.org/10.3168/jds.2013-7378.

Silva N.C., F. F. Guimarães, M. P. Manzi, P. E. Budri, E. Gómez-Sanz, D. Benito, H. Langoni, V. L: Rall, and C. 2013. Molecular characterization and clonal diversity of methicillin-susceptible *Staphylococcus aureus* in milk of cows with mastitis in Brazil. J Dairy Sci. 96(11):6856-62. https://doi.org/10.3168/jds.2013-6719.

Simjee S., J. Amedeo, A. M. Barletta, and A. Pridmore. 2011. Effect of the combination of Tylan 200® & Penicillin G in the treatment of mastitis caused by *Staphylococcus aureus* and/or *Streptococcus uberis*. Pages 294-294 in Udder Health and Communication. H. Hogeveen and T. J. G. M. Lam, Wageningen Academic Publishers, Wageningen.

Société française de microbiologie. 2018. Comité de l'antibiogramme de la société française de microbiologie. Groupe de travail antibiogramme vétérinaire. Recommandations vétérinaires 2018.

Sol J., O. C. Sampimon, J. J. Snoep, and Y. H. Schukken. 1997. Factors associated with bacteriological cure during lactation after therapy for subclinical mastitis caused by *Staphylococcus aureus*. J Dairy Sci. 80(11):2803-8.

Sung, J. M. L., D. H. Lloyd, and J. A. Lindsay. 2008. *Staphylococcus aureus* host specificity: comparative genomics of human versus animal isolates by multi-strain microarray. Microbiology. 154:1949–1959. https://doi.org/10.1099/mic.0.2007/015289-0.

Sutcliffe, J., T. Grebe, A. Tait-Kamradt, and L. Wondrack. 1996. Detection of erythromycin-resistant determinants by PCR. Antimicrob Agents Chemother. 40:2562-2566.

Tenhagen, B. A., G. Koster, J. Wallmann, and W. Heuwieser. 2006. Prevalence of mastitis pathogens and their resistance against antimicrobial agents in dairy cows in Brandenburg, Germany. J. Dairy Sci. 89:2542–2551. https://doi.org/10.3168/jds.S0022-0302(06)72330-X.

USDA. 2007. Part V: Changes in dairy cattle health and management practices in the United States, 1996–2007. National Animal Health Monitoring Service, ed. USDA, Washington, DC.

van Wamel, W. J. B., S. H. M. Rooijakkers, M. Ruyken, K. P. M. van Kessel, and J. A. G. van Strijp. 2006. The innate immune modulators staphylococcal complement inhibitor and chemotaxis inhibitory protein of *Staphylococcus aureus* are located on β-hemolysin converting bacteriophages. J. Bacteriol. 188:1310–1315. https://doi.org/10.1128/JB.188.4.1310-1315.2006.

Walker, R. D. 2006. Antimicrobial susceptibility testing methods and interpretation of results. Pages 11-26 in Antimicrobial Therapy in Veterinary Medicine. S. Guiguere, J. F. Prescott, J. D. Baggot, R. D. Walker, and P.M. Dowling. ed. Blackwell Publishing. Ames. IA.

Wenz, J. R., G. M. Barrington, F. B. Garry, R. P. Dinsmore, and R. J. Callan. 2001. Use of systemic disease signs to assess disease severity in dairy cows with acute coliform mastitis. J Am VetMed Assoc. 218(4):567–72.

WHO. 2017. WHO guidelines on use of medically important antimicrobials in food-producing animals. https://www.who.int/foodsafety/areas_work/antimicrobial-resistance/ cia_ guidelines/en/.

WHO. 2019. WHO list of critically important antimicrobials (WHO CIA list). https://www.who.int/foodsafety/publications/antimicrobials-sixth/en/.

Xu, S.X., K. J. Gilmore, P. A. Szabo, J. J. Zeppa, M. L. Baroja, S. M. Haeryfar, and J. K. McCormick. 2014. Superantigens subvert the neutrophil response to promote abscess formation and enhance *Staphylococcus aureus* survival in vivo. Infect. Immun. 82:3588–3598. https://doi.org/10.1128/IAI.02110-14

Part II: *Streptococcus uberis*

Streptococcus uberis is a gram-positive and catalase-negative coccus belonging to the order *Lactobacillales* and the family *Streptococcaceae* (Krömker et al., 2014). The genus *Streptococcus* is comprised of both contagious and environmental mastitis agents responsible for clinical and subclinical forms with high economic impact on dairy industry's profits (Shome et al., 2012; Tian et al., 2019). *Strep. uberis* is predominantly classified as an environmental pathogen and its primary reservoir is the dairy environment (Zadoks et al., 2005a; Lopez-Benavides et al., 2007; Ericsson et al., 2009), but some strains show a contagious behavior (Wald et al., 2020).

Molecular genotyping has promoted our understanding of the dynamics of *Strep. uberis* mastitis at herd level and has provided meaningful information for practical management. Different subtyping techniques have given a valuable aid in identifying specific strains likely capable of cow-to-cow spread and in formulating appropriate strategies for their control and treatment. Previous studies demonstrated that *Strep. uberis* isolated from IMIs was heterogeneous (Reyes et al., 2019; Wente et al., 2019; Leelahapongsathon et al., 2020), but clonal strains in different cows were identified by MLST and RAPD-PCR, suggesting their contagious nature (Davies et al., 2016; Tomazi et al., 2019). The RAPD-PCR represents a rapid and inexpensive tool to characterize *Strep. uberis*, and to identify closely related strains within a dairy herd (Zadoks et al., 2003). The disadvantage of this method is its poor reproducibility as minimal changes in the PCR conditions can lead to variations in the RAPD types obtained. The differences detected in *Strep. uberis* strains may contribute to understanding the modes of a pathogen's dissemination within a single farm but can be hardly compared with other studies (Tabit, 2016).

Despite high bacterial diversity, four housekeeping genes, encoding chaperonin or heat shock protein (*cpn60*), glyceraldehyde-3-phosphate dehydrogenase (*gapC*), superoxide dismutase (*sodA*) and elongation factor Tu (*tuf*), are highly conserved in *Strep. uberis* populations. (Zadoks et al., 2005b). The detection of their presence can be used to confirm the identification of isolates as *Strep. uberis*

(Shome et al., 2012; Pyatov et al., 2017). The combination of other genes involved in the adhesion to bovine mammary epithelial cells, in the invasion of mammary tissues and in the evasion of mammary immune response (*cfu, lbp, hasA, hasB, hasC, oppF, pauA, sua*) can be associated with *Strep. uberis* pathogenicity. Among them, the *sua* gene encodes the *Strep. uberis* adhesion molecule (SUAM), a potential adherence determinant with affinity for lactoferrin (Almeida et al., 2015). As well as SUAM, the iron-binding protein produced by *lbp* aids in adherence to and internalization into bovine mammary epithelial cells (Fang and Oliver, 1999). The plasminogen activator A, responsible for the acquisition of essential nutrients from milk casein, confers an advantage with respect to *Strep. uberis* survival and colonization in nutritionally limited environments. Although the activation of bovine plasminogen is not strictly required for development of IMI (Ward et al., 2003), the *pauA* was found with high prevalence in *Strep. uberis* strains (Ward and Leigh, 2004). The distribution of *oppF* is similarly high among *Strep. uberis* isolates from bovine mastitis (Boonyayatra et al., 2018); this gene encodes for the oligopeptide permease involved in the active transport of solutes across the cytoplasmic membrane (Smith et al., 2002). The genes of the *has* operon (*hasABC*), conferring resistance to phagocytosis (Parin et al., 2017), are differently distributed among *Strep. uberis* isolated from bovine mastitis, with the *hasA* and the *hasB* more frequently reported in association with clinical infections (Boonyayatra et al., 2018) than the *hasC*, that has been related to subclinical cases (Reinoso et al., 2011; 2015). The *cfu* gene codes for the CAMP factor that is responsible for the formation of pores in host-cell membranes and the evasion of host immune defenses (Lasagno et al. 2011). A clear understanding of the contribution of each virulence factor to the disease is still lacking. *Strep. uberis* strains positive for the above genes could be more virulent and have a greater probability of causing mastitis, suggesting that a "complex multigene arrangement" can influence the clinical outcome more than the presence of a specific factor (Hossain et al., 2015). Multiplex PCR represents a reliable molecular tool for the detection of the relationship among *Strep. uberis* virulence-associated genes.

A multiplex PCR-based diagnostic test can also be useful to investigate the occurrence of AMR genes (Pyatov et al., 2017), whose different combinations are associated with MDR (Reyes et al., 2019). In

streptococci, an increase in antimicrobial resistance has been reported as a consequence of the use of drugs to control and treat bovine streptococcal infections (Pol and Ruegg, 2007). The β-lactams, especially penicillin and cephalosporins, are frequently used to cure clinical cases (Saini et al., 2012). Penicillin has been recommended as the first line antibiotic for several decades because of the high sensibility of *Strep. uberis* to this active substance (Haenni et al., 2010). The ability of *Strep. uberis* to develop penicillin resistance mechanisms, mediated by PBPs and encoded by *pbp* genes (McDougall et al., 2020), has been reported (Tomazi et al., 2019). *Strep. uberis* isolates still exhibit a good susceptibility to the cephalosporins (Käppeli et al., 2019), although a previous study documented a slow increase in ceftiofur resistance (Tomazi et al., 2019). Reports have also shown *Strep. uberis* resistance to macrolides, lincosamides and tetracyclines (de Jong et al., 2018; Käppeli et al., 2019; Reyes et al.,2019). Macrolides, especially erythromycin, are commonly used to treat bovine mastitis but a growing number of resistant *Strep. uberis* strains have been detected, due to the diffusion of macrolide resistance genes (*ermA, ermB, ermC, ermT, msrA* and *msrD*; Lüthje and Schwarz, 2007; Feßler et al., 2010). Among them, the *ermB*, responsible for cross-resistance to macrolides, lincosamides and streptogramin B antibiotics (MLS_B phenotype; Haenni et al., 2011), has seen with the highest frequency (Entorf et al., 2016). Other important resistance mechanisms to macrolides and lincosamides are mediated by *linB* gene, the *mefA* via drug efflux pump or the enzymatic inactivation encoded by *lnuD* (Schmitt-Van de Leemput and Zadoks, 2007; Petinaki et al., 2008). An additional concern is the presence of tetracycline resistance determinants (*tetK, tetL, tetM, tetS* or *tetO*; Kaczorek et al., 2017). A close surveillance of *Strep. uberis* AMR can help make choices for an adequate antibiotic treatment and avoid MDR emergence within dairy herds.

References mentioned in the Part II are reported in the Bibliography.

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Technical Note: **Development of Multiplex PCR Assays for the Molecular Characterization of *Streptococcus uberis* Strains Isolated from Bovine Mastitis**

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Abstract

Streptococcus uberis is an important causative agent for clinical and subclinical mastitis in dairy cattle. The aim of this study was to develop 2 multiplex PCR assays (mPCR) for the simultaneous detection of virulence factors and housekeeping genes for use when investigating the genetic variability and distribution of *Strep. uberis* virulence factors. The *tuf*, *cpn60*, *pauA*, *sodA*, *sua*, *oppF*, and *gapC* genes were grouped in assay 1 (mPCR1) and the *hasA*, *hasB*, and *hasC* genes were included in assay 2 (mPCR2). The detection limits were 11.8 pg and 5.9 pg of DNA for mPCR1 and mPCR2, respectively. The 2 mPCR assays were validated with 56 *Strep. uberis* strains isolated from mastitis milk samples collected from different bovine herds in northern Italy. Results revealed that *gapC* and *oppF* were detected in 98.2% of the strains, whereas *sua* and *hasC* genes were detected in 94.6 and

89.2% of the strains, respectively. The most common pattern was *gapC*+, *oppF*+, *cpn60*+, *sua*+, *sodA*+, *pauA*+, *tuf*+, *hasA*+, *hasB*+, and *hasC*+, which appeared in 59% of the strains analyzed. The molecular assays developed in the present study represent a powerful tool for the evaluation of virulence pattern distribution in *Strep. uberis* strains associated with intramammary infections.

Keywords: *Streptococcus uberis*, virulence factor, mastitis, multiplex PCR

Technical Note

Mastitis is a common disease in dairy cattle and the cause of important economic losses for the dairy industry (Liang et al., 2017). Among mastitis pathogens, *Streptococcus uberis* can colonize different dairy environments (Zadoks et al., 2005) and is implicated in clinical and subclinical IMI during lactation and the dry period (Reinoso et al., 2011), representing a potential risk factor for dairy cattle. It has been estimated that *Strep. uberis* is responsible for 14 to 26% of clinical mastitis cases in Canada, the United States, and the Netherlands, and it is the main cause of clinical mastitis in New Zealand and Australia (Collado et al., 2018). It has also been identified as being responsible for a large part of clinical mastitis in several European countries such as Belgium, Germany, Italy, and the UK (Bradley et al., 2007; Krömker et al., 2014). The control of this environmental microorganism can be particularly problematic (Boonyayatra et al., 2018). *Streptococcus uberis* has several virulence genes; for example, the hyaluronic acid capsule genes (*hasA*, *hasB*, and *hasC*; Ward et al., 2001), the plasminogen activator A gene (*pauA*; Rosey et al., 1999), and the *Strep. uberis* adhesion molecule gene (*sua*; Almeida et al., 2006). These genes all contribute to making this microorganism contagious, as well as having a role in its pathogenicity. Many studies (Yuan et al., 2014; Perrig et al., 2015; Loures et al., 2017) have shown high prevalence of these virulence genes in *Strep. uberis* strains harvested from several regions of the world. The *pauA* and *sua* genes seem to be highly conserved across *Strep. uberis* strains (Perrig et al., 2015), and have been chosen as target genes for detection in milk by PCR assays (Gillespie and Oliver, 2004). Other virulence factors such as *gapC* (Pancholi and

Fischetti, 1993), which encodes glyceraldehyde-3-phosphate dehydrogenase, or *oppF* (Smith et al., 2002), which is involved in the acquisition of essential amino acids from milk during bacterial growth, have previously been studied for their association with pathogenesis in IMI. A clear understanding of these virulence genes as key factors for mastitis development is still lacking (Boonyayatra et al., 2018). From previous studies (Perrig et al., 2015; Reinoso et al., 2015), it appears that in any given herd, only a limited group of *Strep. uberis* strains colonize the bovine mammary gland, resulting in cow-to-cow infection, whereas other strains appear to be less suited for this environment (Tassi et al., 2013). Different techniques have been used to discriminate individual strains of *Strep. uberis*. Random amplified polymorphic DNA (RAPD; Wieliczko et al., 2002), pulsed-field gel electrophoresis (PFGE; Reinoso et al., 2015) and multilocus sequence typing (MLST; Pullinger et al., 2006) have all been developed for epidemiological and genotypic studies. Species-specific PCR assay targeting the 16S rRNA gene was used by Hassan et al. (2001) to unambiguously detect *Strep. uberis* from a phenotypically identical species, *Strep. parauberis*. This PCR protocol had been widely used to identify *Strep. uberis* isolated from mastitis cases before the genotyping methods listed above. Multiplex PCR (**mPCR**) is an additional molecular tool for epidemiological studies and rapid characterization of *Strep. uberis* strains isolated from bovine mastitis milk (Boonyayatra et al., 2018). The aim of this study was to develop 2 low-cost and fast mPCR assays for the simultaneous detection of 10 genes. Included, from the Italian dairy herds, were virulence factors and housekeeping genes helpful for investigating the genetic variability of *Strep. uberis* and the distribution of its virulence factors in isolates. The virulence factors included in these 2 mPCR assays are widely studied (Parin et al., 2017; Boonyayatra et al., 2018) and are thought to represent key factors in the invasion process of mammalian epithelial tissue for *Strep. uberis*.

To optimize the mPCR protocol, the reference *Strep. uberis* ATCC 9927 strain (LGC Promochem, Middlesex, UK) was used. The mPCR assays were then validated on 56 isolates from composite subclinical mastitis milk samples. These samples were collected between January 2016 and August 2017 from 12 bovine herds in northern Italy. Those farms were chosen because of their large herd

size, a monthly incidence of *Strep. uberis* mastitis of 5 to 6%, availability of reliable health records, and access to microbiological diagnosis of milk samples at the University of Milan. The samples were collected aseptically and the isolation of *Strep. uberis* strains was performed by plating 10 μL of bovine mastitis milk onto trypticase soy agar (TSA) containing 5% sheep blood and 0.1% esculin (bioMérieux, Marcy l'Étoile, France). Plates were then incubated aerobically for 24 to 48 h at 37°C. Preliminary identification of *Strep. uberis* was based on colony morphology, esculin splitting, and catalase testing (National Mastitis Council, 2017). All streptococcal isolates were identified at species level by the API 20 Strep (bioMérieux). Genomic DNA was extracted from pure cultures, as previously described by Cremonesi et al. (2006), without a pretreatment step, using a method based on the combination of a chaotropic agent, guanidium thiocyanate, with silica particles to obtain bacterial cell lysis and nuclease inactivation. The concentration of the purified DNA was determined using NanoDrop 2000 (ThermoFisher Scientific Inc., Waltham, MA) and stored at −20°C until further use. The target genes of *Strep. uberis* included in the mPCR were *gapC*, *oppF*, *cpn60*, *sua*, *sodA*, *pauA*, *tuf*, *hasA*, *hasB*, and *hasC*. All details concerning the oligonucleotides, including product size, primer sequences, and GenBank accession numbers, are summarized in Table 1. All primers of this study were designed using Primer 3 software (http://primer3.ut.ee/), except for primer *cpn60*, which was previously described (Dmitriev et al., 2006). The *in silico* specificity was checked by using the BLAST software tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The primers were synthesized by ThermoFisher Scientific. The oligonucleotides were chosen based on similar melting temperatures to use the same amplification protocol with minimal interactions, resulting in different-sized products distinguishable by agarose gel electrophoresis. Using these criteria, 2 mPCR assays were optimized: *gapC*, *oppF*, *cpn60*, *sua*, *sodA*, *pauA*, and *tuf* were grouped in mPCR1 and *hasA*, *hasB*, and *hasC* in mPCR2.

Initially, both mPCR assays were set up using genomic DNA extracted from the reference *Strep. uberis* ATCC 9927 strain. The mPCR assays, prepared in 0.2-mL tubes, were performed in a 25-μL volume with *sodA* primers at 1.6 μ*M* and all other primers at 0.8 μ*M* (ThermoFisher Scientific, Milan,

Italy), PCR master mix 10× (AccuPrime, Invitrogen, Minneapolis, MN), 1 U of AccuPrime *Taq* DNA Polymerase (Invitrogen), and 2 μL of DNA (~40 ng/μL). Amplifications were carried out in a thermocycler (Applied Biosystems, Foster City, CA) with the following program: 94°C for 5 min followed by 30 cycles of 94°C for 1 min, 56°C for 1 min, 68°C for 1 min, and finally a step of 72°C for 7 min. The amplified PCR products were visualized simultaneously by standard gel electrophoresis in a 3% agarose gel (GellyPhor, Euroclone, Milan, Italy), stained with ethidium bromide (0.05 mg/mL; Sigma Aldrich, Milan, Italy). A molecular size marker (100-bp DNA ladder; Finnzymes, Espoo, Finland) was loaded in each agarose gel. The DNA bands were visualized on a UV transilluminator (BioView Ltd., Nes Ziona, Israel). The sensitivity of the 2 mPCR assays was tested using a 2-fold serial dilution of the ATCC 9927 reference strain genomic DNA from 96 ng to 1.5 pg. The PCR products obtained by the 2 mPCR assays were analyzed and quantified using the Agilent BioAnalyzer 2100, applying the DNA 500 LabChip kit (Agilent Technologies, Palo Alto, CA).

Table 1. Multiplex PCR primers used in this study, including gene targets, amplicon size, primer sequences (For, forward; Rev, reverse), and gene sequence accession numbers

Multiplex PCR	Target gene	Amplicon size (bp)	Primer sequence	GenBank accession number	Reference
Reaction 1	*gapC*	505	For:5′GCTCCTGGTGGAGATGATGT-3′ Rev: 3′-AACCGTAAGCCATACCGATG-5′	GU392494	This study
	oppF	454	For: 5′-TCAGAGATATTGTTGCTGAAGGA-3′ Rev: 3′-GGCTCTGGAATTGCTGAAAG-5′	GU392621	This study
	cpn60	400	For: 5′-TCGCGGTATTGAAAAAGCAACAT-3′ Rev: 3′-TGCAATAATGAGAAGGGGACGAC-5’	AF485804	Dmitriev et al., 2006
	sua	350	For: 5′-GCAACATTGGCACCTACAAA-3′ Rev: 3′-GCAGCTGTTACCTCGTCAGA-5′	LN885239.1	This study
	sodA	280	For: 5′-TGATAAAGAAACAATGACCCTTCA-3’ Rev: 3′-TGCATCAAAAGAACCAAATGC-5′	GU392754.1	This study
	pauA	205	For: 5′-TGACGAGTTTCGAAAAATTGC-3′ Rev: 3′-ACCGAGTTCTTTTCCGGATT-5′	KT006562.1	This study
	tuf	143	For: 5′-TCCTTCTTTCACGCCAAGTT-3′ Rev: 3′-GTCATCACCTGGGAAATCGT-5′	GU392973	This study
Reaction 2	*hasA*	599	For: 5′-AAATGGCTTTGGAGACCAAG-3′ Rev: 3′-CAACACTTGGTGTGGCTAATAA-5′	AM946015.1	This study
	hasB	400	For: 5′-CGATCAAGCATTTAGGGATG-3′ Rev: 3′-AGCCTCTGCTGAACCCATAA-5′	AJ242946	This study
	hasC	193	For: 5′-AGGCTTAGGGGATGCTGTTT-3′ Rev: 3′-GGATACGTCATCGTGAGGAAC-5′	AJ400707	This study

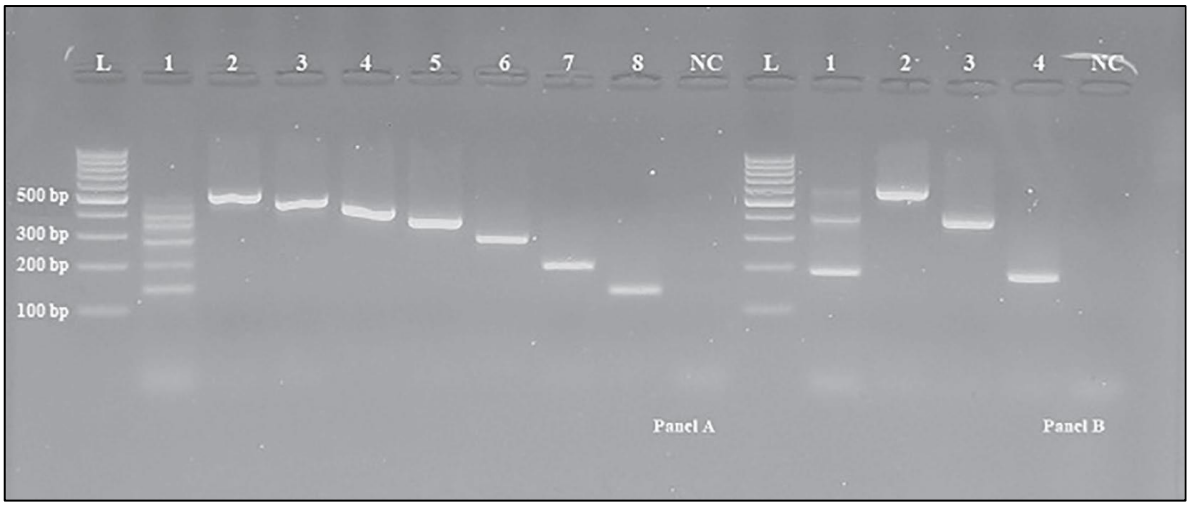

Figure 1. Optimization of multiplex (m)PCR reactions using genomic DNA of *Streptococcus uberis* ATCC 9927 strain. (A) Lane 1 = mPCR1 with all 7 genes; lanes 2–8 = uniplex PCR reactions for each gene: *gapC* (505 bp), *oppF* (454 bp), *cpn60* (400 bp), *sua* (350 bp), *sodA* (280 bp), *pauA* (205 bp), *tuf* (143 bp), respectively; lane L: 100-bp DNA ladder; lane NC = negative control. (B) Lane 1 = mPCR2 with 3 genes; lanes 2–4 = uniplex PCR reactions for each gene: *hasA* (599 bp), *hasB* (400 bp), and *hasC* (193 bp), respectively; lane L = 100-bp DNA ladder; lane NC = negative control.

Using the ATCC reference strain, the mPCR assays were successfully optimized and the desired amplicons were obtained in both reactions, as shown in Figure 1. In reaction 1, 7 bands with sizes of 505, 400, 369, 350, 280, 205, and 143 bp, corresponding to *gapC*, *oppF*, *cpn60*, *sua*, *sodA*, *pauA*, and *tuf* genes, were distinguished without interactions among them, (Figure 1A). In reaction 2, 3 bands with sizes of 599, 400, and 193 bp were obtained, corresponding to *hasA*, *hasB*, and *hasC* genes, respectively (Figure 1B). As shown in lane 1 (Figure 1A and 1B), the primer concentrations used in the single reaction resulted in approximately equal yields for all amplification products. To check the assays' sensitivity and verify similar results between multiplex and simplex PCR, 2-fold serial dilutions were tested starting from the *Strep. uberis* ATCC 9927 genomic DNA reference strain. The sensitivity of the 2 mPCR assays was 23.5 and 11.8 pg, for mPCR1 and mPCR2, respectively, corresponding to approximately 20 cfu/mL for both reactions (Figure 2), which confirms that the standardized assays allowed amplification of all putative and known virulence-associated genes of *Strep. uberis*. The limit of detection (**LOD**) of the 2 mPCRs developed in this work was comparable to those of previous studies. For example, Wang and Liu (2015) optimized a loop-mediated isothermal amplification (LAMP)-PCR for mastitis pathogens, including *Strep. uberis*, with an LOD of 0.1 pg of DNA. Phuektes and coworkers (2001) reported an LOD of 50 pg for their mPCR assay

to identify *Strep. uberis*, whereas Shome et al. (2011) improved the sensitivity of an mPCR assay for the detection of mastitis pathogens including *Strep. uberis* and reached an LOD of 10 fg.

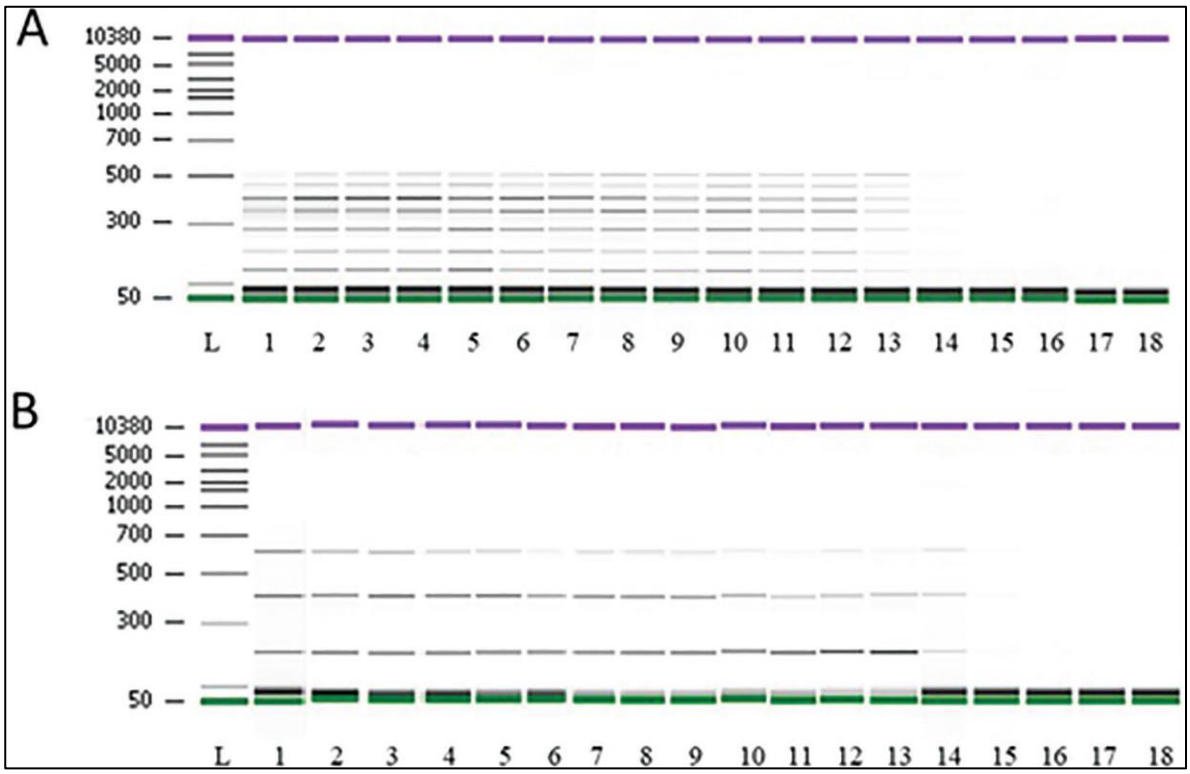

Figure 2. Sensitivity of multiplex (m)PCR analysis of different DNA amounts derived from *Streptococcus uberis* ATCC 9927 strain: (A) reaction 1, (B) reaction 2. The concentration of each DNA fragment was calculated using the Agilent 2100 Bioanalyzer software (Agilent Technologies, Palo Alto, CA). The gel-like image shows the mPCR results obtained using 96.4 ng (lane 1), 48.2 ng (lane 2), 24.1 ng (lane 3), 12.0 ng (lane 4), 6.0 ng (lane 5), 3.0 ng (lane 6), 1.5 ng (lane 7), 0.75 ng (lane 8), 0.4 ng (lane 9), 0.2 ng (lane 10), 94.1 pg (lane 11), 47.1 pg (lane 12), 23.5 pg (lane 13), 11.8 pg (lane 14), 5.9 pg (lane 15), 2.9 pg (lane 16), and 1.5 pg (lane 17) of DNA from the reference strain; lane 18: negative control; lane L = DNA 500 ladder.

All 56 *Strep. uberis* isolates harbored at least one virulence-associated gene and all were positive for *tuf*, *sodA*, and *cpn60* genes. The mPCR assays detected the *gapC* and *oppF* genes in 98.2% of the strains, whereas the *sua* gene was found in 53 strains (94.6%). These virulence genes were commonly detected among the *Strep. uberis* strains, as previously described (Reinoso et al., 2011; Parin et al., 2017; Boonyayatra et al., 2018). The higher distribution of these genes among the *Strep. uberis* strains involved in clinical and subclinical mastitis is due to their role in *Strep. uberis* growth in milk (*oppF*; Smith et al., 2002) or to their involvement in virulence (*gapC* and *sua* genes; Boonyayatra et al., 2018).

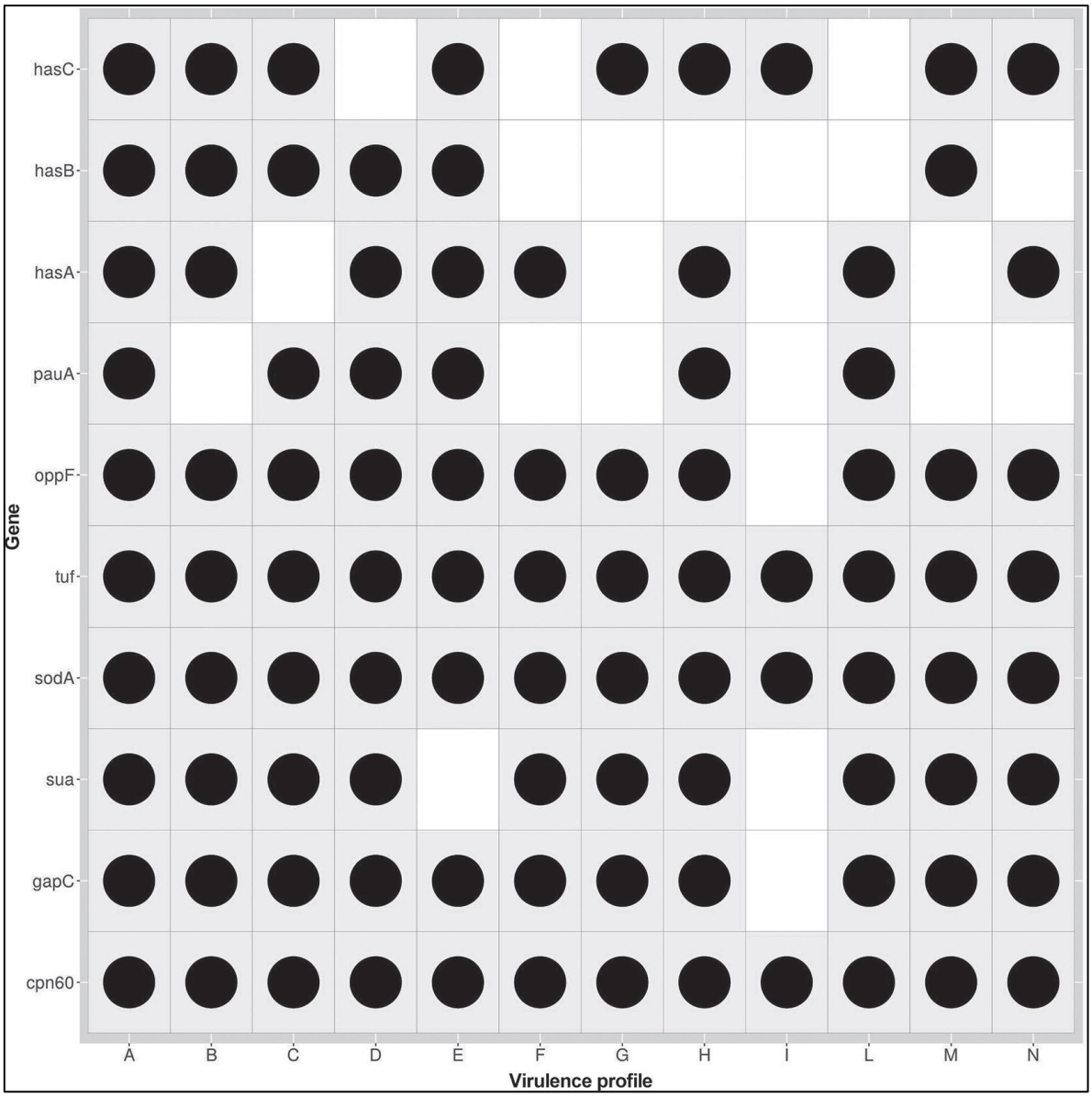

Figure 3. The distribution of genes investigated in virulence profiles. Each profile is indicated by letters from A to N and the presence of a given gene is shown by a black circle. Profile A was found in 59% of isolates (33/56); profiles B and C were found in 7.1% of the isolates (4/56); profile D was found in 5.4% of the isolates (3 out of 56); profiles E, F, G, and H were each found in 3.5% of the isolates (2/56); and profiles I, L, M, and N were each found in 1.7% of the strains (1/56).

The *hasC*, *hasA*, and *hasB* genes were detected in 50 (89.2%), 48 (85.7%), and 47 (83.9%) isolates, whereas the *pauA* gene was detected in 45 (80.3%) isolates. These results concur with previous studies (Reinoso et al., 2011; Boonyayatra et al., 2018) that reported a higher frequency of *hasC* than of *hasA* and *hasB.* As previously published by Reinoso et al. (2011) and Boonyayatra et al. (2018), differences in frequency among these 3 genes arise from the fact that the hyaluronic acid capsule (coded by *hasABC* genes) of *Strep. uberis* may not have a primary role in mammary gland infection.

Also, noncapsulated *Strep. uberis* isolates can induce mastitis by resistance to neutrophil phagocytosis (Field et al., 2003).

The distribution of virulence-associated genes revealed 12 virulence profiles, labeled A to N (Figure 3). The most common pattern was *gapC*+, *oppF*+, *cpn60*+, *sua*+, *sodA*+, *pauA*+, *tuf*+, *hasA*+, *hasB*+, and *hasC*+ (A profile) where *cpn60*, *sodA*, and *tuf* genes are housekeeping genes (Zadoks et al., 2005). Profile A included 59% (33/56) of isolates and was prevalent in 50% of the herds analyzed (6/12). The remaining 41% (23/56) of isolates were distributed in 11 profiles with 14% of isolates presenting pattern B (*gapC*+, *oppF*+, *cpn60*+, *sua*+, *sodA*+, *tuf*+, *hasA*+, *hasB*+, *hasC*+; 7%) or C (*gapC*+, *oppF*+, *cpn60*+, *sua*+, *sodA*+, *pauA*+, *tuf*+, *hasB*+, *hasC*+; 7%). Although a small group of isolates was analyzed, our results showed a large genetic variability of *Strep. uberis* isolates, as previously published by Boonyayatra et al. (2018) and Reinoso et al. (2011) for 88 and 78 isolates, respectively. Some of these genes, such as *sua*, *pauA*, and *gapC*, encode virulence factors involved in the survival of the microorganism in the host environment, in its evasion of host tissue, and in its internalization in mammary gland cells, suggesting that isolates with pattern A could be more virulent and have a greater probability of causing mastitis (Reinoso et al., 2011; Boonyayatra et al., 2018). Further studies should be carried out on more isolates to reinforce these findings. Finally, to our knowledge, only a few studies have described mPCR assays for the molecular characterization of *Strep. uberis* strains (Parin et al., 2017; Boonyayatra et al., 2018). Boonyayatra et al. (2018) analyzed 11 genes in 3 different mPCR reactions that used 3 different annealing temperatures, whereas Parin et al. (2017), after species identification by the 16S rRNA gene, detected 10 virulence genes in a single multiplex amplification reaction. They probably could not distinguish between the *hasB* and *hasC* virulence factors, because these 2 genes have amplification products of the same size (300 bp). The mPCR assays developed in the present study used some housekeeping genes—*cpn60*, *soda*, and *tuf*—in order to unambiguously distinguish *Strep. uberis* from *Strep. parauberis*, a genetically closely related species, avoiding the need for any additional genotyping steps. It may be possible to analyze the same virulence genes, previously described, by reducing the time of analysis and the cost of the assay.

These assays used only one amplification program, making the approach less costly and faster than previously described methods (Parin et al., 2017; Boonyayatra et al., 2018). This approach will potentially be very useful for the characterization of *Strep. uberis* in epidemiological studies, offering the ability to quickly obtain relevant information on the pathogenicity of isolates and progression of herd infections for a disease of growing concern.

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References

Almeida, R.A., D.A. Luther, H.M. Park, and S.P. Oliver. 2006. Identification, isolation, and partial characterization of a novel *Streptococcus uberis* adhesion molecule (SUAM). Vet. Microbiol. 115:183–191.

Boonyayatra, S., P. Tharavichitkul, and S.P. Oliver. 2018. Virulence-associated genes and molecular typing of *Streptococcus uberis* associated with bovine mastitis in northern Thailand. Turk. J. Vet. Anim. Sci. 42:73–81.

Bradley, A.J., K.A. Leach, J.E. Breen, L.E. Green, and M.J. Green. 2007. Survey of the incidence and aetiology of mastitis on dairy farms in England and Wales. Vet. Rec. 160:253–257.

Collado, R., C. Montbrau, M. Sitjà, and A. Prenafeta. 2018. Study of the efficacy of a *Streptococcus uberis* mastitis vaccine against an experimental intramammary infection with a heterologous strain in dairy cows. J. Dairy Sci. 101:10290–10302. https://doi.org/10.3168/jds.2018-14840.

Cremonesi, P., B. Castiglioni, G. Malferrari, I. Biunno, C. Vimercati, P. Moroni, S. Morandi, and M. Luzzana. 2006. Technical note: Improved method for rapid DNA extraction of mastitis pathogens directly from milk. J. Dairy Sci. 89:163–169.

Dmitriev, A., M. Bhide, and I. Mikula. 2006. *cpn60* gene-based multiplex-PCR assay for simultaneous identification of streptococcal species. Acta Vet. Brno 75:235–240.

Field, T.R., P.N. Ward, L.H. Pedersen, and J.A. Leigh. 2003. The hyaluronic acid capsule of *Streptococcus uberis* is not required for the development of infection and clinical mastitis. Infect. Immun. 71:132–139.

Gillespie, B.E., and S.P. Oliver. 2004. Comparison of an automated ribotyping system, pulsed-field gel electrophoresis and randomly amplified polymorphic DNA fingerprinting for differentiation of *Streptococcus uberis* strains. Biotechnology (Faisalabad) 3:165–172.

Hassan, A.A., I.U. Khan, A. Abdulmawjood, and C. Lämmler. 2001. Evaluation of PCR methods for rapid identification and differentiation of *Streptococcus uberis* and *Streptococcus parauberis*. J. Clin. Microbiol. 39:1618–1621.

Krömker, V., N.T. Grabowski, and J. Friedrich. 2014. New infection rate of bovine mammary glands after application of an internal teat seal at dry-off. J. Dairy Res. 81:54–58. https://doi.org/10.1017/S0022029913000599.

Liang, D., L.M. Arnold, C.J. Stowe, R.J. Harmon, and J.M. Bewley. 2017. Estimating US dairy clinical disease costs with a stochastic simulation model. J. Dairy Sci. 100:1472–1486. https://doi.org/10.3168/jds.2016-11565.

Loures, R.A., U.D. Pereira, G.D. de Carvalho-Castro, G.F. Mian, D.A.D. Custodio, J.R. da Silva, and G.M. da Costa. 2017. Genetic diversity and virulence genes in *Streptococcus uberis* strains isolated from bovine mastitis. Semin. Cienc. Agrar. 38:2595–2605. https://doi.org/10 .5433/1679-0359.2017v38n4Supl1p2595.

National Mastitis Council. 2017. Laboratory handbook on bovine mastitis. National Mastitis Council, New Prague, MN.

Pancholi, V., and V.A. Fischetti. 1993. Glyceraldehyde-3-phosphate dehydrogenase on the surface of group A streptococci is also an ADP-ribosylating enzyme. Proc. Natl. Acad. Sci. USA 90:8154–8158.

Parin, U., S. Kirkan, E. Cicek, and H.T. Yuksel. 2017. Detection of virulence genes in *Streptococcus uberis* isolated from bovine mastitis in Aydin province by multiplex polymerase chain reaction. F. Ü. Sağ. Bil. Vet. Derg. 31:213–219.

Perrig, M.S., M.B. Ambroggio, F.R. Buzzola, I.S. Marcipar, L.F. Calvinho, C.M. Veaute, and M.S. Barbagelata. 2015. Genotyping and study of the *pauA* and *sua* genes of *Streptococcus uberis* isolates from bovine mastitis. Rev. Argent. Microbiol. 47:282–294. https://doi.org/10.1016/j .ram.2015.06.007.

Phuektes, P., P.D. Mansell, and G.F. Browning. 2001. Multiplex polymerase chain reaction assay for simultaneous detection of *Staphylococcus aureus* and streptococcal causes of bovine mastitis. J. Dairy Sci. 84:1140–1148.

Pullinger, G.D., M. Lopez-Benavides, T.J. Coffey, J.H. Williamson, R.T. Cursons, E. Summers, J. Lacy-Hulbert, M.C. Maiden, and J.A. Leigh. 2006. Application of *Streptococcus uberis* multilocus sequence typing: analysis of the population structure detected among environmental and bovine isolates from New Zealand and the United Kingdom. Appl. Environ. Microbiol. 72:1429–1436.

Reinoso, E.B., M.C. Lasagno, S.A. Dieser, and L.M. Odierno. 2011. Distribution of virulence-associated genes in *Streptococcus uberis* isolated from bovine mastitis. FEMS Microbiol. Lett. 318:183–188. https://doi.org/10.1111/j.1574-6968.2011.02258.x.

Reinoso, E.B., M.C. Lasagno, and L.M. Odierno. 2015. Genetic patterns of *Streptococcus uberis* isolated from bovine mastitis. Rev. Argent. Microbiol. 47:108–111. https://doi.org/10. 1016/j.ram.2015.02.001.

Rosey, E.L., R.A. Lincoln, P.N. Ward, R.J. Yancey Jr., and J.A Leigh. 1999. PauA: A novel plasminogen activator from *Streptococcus uberis*. FEMS Microbiol. Lett. 178:27–33.

Shome, B.R., S. Das Mitra, M. Bhuvana, N. Krithiga, D. Velu, R. Shome, S. Isloor, S.B. Barbuddhe, and H. Rahman. 2011. Multiplex PCR assay for species identification of bovine mastitis pathogens. J. Appl. Microbiol. 111:1349–1356. https://doi.org/10.1111/j.1365-2672.2011.05169.x.

Smith, A.J., A.J. Kitt, P.N. Ward, and J.A. Leigh. 2002. Isolation and characterization of a mutant strain of *Streptococcus uberis*, which fails to utilize a plasmin derived beta-casein peptide for the acquisition of methionine. J. Appl. Microbiol. 93:631–639.

Tassi, R., T.N. McNeilly, J.L. Fitzpatrick, M.C. Fontaine, D. Reddick, C. Ramage, M. Lutton, Y.H. Schukken, and R.N. Zadoks. 2013. Strain-specific pathogenicity of putative host-adapted and nonadapted strains of *Streptococcus uberis* in dairy cattle. J. Dairy Sci. 96:5129–5145. https://doi.org/10.3168/jds.2013-6741.

Wang, D., and Y. Liu. 2015. Development of primer sets for loop-mediated isothermal amplification that enables rapid and specific detection of *Streptococcus dysgalactiae*, *Streptococcus uberis* add *Streptococcus agalactiae*. Int. J. Environ. Res. Public Health 12:5735–5742. https://doi.org/10.3390/ijerph120605735.

Ward, P.N., T R. Field, W.G. Ditcham, E. Maguin, and J.A. Leigh. 2001. Identification and disruption of two discrete loci encoding hyaluronic acid capsule biosynthesis genes *hasA*, *hasB*, and *hasC* in *Streptococcus uberis*. Infect. Immun. 69:392–399.

Wieliczko, R.J., J.H. Williamson, R.T. Cursons, S.J. Lacy-Hulbert, and M.W. Woolford. 2002. Molecular typing of *Streptococcus uberis* strains isolated from cases of bovine mastitis. J. Dairy Sci. 85:2149–2154.

Yuan, Y., O.K. Dego, X. Chen, E. Abadin, S. Chan, L. Jory, S. Kovacevic, R.A. Almeida, and S.P. Oliver. 2014. Short communication: Conservation of *Streptococcus uberis* adhesion molecule and the *sua* gene in strains of *Streptococcus uberis* isolated from geographically diverse areas. J. Dairy Sci. 97:7668–7673. https://doi.org/10.3168/jds.2013-7637.

Zadoks, R.N., L.L. Tikofsky, and K.J. Boor. 2005. Ribotyping of from a dairy's environment, bovine feces and milk. Vet. Microbiol. 109:257–265.

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Genotyping and Antimicrobial Susceptibility Profiling of *Streptococcus uberis* Isolated from a Clinical Bovine Mastitis Outbreak in a Dairy Farm

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Abstract

Streptococcus uberis, an environmental pathogen responsible also for contagious transmission, has been increasingly implicated in clinical mastitis (CM) cases in Europe. We described a 4-month epidemiological investigation of *Strep. uberis* CM cases in an Italian dairy farm. We determined molecular characteristics and phenotypic antimicrobial resistance of 71 *Strep. uberis* isolates from dairy cows with CM. Genotypic variability was investigated via multiplex PCR of housekeeping and

virulence genes, and by RAPD-PCR typing. Antimicrobial susceptibility was assessed for 14 antimicrobials by MIC assay. All the isolates carried the 11 genes investigated. At 90% similarity, two distinct clusters, grouping 69 of the 71 isolates, were detected in the dendrogram derived from the primer ERIC1. The predominant cluster I could be separated into two subclusters, containing 38 and 14 isolates, respectively. *Strep. uberis* strains belonging to the same RAPD pattern differed in their resistance profiles. Most (97.2%) of them were resistant to at least one of the drugs tested, but only 25.4% showed a multidrug resistance phenotype. The highest resistance rate was observed for lincomycin (93%), followed by tetracycline (85.9%). This study confirmed a low prevalence of β-lactam resistance in *Strep. uberis*, with only one isolate showing resistance to six antimicrobial classes, including cephalosporins.

Keywords: *Streptococcus uberis*; cow; clinical mastitis; RAPD; MIC

Introduction

Streptococcus uberis is primarily classified as an environmental pathogen causing about one-third of all intramammary infection (IMI) cases in lactating and nonlactating cows worldwide (Bradley et al., 2007; Botrel et al., 2010). Recently, an increased prevalence of *Strep. uberis* isolates associated with clinical mastitis (CM) has been reported in several European countries (Verbeke et al., 2014; Poutrel et al., 2018).

Although its primary reservoir is the dairy farm environment (Zadoks et al., 2005; Lopez-Benavides et al., 2007; Ericsson et al., 2009), some *Strep. uberis* strains show a contagious transmission mode (Wente et al., 2019; Wald et al., 2020), deserving further investigation. Molecular genotyping contributes to understanding the modes of dissemination and can be useful in epidemiological studies of *Strep. uberis* mastitis. The most commonly used subtyping methods are MLVA (Multiple Loci VNTR Analysis; VNTR, Variable Number of Tandem Repeats), MLST (MultiLocus Sequencing typing), PFGE (Pulsed Field Gel Electrophoresis), and RAPD-PCR (Random Amplified Polymorphic DNA-PCR; Gilbert et al., 2006; Reyes et al., 2019; Wente et al., 2019; Leelahapongsathon et al.,

2020). Generally, *Strep. uberis* has a nonclonal population structure but clonal strains in different cows have been detected (Davies et al., 2016). Zadoks and collaborators (2003) applied RAPD-PCR to demonstrate the genetic homogeneity of *Strep. uberis* within some farms. Recently, this latter technique was shown to be reliable in typing *Strep. uberis* and identifying clonal strains in different cows within herds (Tomazi et al., 2019). Despite bacterial diversity, most *Strep. uberis* strains share the same combination of highly conserved virulence genes (*hasA, hasB, hasC, oppF, pauA, sua)* (Gilchrist et al., 2013; Almeida et al., 2015; Boonyayatra et al.,2018), strongly associated with the pathogenesis of IMI. The two multiplex PCR (mPCR) assays developed by Calonzi and collaborators (2020) allow the detection of these genes for a rapid characterization of *Strep. uberis* strains. Additionally, the *lbp*-producing iron-binding protein aids in adherence to and internalization into bovine mammary epithelial cells (Fang et al., 1999).

Antimicrobial therapy is still the primary strategy to control and treat bovine IMI. The widespread and sometimes inappropriate use of drugs to cure clinical cases has led to an increasing diffusion of antimicrobial resistance (AMR) in Streptococci, resulting in health problems at the herd level (Pol and Ruegg, 2007). Although β-lactam antibiotics, especially penicillin and cephalosporins, are frequently recommended to treat clinical IMI (Saini et al., 2012), an increased ability of the bacterium to develop resistance to cephalosporins has been reported (Tomazi et al., 2019). Recent reports have also shown the resistance of *Strep. uberis* to macrolides, lincosamides, and tetracyclines (de Jong et al., 2018; Käppeli et al., 2019; Reyes et al., 2019).

We investigated the epidemiology of *Strep. uberis* CM cases in a dairy farm during a 4-month study period. We determined the genotypes, the virulence profiles and the AMR patterns of 71 *Strep. uberis* strains in order to understand their heterogeneity and the mode of transmission within the herd.

Results

Virulence Profiling

The four housekeeping genes investigated (*cpn60, gapC sodA* and *tuf*) and the combination of the seven virulence-associated genes (*lbp, hasA, hasB, hasC, oppF, pauA, sua*) were detected in 100% of the analyzed isolates.

Genotyping

The 71 *Strep. uberis* isolates were characterized by RAPD-PCR analysis with primer ERIC1. As isolates with a similarity coefficient equal to or higher than 90% can be considered as closely related, the amplification profiles identified clonal strains in different cows and displayed conserved patterns within the herd. At 90% similarity, two distinct clusters were detected in the dendrogram derived from ERIC1 (Figure 1). These two major clusters, indicated with roman numerals (I and II), grouped 69 of the 71 *Strep. uberis* isolates. A unique cluster (cluster I) was predominant among the herd isolates (73.3%) and could be separated into two subclusters (Ia and Ib), containing 38 and 14 isolates, respectively. Cluster II included 17 (23.9%) isolates, while the remaining two (2.8%) isolates did not enter any cluster.

Antimicrobial Profiling

The phenotypic results obtained by MIC assay (Table 1) revealed that the 71 *Strep. uberis* isolates had different degrees of resistance to the 14 active substances and most (97.2%) were not inhibited by at least one of the drugs tested, showing the highest resistance rate to lincomycin (93%) and tetracycline (85.9%). Analyzing the resistance to multiple classes of antimicrobials, 14.1% of the *Strep. uberis* isolates were resistant to one antimicrobial class, but most were resistant to two (57.7%),

or three and more classes (25.4%). In particular, the percentage of resistance to four and five antimicrobial classes was 7.1% and 2.8% respectively, and only one isolate was classified as resistant to six different classes. Table 1 reports the distribution of the MIC inhibiting the growth of 50% and 90% of the isolates (MIC_{50} and MIC_{90}) for all the antimicrobials tested, and shows the highest inhibiting concentrations for lincomycin and tetracycline, with MIC_{90} values even higher than the resistance breakpoints. The lowest inhibiting concentrations were for erythromycin and most of the β-lactams. Low levels of resistance (8.5%) were found to erythromycin, and also to fluoroquinolones (enrofloxacin). Resistance to β-lactam antibiotics was present, with 11 (15.5%) isolates resistant to penicillin, three (4.2%) to oxacillin, one to first-generation (cefazolin) and fourth-generation (cefquinome) cephalosporins, and two isolates resistant to third-generation (cefoperazone and ceftiofur).

Distribution of Antimicrobial Resistance Between Genotypic Clusters

Table 2 shows the differences in antimicrobial resistance observed between the two clusters obtained by the RAPD-PCR analysis. The distribution of MIC_{50} and the MIC_{90} values for florfenicol, lincomycin and tetracycline was identical throughout the RAPD clusters, while there were differences for the other antimicrobials tested. The cluster I isolates showed significantly higher MIC values than those of cluster II for amoxicillin plus clavulanate (p value = 0.034) and for ceftiofur (p value = 0.01) (Table S1).

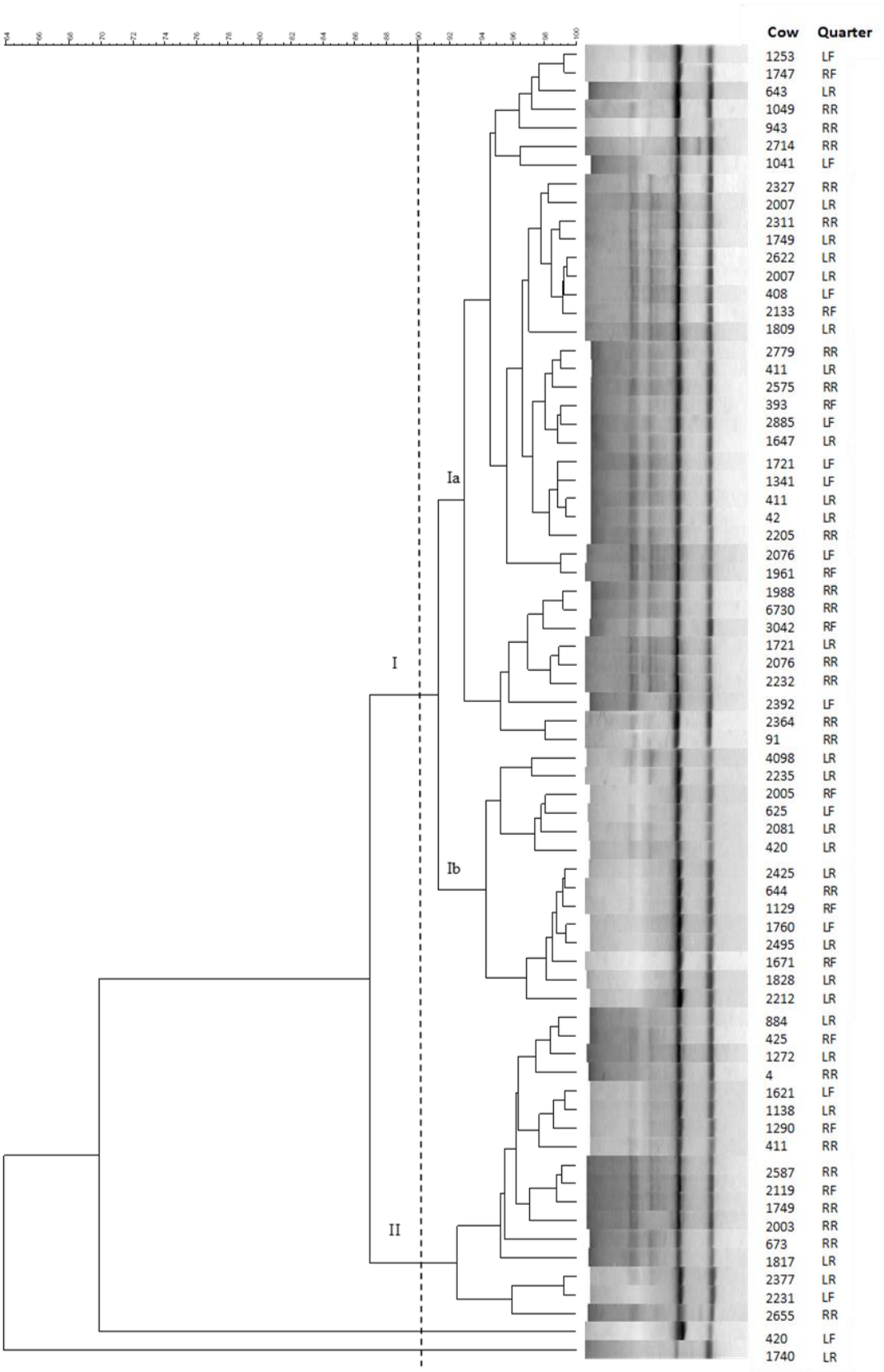

Figure 1. Unweighted pair group method with arithmetic averages (UPGMA)-based dendrogram derived from the RAPD-PCR profiles generated with primer ERIC1 of the 71 *Strep. uberis* strains isolated from 71 quarters of different cows.

Table 1. Antimicrobials tested, dilution range, breakpoints values, percentage of susceptible, intermediate and resistant *Strep. uberis* isolates, MIC inhibiting the growth of at least 50% (MIC_{50}) and 90%(MIC_{90}) of the 71 isolates analyzed.

Antimicrobials	Range (µg/mL)	Breakpoints (µg/mL) and Susceptibility						MIC_{50}	MIC_{90}	Reference
		S[1]	[%]	I[2]	[%]	R[3]	[%]	(µg/mL)	(µg/mL)	
AMC [4]	0.064/0.032–64/32	≤0.25/0.125	98.6	0.5/0.25	0	>1/0.5	1.4	0.25/0.125	0.25/0.125	(CLSI, 2018a)
AMP [5]	0.016–16	≤0.25	98.6	0.5	0	>2	1.4	0.25	0.25	(CLSI, 2018a)
CEZ [6]	0.125–32	≤2	98.6	4	0	>8	1.4	0.5	0.5	(CLSI, 2018a)
CPZ [7]	0.125–16	≤2	97.2	4	1.4	>8	1.4	1	2	(Feβler, et al., 2012)
CEQ [8]	0.125–8	≤2	98.6			>4	1.4	≤0.125	0.25	(Lang et al., 2002)
CEF [9]	0.125–32	≤2	98.6	4	0	>8	1.4	0.5	1	(CLSI, 2018a)
ENRO [10]	0.016–8	≤0.5	91.6	1–2	7	>4	1.4	0.5	0.5	(CLSI, 2018a)
ERY [11]	0.125–8	≤0.25	91.6	0.5	2.8	>1	5.6	≤0.125	≤0.125	(CLSI, 2018a)
FLL [12]	0.064–64	≤2	94.4	4	5.6	>8	0	2	2	(CLSI, 2018a)
LIN [13]	1–8	≤2	7	4–8	11.3	>18	81.7	>8	>8	(CASFM, 2019)
OXA [14]	0.125–4	≤2	95.8			>4	4.2	2	2	(CASFM, 2019)
PEN [15]	0.0625–16	≤0.125	84.5	0.25–2	12.7	>4	2.8	0.125	0.25	(CLSI, 2018a)
TET [16]	0.032–16	≤2	14.1	4	2.8	>8	83.1	>16	>16	(CLSI, 2018a)
T/S [17]	0.016/0.304–32/608	≤1/19	98.6			> 2/38	1.4	0.062/1.18	0.125/2.37	(EUCAST, 2020)

[1]Susceptible, [2]Intermediate, [3]Resistant, [4]Amoxicillin/clavulanic acid, [5]Ampicillin, [6]Cefazolin, [7]Cefoperazone, [8]Cefquinome, [9]Ceftiofur, [10]Enrofloxacin, [11]Erythromycin, [12]Florfenicol, [13]Lincomycin, [14]Oxacillin, [15]Penicillin, [16]Tetracycline, [17]Trimethoprim/sulfamethoxazole.

Table 2. Distribution of the MIC_{50} and MIC_{90} for the 14 antimicrobial agents between the genotypic clusters, and cumulative percentage of *Strep. uberis* strains inhibited by their relative concentrations.

Antimicrobial Agents	Cluster I				Cluster II			
	MIC_{50}		MIC_{90}		MIC_{50}		MIC_{90}	
	(µg/mL)	[%]	(µg/mL)	[%]	(µg/mL)	[%]	(µg/mL)	[%]
AMC [1]	0.25/0.125	98	0.25/0.125	98	0.125/0.064	53	0.25/0.125	100
AMP [2]	0.25	98	0.25	98	0.125	53	0.25	100
CEZ [3]	0.5	92	0.5	92	0.5	88	1	100
CPZ [4]	1	73	2	96	1	65	2	100
CEQ [5]	0.25	96	0.25	96	≤0.125	71	0.25	94
CEF [6]	0.5	69	1	96	0.5	94	0.5	94
ENRO [7]	0.5	88	1	94	0.5	100	0.5	100
ERY [8]	≤0.125	90	≤0.125	90	≤0.125	94	≤0.125	94
FLL [9]	2	94	2	94	2	94	2	94
LIN [10]	>8	100	>8	100	>8	100	>8	100
OXA [11]	2	96	2	96	2	94	2	94
PEN [12]	0.125	81	0.25	96	0.125	94	0.125	94
TET [13]	>16	100	>16	100	>16	100	>16	100
T/S [14]	0.062/1.18	83	0.125/2.37	96	0.062/1.18	94	0.062/1.18	94

[1]Amoxicillin/clavulanic acid, [2]Ampicillin, [3]Cefazolin, [4]Cefoperazone, [5]Cefquinome., [6]Ceftiofur, [7]Enrofloxacin, [8]Erythromycin, [9]Florfenicol, [10]Lincomycin, [11]Oxacillin, [12]Penicillin, [13]Tetracycline, [14]Trimethoprim/sulfamethoxazole.

Discussion

A 4-month retrospective cohort study was undertaken to characterize 71 *Strep. uberis* isolates obtained from CM cases at an Italian dairy farm. The genetic analyses revealed that *Strep. uberis* mastitis cases in this herd could be linked to a relatively restricted number of cow-adapted strains, grouped in the same genotypic cluster. In the present study, the genetic similarity of *Strep. uberis* strains analyzed was evident in the PCR analysis, revealing that their virulence profile was characterized by a particular combination of genes that may be responsible for the high incidence of CM cases. The genes *sua, lbp* and *pauA* could express potential adherence determinants enhancing the ability to cause clinical disease (Almeida et al., 2015; Hossain et al., 2015). The high prevalence of such genes was in agreement with a previous study (Fessia et al., 2019), demonstrating that they may confer *Strep. uberis* an advantage in survival and colonization in nutritionally limited environments (Moshynskyy et al., 2003; Ward et al., 2003). The *oppF* is similarly important during bacterial growth in milk (Smith et al., 2002) because of its role in the acquisition and utilization of essential amino acids from milk. The *has* operon (*hasABC*), conferring resistance to phagocytosis, may play a role in the development of IMI (Parin et al., 2017), although not strictly required in the onset of infection (Ward et al., 2009).

The genetic similarity of *Strep. uberis* strains within the herd was confirmed by RAPD-PCR, a rapid and inexpensive tool to discriminate individual strains of *Strep. uberis* (Tomazi et al., 2019). In our study, RAPD-PCR analysis identified closely related *Strep. uberis* strains in different cows. The use of the primer ERIC1 detected two major clusters (I and II) and grouped most (73.3%) of the strains in cluster I, similar to what was reported in a previous paper (Zadoks et al., 2003). Despite the high level of genetic relatedness, the remaining 26.7% of *Strep. uberis* strains were grouped in a minor genotypic cluster or did not belong to any cluster.

The clear prevalence of a single RAPD cluster might suggest the predominance of a contagious behavior (Leelahapongsathon et al., 2020), although an environmental transmission should not be

ruled out (Wente et al., 2019). The minor cluster might group the strains coming from extramammary sites (Reyes et al., 2019), including manure and bedding materials (Lopez-Benavides et al., 2007; Ericsson et al., 2009), with less opportunities to be transmitted from cow to cow. Overall, the spreading of *Strep. uberis* infections might originate from the exposure of the teat to the bovine reservoir, as well as to a common environmental source, because of the ability of *Strep. uberis* to persist in various spots of the dairy environment (Zadoks et al., 2005). The coexistence of these two modes of transmission might be the reason for the high number of *Strep. uberis* CM cases within the studied herd, as recently suggested (Wald et al., 2020). Control measures focused on the improvement of environmental hygiene (Krömker et al., 2014) may not be sufficient and different management decisions can be recommended on each farm (Wente et al., 2019; Wald et al., 2020). Accordingly, the identification of strains likely to be responsible for cow-to-cow spread within a herd might help with the prevention strategies for the control of *Strep. uberis* mastitis (Phuektes et al., 2001).

The *Strep. uberis* strains belonging to the same RAPD pattern differed in their resistance profiles, as previously found by Tomazi and colleagues (2019).

The most common (81.7%) resistance phenotype was for lincosamides (lincomycin) and tetracyclines. In line with previous findings (Tian et al., 2019), we observed a high (85.9%) resistance rate and MIC_{90} of *Strep. uberis* for tetracycline, although formulations containing tetracycline are not used to treat CM in Italy, but rather for other cattle diseases and in different food-production animals (Gajda et al., 2012; Saini et al., 2012). The resulting increase of tetracycline resistance in *Strep. uberis* from IMI might reflect the environmental nature of this pathogen. Here, most isolates (93%) showed high MIC_{90} values and resistance to lincosamides, in agreement with other European data (Haenni et al., 2010a; Rato et al., 2013). The lincosamide resistance mechanism can be due to mobile genetic elements (MGEs). Among them, multiple integrative and conjugative elements were identified as carriers of resistance determinants, leading to cross-resistance to lincosamides and macrolides (Haenni et al., 2011; Ruegg et al., 2015). Of the *Strep. uberis* isolates resistant to lincosamides, only six were resistant also to erythromycin, a representative of macrolides. These results, similarly to

those reported in Poland (Kaczorek, et al., 2017), showed that erythromycin resistance rate was lower than in other European countries (Thomas et al., 2015; de Jong et al., 2018). Our data suggest that macrolides, the third-line antimicrobials recommended for the treatment of *Strep. uberis* infections (Käppeli et al., 2019), can be used in the herd, as most of the *Strep. uberis* strains displayed an L-phenotype (phenotypic resistance to lincosamides coupled with sensitivity to macrolides), according to Haenni and collaborators (2011).

Overall, we found 25.4% of the *Strep. uberis* isolates had a multidrug resistance phenotype. On the contrary, Tian and collaborators (2019) by using the Kirby Bauer disc diffusion test, reported a higher percentage (100%) of multiresistant streptococci in China. These discrepancies in antimicrobial resistance among countries could be due to the treatment of bovine IMI with different antimicrobials in diverse geographical areas (Tomazi et al., 2019), but also to the use of diverse susceptibility tests and interpretive criteria for phenotypic results (Entorf et al., 2016). It is difficult to accurately assess the level of antimicrobial resistance in mastitis pathogens using only clinical cut-off values, often adopted from other animal species, other groups of bacteria, or human medicine standards (McDougall et al., 2014; Entorf et al., 2016; Kaczorek et al., 2017). For this reason, the comparison of the MIC_{50} and MIC_{90} could provide useful information about the level of resistance, and it should always be performed when antimicrobial susceptibility and resistance data are analyzed. In our study, a good example of that is provided by the results of penicillin. This antimicrobial, considered as the most effective drug for treatment of *Strep. uberis* IMI (Haenni et al., 2010b; Käppeli et al., 2019), showed the highest resistance rate (15.5%) among β-lactams, but it had the same MIC_{90} of ampicillin and amoxicillin, which displayed a much lower resistance rate (1.4%). A misclassification of intermediate isolates to penicillin, due to the clinical breakpoints adopted, could probably be the source of this difference between the rate of resistance and the value of MIC_{90} for these antimicrobials, which share the same mechanisms of antimicrobial resistance against Streptococci.

We found low levels of resistance also to cephalosporins, with only one *Strep. uberis* isolate displaying the widest resistance profile (six antimicrobial classes). This pattern of resistance to all β-

lactams, including cephalosporins, could be associated with amino-acid substitutions in one or more of the penicillin-binding proteins (PBPs) encoded by *pbp* genes, whose variants might reduce the clinical efficacy of different β-lactams (McDougall et al., 2020).

Materials and Methods

Herd

The herd is located in Lombardy (Northern Italy) and during the study it consisted of approximately 1400 lactating Holstein Friesian cows housed in free stalls, with an average daily milk production of 36 kg/cow and a bulk tank SCC of 268,000 cells/mL. Bedding in all lactating groups was chopped straw except in the groups of fresh and dry cows, where fresh-cut straw and rice husks were used. The feed consisted of a total mixed ration (TMR) of corn silage, alfalfa haylage, wheat straw, corn grain and protein mix. Cows were milked 3 times daily in 3 different parallel parlors (13+13). The farm is not under a DHIA (Dairy Herd Improvement Association) control program.

Sample Collection

From November of 2019 to February of 2020, a total of 422 milk samples were recovered from a quarter of clinical cases. Milk samples were collected by farm personnel trained to detect CM based on visibly abnormal milk (color, clots, blood) or udder changes (redness, heat, swelling, or pain) (Wenz et al., 2001; Ruegg, 2011). After disinfection of teat ends and discarding the first streams of fore-milk, milk was collected in 10 mL sterile vials labeled with the cow number and quarter. Milk samples were immediately stored at −20 °C and were shipped weekly to the Dipartimento di Medicina Veterinaria (DiMeVet) at the University of Milan.

Bacteriological Culture and MALDI-TOF Confirmation

For bacteriological analysis, 100 µL of milk was plated onto blood agar plates containing 5% defibrinated sheep blood (Microbiol, Cagliari, Italy). Plates were incubated aerobically at 37°C and evaluated after 24 and 48 h. Bacteria were identified according to the National Mastitis Council guidelines (NMC, 2017). Catalase-negative and Gram-positive cocci were identified as Streptococci, and species were differentiated by further biochemical tests (growth in 6.5% NaCl broth, esculin hydrolysis, fermentation of ribose, sorbitol, sucrose, and inulin). A total of 76 isolates from quarter milk samples collected at the first CM case from 76 different cows were identified as *Strep. uberis*. Matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) was used to confirm the identification of isolates as *Strep. uberis* at the DiMeVet microbiology laboratory. Isolates were freshly cultured on blood agar plates and cell material from an isolated colony was deposited on the target plate using a toothpick. Samples were overlaid with 1 µL of α-cyano-4-hydroxycinnamic acid in 50% acetonitrile with 2.5% trifluoroacetic acid (Bruker Daltonik GmbH, Bremen, Germany). The spectra were acquired with a microFlex™ mass spectrometer (Bruker Daltonik GmbH) in the positive mode. Bacterial Test Standard (Bruker Daltonik GmbH) was used for Instrument Calibration. Spectra were automatically interpreted by the database MBT Compass® 4.1. A log (score) ≥ 1.7 was the threshold for the genus level identification and a log (score) ≥ 2.0 was the threshold for the species level identification. Out of the original 76 isolates, 1 isolate did not grow and another isolate was classified as *Streptococcus* spp. at the genus level with MALDI score <2.0, while the other 3 were identified as *Streptococcus dysgalactiae*. The remaining 71 strains were confirmed as *Strep. uberis* and were included in the study.

Molecular Characterization

DNA was extracted from the 71 confirmed *Strep. uberis* isolates following the protocol described by Cremonesi and collaborators (2006). After concentration and quality determination with a NanoDrop ND-1000, the DNA was amplified via mPCR to verify the presence of 4 housekeeping genes (*cpn60, gapC, sodA* and *tuf*) and to determine the occurrence of 6 genes related to virulence (*hasA, hasB, hasC, oppF, pauA, sua*), according to Calonzi and collaborators (2020). All the isolates were further characterized by a standard PCR assay to investigate *lbp* (lbp-FOR: 5′-GAGGCTGGCAACAAAGAACT-3′; lbp-REV: 5′-GCTTGTGCTTGGTTGTTTTG-3′). The in silico specificity was checked by using the BLAST software tool (https://blast.ncbi.nlm.nih.gov/Blast .cgi; accessed on 1 November 2019). The primers were synthesized by Thermo Fisher Scientific. The following cycling conditions were used 94 °C for 5 min, followed by 30 cycles of 94 °C for 1 min, 56 °C for 1 min and 72 °C for 1 min, and final extension of 72 °C for 7 min. The 25-µL reaction sample contained 12.5 µL of PCR Master Mix 2× (Thermo Fisher Scientific Waltham, MA, United States), 10.1 µL of Nuclease-free water, and 0.2 µL of each primer (100 µ*M*) and 2 µL of genomic DNA (5 ng/µL). Ten microliters of PCR product was electrophoresed on 2% agarose gel added with ethidium bromide (0.05 mg/mL; Sigma Aldrich, Milan, Italy). A 100 bp DNA ladder (Finnzymes, Espoo, Finland) was included in each gel. The results were visualized on an UV transilluminator (BioView Ltd., Nes Ziona, Israel). The positive control used in this study was *Strep. uberis* ATCC 9927 strain.

All the isolates were typed by RAPD-PCR analysis performed with primer ERIC1 (5'-ATGTAAGCTCCTGGGGATTCAC-3'). Amplification conditions, electrophoresis, and analysis of the amplification products were the same as those described by Schmitt-Van de Leemput and Zadoks (2007).

Cluster Analysis

Grouping of the RAPD-PCR profiles was carried out using the BioNumeric 5.1 software package (Applied Maths, Sint-Martens-Latem, Belgium). The resulting dendrogram was created by the unweighted pair group method with arithmetic averages (UPGMA) cluster analysis; strains sharing the same number and the same size of PCR bands were considered genetically identical, while any relationship >90% and <100%, was defined as closely related.

Antimicrobial Susceptibility Testing

The Minimum Inhibitory Concentration (MIC) of 14 antimicrobials was determined using the broth dilution test according to the procedure described in the Clinical and Laboratory Standards Institute (CLSI) guidelines VET015th edition (CLSI, 2018b). MIC was evaluated with a customized commercial microdilution MIC system (Micronaut-S MIC Mastitis, Merlin Diagnostika, GmbH, Bornheim, Germany) used for routine laboratory testing of mastitis isolates. The MIC value of each isolate, expressed as μg/mL, was defined as the lowest concentration of the antimicrobial agent that completely inhibited the growth after the incubation period. *Streptococcus pneumoniae* ATCC 49619 was used as a quality control strain in each MIC batch, according to the reference values provided by CLSI VET08 guidelines, and a double-positive control was used for each plate. The antimicrobials were selected based on their activity against dairy cattle pathogens and on their registrations. The selected antimicrobials included 8 β-lactams (penicillin, ampicillin, oxacillin, amoxicillin plus clavulanate, cefazoline, ceftiofur, cefoperazone, cefquinome), enrofloxacin, erythromycin, florfenicol, lincomycin, tetracycline, and trimethoprim plus sulphametoxazole. Results were interpreted using available CLSI resistance breakpoints according to VET08 4th edition guidelines (CLSI, 2018a), the Comitè de l'Antibiogramme de la Sociètè Française de Microbiologie guidelines (CASFM, 2019), the European Committee on Antimicrobial Susceptibility Testing guidelines

(EUCAST, 2020) and the breakpoints reported in the literature (Lang et al., 2002; Feβler et al., 2012), when specific standards were not established by any international recognized guidelines. The criteria used for the selections of the breakpoints were cattle, when available, human and other animal species breakpoints. The isolates with an intermediate MIC were classified as resistant. The MIC_{50} and MIC_{90} was calculated for each antimicrobial. MIC plate reading was performed manually and the last concentration of antimicrobial that did not show turbidity or a deposit of cells at the bottom of the well was recorded.

Statistical Analysis

Statistical analyses were performed using SPSS 27.0 Statistics for Windows (IBM, Armonk, NY, USA). Descriptive statistics of MIC values in different clusters were expressed as a mean±SD. Normality of MIC value data distribution was assessed by the Shapiro–Wilk test. Since data were not normally distributed, the comparison of MIC values in different clusters was assessed using a nonparametric test (U Mann–Whitney) for 2 independent samples. p values < 0.05 were considered statistically significant.

Conclusions

In conclusion, *Strep. uberis* isolated from CM carried a combination of virulence genes, that might be linked to strains with a greater probability of causing clinical infections. The RAPD-PCR analysis carried out with ERIC1 showed a high frequency of closely related strains, whose occurrence might suggest their contagious nature. This study indicated that this molecular method can be a useful tool for investigating *Strep. uberis* mastitis. Even though the RAPD types obtained cannot be compared with those reported in other studies, this technique can provide insights about the epidemiology of *Strep. uberis* within the single dairy farm and help the understanding of clinical cases associated with

this pathogen at the herd level. Some differences in the MIC values were found between clusters. AMR was widespread and multidrug resistant isolates were present but not prevalent. Our study underlines the need to consider MIC_{50} and MIC_{90} values when making farm management decisions because of the lack of breakpoints specific for mastitis pathogens. Genotypic and AMR results supported the concept that few *Strep uberis* isolates might be prevalent in the dairy herd; strategies aimed to control contagious mastitis may be useful to reduce *Strep. uberis* spreading within the farm. Surveillance data can be meaningful for practical management and helpful for the identification of the most appropriate antibiotic agents.

Supplementary Materials

The following are available online at https://www.mdpi.com/article/10.3390/antibiotics10060644/s1, Table S1: Descriptive statistics of MIC values for the 14 antimicrobial agents and comparison of their distribution in the two genotypic clusters.

Author Contributions

Conceptualization, V.M., P.M., P.C., L.B.A., D.C.K.L. and A.B.; methodology, V.M., B.C., and C.L.; software, S.M., V.B. and A.B.; validation, V.M., C.L., and P.C.; formal analysis, A.B., S.M., and V.B.; investigation, C.L., V.M.; resources, P.M., P.C., and B.C.; data curation, V.M., C.L., A.B. and S.M.; writing—original draft preparation, M.F.A., V.M., P.C., and R.P.; writing—review and editing, M.F.A., V.M., P.C., and R.P.; funding acquisition, P.M. All authors have read and agreed to the published version of the manuscript.

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Conflict of Interest

The authors declare no conflict of interest.

References

Almeida, R.A., O.K. Dego, S.I. Headrick, M.J. Lewis, and S.P. Oliver. 2015. Role of *Streptococcus uberis* adhesion molecule in the pathogenesis of *Streptococcus uberis* mastitis. Vet Microbiol. 179(3-4):332-5. https://doi.org/10.1016/j.vetmic.2015.07.005.

Boonyayatra, S., P. Tharavichitkul, and S.P. Oliver. 2018. Virulence-associated genes and molecular typing of *Streptococcus uberis* associated with bovine mastitis in northern Thailand. Turk. J. Vet. Anim. Sci. 42:73–81. https://doi.org/10.3906/vet-1704-75.

Botrel, M.A., M. Haenni, E. Morignat, P. Sulpice, J.Y. Madec, and D. Calavas. 2010. Distribution and antimicrobial resistance of clinical and subclinical mastitis pathogens in dairy cows in Rhône-Alpes, France. Foodborne Pathog Dis 7:479–487. https://doi.org/10.1089/fpd.2009.0425.

Bradley, A.J., K.A. Leach, J.E. Breen, L.E. Green, and M.J. Green. 2007. Survey of the incidence and aetiology of mastitis on dairy farms in England and Wales. Vet Rec. 160(8):253–258.

Calonzi, D., A. Romano, V. Monistero, P. Moroni, M.V. Luini, F. Biscarini, B. Castiglioni, and P. Cremonesi. 2020. Technical note: Development of multiplex PCR assays for the molecular characterization of *Streptococcus uberis* strains isolated from bovine mastitis. J Dairy Sci. 103(1):915-921. https://doi.org/10.3168/jds.2019-16823.

CASFM. 2019. Comité de l'antibiogramme de la société française de microbiologie. Groupe de travail antibiogramme vétérinaire. Recommandations vétérinaires 2019. SFM 36 avenue Jean Moulin 75014 Paris.

CLSI. 2018a. Performance Standards for Antimicrobial Disk and Dilution Susceptibility Tests for Bacteria Isolated from Animals. 4th ed. CLSI supplement VET 08. Clinical and Laboratory Standards Institute, Wayne, PA.

CLSI. 2018b. Performance Standards for Antimicrobial Disk and Dilution Susceptibility Tests for Bacteria Isolated from Animals. 5th Ed. CLSI standard Vet 01. Clinical and Laboratory Standards Institute, Wayne, PA.

Cremonesi, P., B. Castiglioni, G. Malferrari, I. Biunno, C. Vimercati, P. Moroni, S. Morandi, and M. Luzzana. 2006. Technical Note: Improved method for rapid DNA extraction of mastitis pathogens directly from milk. Journal of Dairy Science. 89:163–169. https://doi.org/10 .3168/jds.S0022-0302(06)72080-X.

Davies, P.L., J.A. Leigh, A.J. Bradley, S.C. Archer, R.D. Emes, and M.J. Green. 2016. Molecular epidemiology of *Streptococcus uberis* clinical mastitis in dairy herds: strain heterogeneity and transmission. J Clin Microbiol. 54(1):68-74. https://doi.org/10.1128/JCM.01583-15.

de Jong, A., F.E. Garch, S. Simjee, H. Moyaert, M. Rose, M. Youala, E. Siegwart, and VetPath Study Group. 2018. Monitoring of antimicrobial susceptibility of udder pathogens recovered from cases of clinical mastitis in dairy cows across Europe: VetPath results. Vet Microbiol. 213:73-81. https://doi.org/10.1016/j.vetmic.2017.11.021.

Entorf, M., A.T. Feßler, H. Kaspar, K. Kadlec, T. Peters, and S. Schwarz. 2016. Comparative erythromycin and tylosin susceptibility testing of streptococci from bovine mastitis. Vet Microbiol. 194:36-42. https://doi.org/10.1016/j.vetmic.2015.12.003.

Ericsson Unnerstad, H., A. Lindberg, K. Persson Waller K, T. Ekman, K. Artursson, M. Nilsson-Ost, and B. Bengtsson. 2009. Microbial aetiology of acute clinical mastitis and agent-specific risk factors. Vet Microbiol. 137:90–97. https://doi.org/10.1016/j.vetmic.2008.12.005.

EUCAST. 2020. European Committee on Antimicrobial Susceptibility Testing. Breakpoint tables for interpretation of MICs and zone diameters. Version 10.0. http://www.eucast.org.

Fang, W., and S.P. Oliver. 1999. Identification of lactoferrin-binding proteins in bovine mastitis-causing *Streptococcus uberis*. FEMS Microbiol. Lett. 176(1);91–96. https://doi.org/10.1111/j.1574-6968.1999.tb13647.x.

Fessia A.S., S.A. Diesera, C.G. Raspantia, and L.M. Odierno. 2019. Genotyping and study of adherence-related genes of *Streptococcus uberis* isolates from bovine mastitis. Microb Pathog. 130:295-301. https://doi.org/10.1016/j.micpath.2019.03.027.

Feβler A. T., H. Kaspar, C. J. Lindeman, M. R. Stegemann, T. Peters, J. Mankertz, J. L. Watts, and S. Schwarz. 2012. A proposal of interpretive criteria for cefoperazone applicable to bovine mastitis pathogens. Vet. Microbiol. 157:226-231. https://doi.org/10.1016/j.vetmic.2011.11.032.

Gajda, A., A. Posyniak, J. Żmudzki, and H. Rożańska. 2012. Occurrence of tetracyclines in tissues and food of animal origin: causes and consequences. Med. Weter. 68:650–655.

Gilbert, F.B., A. Fromageau, J. Lamoureux, and B. Poutrel. 2006. Evaluation of tandem repeats for MLVA typing of *Streptococcus uberis* isolated from bovine mastitis. BMC Vet Res. 2:33. https://doi.org/10.1186/1746-6148-2-33.

Gilchrist, T.L., D.G. Smith, J.L. Fitzpatrick, R.N. Zadoks, and M.C. Fontaine. 2013. Comparative molecular analysis of ovine and bovine *Streptococcus uberis* isolates. J Dairy Sci. 96(2):962-70. https://doi.org/10.3168/jds.2012-5705.

Haenni, M., E. Saras, S. Bertin, P. Leblond, J.Y. Madec, and S. Payot. 2010a. Diversity and mobility of integrative and conjugative elements in bovine isolates of *Streptococcus agalactiae*, *S. dysgalactiae* subsp. *dysgalactiae*, and *S. uberis*. Appl Environ Microbiol. 76(24):7957-65. https://doi.org/10.1128/AEM.00805-10.

Haenni, M., L. Galofaro, M. Ythier, M. Giddey, P. Majcherczyk, P. Moreillon, and J.Y. Madec. 2010b. Penicillin-binding protein gene alterations in *Streptococcus uberis* isolates presenting decreased susceptibility to penicillin. Antimicrob Agents Chemother. 54(3): 1140–1145. https://doi.org/10.1128/AAC.00915-09.

Haenni, M., E. Saras, S. Chaussière, M. Treilles, and J.Y. Madec. 2011. *ermB*-mediated erythromycin resistance in *Streptococcus uberis* from bovine mastitis. Vet J. 189(3):356-8. https://doi.org/10.1016/j.tvjl.2010.06.021.

Hossain, M., S.A. Egan, T. Coffey. P.N. Ward, R. Wilson, J.A. Leigh, and R.D. Emes. 2015. Virulence related sequences; insights provided by comparative genomics of *Streptococcus uberis* of differing virulence. BMC Genomics. 16:334. https://doi.org/10.1186/s12864-015-1512-6.

Kaczorek, E., J. Małaczewska, R. Wójcik, W. Rękawek, and A.K. Siwicki. 2017. Phenotypic and genotypic antimicrobial susceptibility pattern of *Streptococcus* spp. isolated from cases of clinical mastitis in dairy cattle in Poland. J Dairy Sci. 100(8):6442-6453. https://doi.org/10.3168/jds.2017-12660.

Käppeli, N., M. Morach, K. Zurfluh, S. Corti, M. Nüesch-Inderbinen, and R. Stephan. 2019. Sequence types and antimicrobial resistance profiles of *Streptococcus uberis* isolated from ovine mastitis. Front Vet Sci. 6:234. https://doi.org/10.3389/fvets.2019.00234.

Krömker, V., F. Reinecke, J.H. Paduch, and N. Grabowski. 2014. Bovine *Streptococcus uberis* intramammary infections and mastitis. Clin Microbial. 3:157. https://doi.org/10.4172/2327-5073.1000157.

Lang I., M. Rose, E. Thomas, and E. Zschiesche. 2002. A field study of cefquinome for the treatment of pigs with respiratory disease. Revue Méd. Vét. 153:575-580.

Leelahapongsathon, K., Y.H. Schukken, A. Srithanasuwan, and W. Suriyasathaporn. 2020. Molecular epidemiology of *Streptococcus uberis* intramammary infections: Persistent and transient patterns of infection in a dairy herd. J Dairy Sci. 103(4):3565-3576. https://doi.org/10 .3168/jds.2019-17281.

Lopez-Benavides, M.G., J.H. Williamson, G.D. Pullinger, S.J. Lacy-Hulbert, R.T. Cursons, and J.A. Leigh. 2007. Field observations on the variation of *Streptococcus uberis* populations in a pasture-based dairy farm. J Dairy Sci. 90:5558–5566. https://doi.org/10.3168/jds.2007-0194.

McDougall, S., H. Hussein, and K. Petrovski. 2014. Antimicrobial resistance in *Staphylococcus aureus*, *Streptococcus uberis* and *Streptococcus dysgalactiae* from dairy cows with mastitis. N. Z. Vet. J. 62:68–76. https://doi.org/10.1080/00480169.2013.843135.

McDougall, S., L. Clausen, H.J. Ha, I. Gibson, M. Bryan, N. Hadjirin, E. Lay, C. Raisen, X. Ba, O. Restif, J. Parkhill, and M.A. Holmes. 2020. Mechanisms of β-lactam resistance of *Streptococcus uberis* isolated from bovine mastitis cases. Vet Microbiol. 242:108592. https://doi.org/10.1016/j.vetmic.2020.108592.

Moshynskyy, I., M. Jiang, M.C. Fontaine, J. Perez-Casal, L.A. Babiuk, and A.A. Potter. 2003. Characterization of a bovine lactoferrin binding protein of *Streptococcus uberis*. Microb. Pathog. 35: 203–215. https://doi.org/10.1016/s0882-4010(03)00150-5.

National Mastitis Council. 2017. NMC Laboratory handbook on bovine mastitis – Third edition. National Mastitis Council, New Prague, Minnesota, USA.

Parin, U., S. Kirkan, E. Cicek, and H. T. Yuksel. 2017. Detection of virulence genes in *Streptococcus uberis* isolated from bovine mastitis in Aydin province by multiplex polymerase chain reaction. F.Ü. Sağ. Bil. Vet. Derg. 31(3):213–219.

Phuektes, P., P.D. Mansell, R.S. Dyson, N.D. Hooper, J.S. Dick, and G.F. Browning. 2001. Molecular epidemiology of *Streptococcus uberis* isolates from dairy cows with mastitis. J Clin Microbiol. 39: 1460–6. https://doi.org/10.1128/JCM.39.4.1460-1466.2001.

Pol, M., and P.L. Ruegg. 2007. Treatment practices and quantification of antimicrobial drug usage in conventional and organic dairy farms in Wisconsin. J Dairy Sci. 90: 249–261. https://doi.org/10.3168/jds.S0022-0302(07)72626-7.

Poutrel, B., S. Bareille, G. Lequeux, and F. Leboeuf. 2018. Prevalence of mastitis pathogens in France: Antimicrobial susceptibility of *Staphylococcus aureus*, *Streptococcus uberis* and *Escherichia coli*. J. Vet. Sci. Technol. 9:522. https://doi.org/10.4172/2157-7579.1000522.

Rato, M.G., R. Bexiga, C. Florindo, L.M. Cavaco, C.L. Vilela CL, and I. Santos-Sanches. 2013. Antimicrobial resistance and molecular epidemiology of streptococci from bovine mastitis. Vet Microbiol. 161:286–294. https://doi.org/10.1016/j.vetmic.2012.07.043.

Reyes, J., J.C. Rodriguez-Lecompte, A. Blanchard, J.T. McClure, and J. Sánchez. 2019. Molecular variability of *Streptococcus uberis* isolates from intramammary infections in Canadian dairy farms from the Maritime region. Can J Vet Res. 83(3):168-176.

Ruegg, P.L. 2011. Managing mastitis and producing quality milk. Pages 207-232 in Dairy Production Medicine. Risco, C. A. and Melendez P. R., editors. West Sussex (Shire) John Wi-ley & Sons.

Ruegg, P. L., L. Oliveira, W. Jin, and O. Okwumabua. 2015. Phenotypic antimicrobial susceptibility and occurrence of selected resistance genes in gram-positive mastitis pathogens isolated from Wisconsin dairy cows. J. Dairy Sci. 98:4521–4534. https://doi.org/10.3168/jds.2014-9137.

Saini, V., J.T. McClure, D. Léger, S. Dufour, A.G. Sheldon, D.T. Scholl, and H.W. Barkema. 2012. Antimicrobial use on Canadian dairy farms. J Dairy Sci. 95: 1209–1221. https://doi.org/10 .3168/jds.2011-4527.

Schmitt-Van de Leemput, E., and R.N. Zadoks. 2007. Genotypic and phenotypic detection of macrolide and lincosamide resistance in *Streptococcus uberis*. J Dairy Sci. 90(11):5089-96. https://doi.org/10.3168/jds.2007-0101.

Smith, A.J., A.J. Kitt, P.N. Ward, and J.A. Leigh. 2002. Isolation and characterization of a mutant strain of *Streptococcus uberis*, which fails to utilize a plasmin derived beta-casein peptide for the acquisition of methionine. J Appl Microbiol. 93(4):631–639.

Thomas, V., A. de Jong, H. Moyaert, S. Simjee, F. El Garch, I. Morrissey, H. Marion, and M. Vallé. 2015. Antimicrobial susceptibility monitoring of mastitis pathogens isolated from acute cases of clinical mastitis in dairy cows across Europe: VetPath results. Int. J. Antimicrob. Agents. 46:13–20. https://doi.org/10.1016/j.ijantimicag.2015.03.013.

Tian, X.Y., N. Zheng, R.W. Han, H. Ho, J. Wang, Y.T. Wang, S.Q. Wang, H.G. Li, H.W. Liu, and Z.N. Yu. 2019. Antimicrobial resistance and virulence genes of *Streptococcus* isolated from dairy cows with mastitis in China. Microb Pathog. 131:33-39. https://doi.org/10 .1016/j.micpath.2019.03.035.

Tomazi, T., G. Freu, B.G. Alves, A.F. de Souza Filho, M.B. Heinemann, and M. Veiga Dos Santos. 2019. Genotyping and antimicrobial resistance of *Streptococcus uberis* isolated from bovine clinical mastitis. PLoS One.14(10):e0223719. https://doi.org/10.1371/journal.pone.0223719.

Verbeke, J., S. Piepers, K. Supre, and S. De Vliegher. 2014. Pathogen-specific incidence rate of clinical mastitis in Flemish dairy herds, severity, and association with herd hygiene. J. Dairy Sci. 97:6926–6934. https://doi.org/10.3168/jds.2014-8173.

Wald, R., M. Baumgartner, J. Gutschireiter, B. Bazzanella, K. Lichtmannsperger, M. Wagner, T. Wittek, and B. Stessl. 2020. Comparison of the population structure of *Streptococcus uberis* mastitis isolates from Austrian small-scale dairy farms and a Slovakian large-scale farm. J Dairy Sci. 103(2):1820-1830. https://doi.org/10.3168/jds.2019-16930.

Ward, P.N., T.R. Field, C.D. Rapier, and J.A. Leigh. 2003. The activation of bovine plasminogen by PauA is not required for virulence of *Streptococcus uberis*. Infect Immun 71(12):7193–7196. https://doi.org/10.1128/iai.71.12.7193-7196.2003.

Ward, P.N., M.T. Holden, J.A. Leigh, N. Lennard, A. Bignell, A. Barron, L. Clark, M.A. Quail, J. Woodward, B.G. Barrell, S.A. Egan, T.R. Field, D. Maskell, M. Kehoe, C.G. Dowson, N.

Chanter, A.M. Whatmore, S.D. Bentley, and J. Parkhill. 2009. Evidence for niche adaptation in the genome of the bovine pathogen *Streptococcus uberis*. BMC Genomics. 10:54. https://doi.org/10.1186/1471-2164-10-54.

Wente, N., D. Klocke, J.H. Paduch, Y. Zhang, M.T. Seeth, V. Zoche-Golob, F. Reinecke, E. Mohr, and V. Krömker. 2019. Associations between *Streptococcus uberis* strains from the animal environment and clinical bovine mastitis cases. J Dairy Sci. 102(10):9360-9369. https://doi.org/10.3168/jds.2019-16669.

Wenz, J. R., G. M. Barrington, F. B. Garry, R. P. Dinsmore, and R. J. Callan. 2001. Use of systemic disease signs to assess disease severity in dairy cows with acute coliform mastitis. J Am VetMed Assoc. 218(4):567–72. https://doi.org/10.2460/javma.2001.218.567.

Zadoks, R.N., B.E. Gillespie, H.W. Barkema, O.C. Sampimon, S.P. Oliver, Y.H. Schukken. 2003. Clinical, epidemiological and molecular characteristics of *Streptococcus uberis* infections in dairy herds. Epidemiol Infect. 130: 335–349. https://doi.org/10.1017/s0950268802008221.

Zadoks, R.N., L.L. Tikofsky, and K.J. Boor KJ. 2005. Ribotyping of *Streptococcus uberis* from a dairy's environment, bovine feces and milk. Vet Microbiol. 109:257–265. https://doi.org/10 .1016/j.vetmic.2005.05.008.

PART III: Other Pathogens and Foodborne Diseases

Dairy cows are considered reservoirs for several mastitis pathogens which can be responsible for the most important human foodborne diseases. The foodborne outbreaks, whose main symptoms include nausea, vomiting, abdominal cramps and diarrhea, can cause significant economic and social losses around the globe. In United States, millions of illnesses, hundreds of thousands of hospitalizations, and thousands of deaths are attributable to food poisoning each year (Scallan et al., 2011). The occurrence of these diseases is strongly linked to the ingestion of contaminated food. In particular, milk is highly nutritious but also highly vulnerable. This product has a neutral pH and represents a suitable medium for survival and growth of several pathogenic and opportunistic microorganisms, including *Listeria monocytogenes, Campylobacter jejuni, Salmonella* spp., *Yersinia enterocolitica, S. aureus,* and enteropathogenic *E. coli* (Paswan and Park, 2020). Nontyphoidal *Salmonella* spp. and *C. jejuni* are important for their widespread prevalence in food animals (Bottieau et al., 2011; Thomas et al., 2020). *Salmonella* spp., as well as *L. monocytogenes,* and *E. coli* O157:H7, is associated with severe infection that results in 93.8 million cases of gastroenteritis and 155,000 worldwide deaths annually (Paswan and Park, 2020).

Serious concerns arise primarily when milk of infected cows is consumed raw or the dairy products are produced and consumed under unsatisfactory hygiene conditions (Ayele et al., 2017; Regasa et al., 2019). Foodborne pathogens can be found in mastitic milk but can also opportunistically contaminate microbiologically safe milk and dairy products at various points of the supply chain. The major sources of contamination are milker's hands, poor hygienic practices during or after milking, unhygienic production and storage processes, food handlers and equipment. The spread of SFP via food handlers is a common and persistent problem worldwide as up to 30-50% of healthy human population carries *S. aureus* within nasal passage (Moroni et al., 2010). As the isolates present in the nose can contaminate hands, the nasal carriers are the main perpetrators of staphylococcal food contamination.

Dairy farm food safety can be maintained by using hygienic standards that can be easily evaluated. The adoption of Hazard Analysis Critical Control Point (HACCP) programs at the farm-level can help limit the microbial contamination of milk, creating a hostile environment for the growth of microorganisms (Ruegg, 2003).

References mentioned in the Part III are reported in the Bibliography.

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Detection Methods

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Abstract

Milk and dairy products could contain a variety of microorganisms, deriving from the environment or from infected udders, becoming an important source of foodborne pathogens. The main microorganisms involved in mastitis are *Staphylococcus aureus, Prototheca* spp., *Mycoplasma* spp. and *Streptococcus* spp., while the principal pathogens responsible for foodborne disease are enterotoxigenic *Staphylococcus aureus, Salmonella* spp., *Listeria monocytogenes, Escherichia coli* VTEC, *Campylobacter* spp. and *Bacillus cereus*. This chapter presents information on characteristics of these pathogens, and illnesses caused by them. An overview in the field of foodborne pathogen identification and detection by using different molecular techniques (multiplex PCR, quantitative PCR, digital PCR and Whole Genome Sequencing) is also reported.

Keywords: *Bacillus cereus*, *Campylobacter* spp., Enterotoxins, *Escherichia coli* VTEC, Foodborne pathogens, *Listeria monocytogenes*, Mastitis, Milk, Molecular detection, *Prototheca* spp., *Salmonella* spp., *Staphylococcus aureus*, *Streptococcus* spp., Virulence genes

Main Pathogens Detected in Milk

Harmful Pathogens for Animal Health

Staphylococcus aureus

Staphylococcus aureus is one of the most important pathogens in veterinary medicine. This species, characterized by pigmented golden-yellow colonies, belongs to the genus *Staphylococcus*, currently comprising more than 50 g-positive bacteria. *S. aureus* is implicated in a large variety of infections in warm-blooded animals, ranging from dermatitis to septicemia. The skin and mucosa of pigs, chickens, sheep, goats and cows represent its principal reservoirs. In dairy ruminants, this microorganism is also recognized as one of the main causes of udder infections, whose primary sources are infected quarters and teat skin.

Although *S. aureus* can survive in the environment and several isolates were previously detected in extramammary sites, it is mainly considered as a contagious pathogen, because the proliferation is strongly related to colonization and invasion of the mammary gland. The transmission occurs between udders during the milking process via the milking machine or the farmer's hands. Especially in dairy cows, *S. aureus* strains are commonly isolated from subclinical intramammary infection (IMI), the most expensive disease in the dairy industry for the important economic impact on milk yield and quality. However, this microorganism can also cause clinical symptoms or chronic mastitis. The severity and the persistence of the outcome are related to the bacterium and, particularly, to its virulence factors, but depend also on the host.

This pathogen has a highly clonal population structure and each clonal lineage carries a particular combination of genes. These genes are classified into a core genome (75%), associated with housekeeping functions common to almost all strains, and an accessory genome (25%), encoding different virulence factors strongly related to the epidemiological and pathogenic properties of the strains; the latter includes core variable genes (10%) and mobile genetic elements (MGEs) (15%). Due to the unique environment of the mammary gland, only specific lineages, characterized by virulence factors relevant to bovine IMI, have adapted to infect cows; conversely, several factors causing diseases in humans are not expressed by isolates of ruminant origin. Bovine *S. aureus* strains can express a wide array of secreted and surface-associated proteins that (1) mediate adherence to extra-cellular matrix and adhesion to host cells and tissues, causing their lysis and destruction, respectively; (2) evade innate and complement-mediated immune responses, including leukocyte migration, phagocytic activity, opsonization, action of immunoglobulins and antimicrobial peptides. The core variable genome comprises genes encoding microbial surface components recognizing adhesive matrix molecules (MSCRAMMs) that are essential to colonize tissue and start the infection. Among them, the fibronectin binding proteins A and B (*fnbpA* and *fnbpB*) cause adhesion between fibronectin and elastin, clumping factors A and B (*clfA* and *clfB*) are fibrin/fibrinogen binding proteins, and collagen-binding adhesin (*cna*) contributes to the adhesion to host's collagen. The successive invasion of host cells involves the expression of the hemolysins. The gene for β-hemolysin (*hlb*) is frequent in bovine isolates as its activity is important in the pathogenesis of mastitis increasing the adherence of bacteria to mammary epithelial cells. The hemolysin gene cluster (*hla, hld, hlg*) is present essentially in all *S. aureus* strains because it belongs to the highly conserved core genome; it plays a primary role in the commensal colonization of mammalian epithelia with their cytolytic and antimicrobial functions. The leukotoxins belong to the bi-component and β-barrel pore-forming toxin family, a group of staphylococcal cytotoxins able to kill mammalian cells. They consist of one class F subunit (HlgB, LukF-PV, LukD, LukF'-PV or LukG) and one class S subunit (HlgA, HlgC, LukS-PV, LukE, LukM, or LukH), whose genes are either core genome- or phage-encoded. Other genes

encoding leukotoxins are lukF-PV + lukS-PV, LukDE, LukGH and LukF'M. The lukF-PV + lukS-PV genes express Panton-Valentine leucocidin (PVL), also known as leukocidin S/F-PV, implicated in mastitis and responsible for leukocyte destruction and tissue necrosis. The lukM-lukF' genes, encoding a PVL variant targeting bovine polymorphonuclear neutrophils (PMNs), are almost exclusively carried by *S. aureus* isolates of ruminant origin. *S. aureus* is also characterized by the expression of a wide array of toxin genes carried by MGEs, such as bacteriophages, plasmids, *S. aureus* pathogenicity islands (SaPI), transposons, staphylococcal chromosomal cassettes (SCC); they can be acquired either by vertical transmission or by horizontal gene transfer. These genes encode enterotoxins that act as Superantigens (SAgs) stimulating a large fraction of T-lymphocytes simultaneously. Although their role in the pathogenesis of bovine mastitis is still unclear, they seem to be involved in the development of IMI. Besides virulence genes, MGEs encode proteins required for antibiotic resistance, such as PBP2a expressed by *mecA* gene, that is included in a mobile staphylococcal cassette chromosome (SCC*mec*) and is responsible for resistance to methicillin. Therefore, staphylococcal mastitis is difficult to treat also because *S. aureus* responds poorly to antimicrobial therapy because of its ability to acquire antimicrobial resistance and to build biofilm. In fact, biofilm formation, mediated by the intercellular adhesion (*ica*) operon grouping the *icaA, icaB, icaC* and *icaD* genes, and by the *bap* gene encoding the biofilm-associated protein, seems to be associated with reduced antimicrobial susceptibility. Moreover, vaccination has not yet been a major prophylactic measure because its efficacy may be strain-specific. As different *S. aureus* strains require separate measures for prevention and treatment, research on their genotypic characteristic and pathogenic properties can be of clinical importance.

The main identification of *S. aureus* is based on the detection of hemolysis and coagulase production. The species is easily distinguished on blood agar, also from coagulase-negative staphylococci (CNS), in which hemolysis is variable and often slow in appearing. Growth on selective media, such as Mannitol salt agar or Baird-Parker agar, can also be used to identify *S. aureus*. This pathogen usually gives positive results in coagulase tube test and in slide agglutination test for clumping factor, that

differs from free coagulase in being cell-bound. This staphylococcal fibrinogen-binding protein can also be detected simultaneously with protein A typically produced by *S. aureus* using another simple slide agglutination test based on latex particles coated with human plasma. On the other hand, *S. aureus* isolates can be rapidly and reliably identified by PCR amplification of the *nuc* gene, which encodes the thermonuclease and it is known to be highly specific for *S. aureus*. Regarding subtyping techniques, *spa* typing is based on DNA sequencing of the variable spacer region of the staphylococcal *spa* gene, while Multilocus Sequence Typing (MLST) requires the sequencing of seven housekeeping genes to discriminate *S. aureus* strains of different clonal complexes (CCs). The CCs mostly detected in isolates from ruminant species are CC8, CC705, CC97, CC479, and CC133, with CC705 being restricted to cows, whereas the CC133 can be found also in sheep and goats.

Mycoplasma spp.

Mycoplasma bovis is the most prevalent and invasive agent of mycoplasma mastitis in dairy cattle, and early detection is critical. *Mycoplasma* mastitis is contagious and affects young and mature animals, and milk productivity. *Mycoplasma* genus belongs to the class Mollicutes, which are characterized by lack of cell wall, low G + C content (23%–40%) and small genome size (0.58–1.4 Mbp). Other *Mycoplasma* have been isolated from milk; *M. alkalescens, M. arginini M. californicum, M. bovigenitalium, M. bovirhinis, M. canadense*, and *Acholeplasma* genus are often isolated.

Mycoplasma spp. are resistant to antibiotic treatment and the role and the pathobiology of these species as mastitis causes are poorly understood. Due to these factors, control the disease and management strategies to reduce spread of bacteria and effect rely on regular bacteriological culture screening of bulk-tank milk or pooled animal milk. When positive cultures are identified, positive animals are culled or segregated (more difficult to handle but a possibility). Bacteriological culture confirmation can take time (6–8 days) and difficult process that can increase the response time of a farm to a *Mycoplasma* outbreak.

Mycoplasma spp. require specific media and CO_2 incubation conditions for isolation and takes time to grow. The molecular diagnostic assay developed a couple of years ago at QMPS (Cornell University) includes a multiplex conventional PCR followed by optional sequencing. Composing the assay are three separate PCR amplifications that use separate amplification mixes but run concurrently in the same thermocycler under the same running conditions. The first assay amplifies *M. bovis uvrC* (*uvrC*) and *Mycoplasma* and *Acholeplasma* 16S rDNA (16S) gene targets and incorporates forward and reverse primer sets. A second assay amplifies the *Mycoplasma* 16S to 23S rDNA intergenic transcribed spacer region (MITS), and a third assay amplifies the *Acholeplasma* 16S to 23S rDNA intergenic transcribed spacer region (AITS). Amplification of a combination of two or more targets is used to identify a specimen DNA as *M. bovis* (*uvrC*,16S, MITS positive; AITS negative), *Mycoplasma* (not *M. bovis*; 16S, MITS positive; *uvrC*, AITS negative), *Acholeplasma* (16S, AITS positive; *uvrC*, MITS negative), or not a *Mycoplasma* (negative 16S, MITS; negative *uvrC*, 16S, MITS; negative 16S, AITS; any single gene target alone positive or all gene targets negative). The *uvrC* gene has been demonstrated to be a specific PCR target for *M. bovis*, with no cross-amplification with non-*M. bovis*. The *uvrC* gene encodes deoxyribodipyrimidine photolyase, an enzyme, which is essential for replication as it is involved with DNA repair, making it a highly stable gene. It is a well conserved but significantly different gene in both *M. bovis* and *M. agalactiae* making it a much more specific target gene than 16S rRNA; however recent studies have since identified point mutations in the *M. bovis uvrC* gene. Validation of the *uvrC* gene as a target for identifying *M. bovis* using real time PCR has been demonstrated on clinical samples from the lung, milk, joint fluid, nasal swabs, bronchoalveolar lavage fluid, tracheal wash fluid, and genes encode an oligopeptide permease and are a member of the ABC-transporter family. The use of this gene for detecting *M. bovis* has been validated in milk samples and nasal swabs, with a limit of detection as low as 1×10^2 CFU mL^{-1} in milk.

Prototheca spp.

Prototheca spp. is algae assigned to the genus *Prototheca*, family Chlorellaceae. These species are ubiquitous in nature, living predominantly in aqueous environments containing decomposing plant material. Within the known *Prototheca* spp., only *P. zopfii*, *P. wickerhamii* and *P. blaschkeae* have been associated with disease in humans and animals. In the past, the genus *Prothoteca* was considered a rare pathogen in dairy cattle and associated with infection in the presence of predisposing factors, such as poor environmental conditions and insufficient milking hygiene; however, cases of clinical and chronic mastitis are increasingly recognized as endemic worldwide. Bovine IMIs are most frequently caused by *P. zopfii* infection, whereas *P. wickerhamii* infection is rarely seen. Almost all *Prototheca* isolates from bovine milk in China, Germany, Italy, Portugal, Poland, and Japan were *P. zopfii* genotype 2, suggesting that it is the principal causative agent. However, *P. blaschkeae* in bovine mastitis was also detected. Recently, based on the 18S rDNA sequence analysis, *P. zopfii* has been divided into three genotypes (1–3), of which *P. zopfii* genotype 3 has been given the status of a new species *Prototheca blaschkeae* sp. nov.

The sequences of *Prototheca* species currently available in public databases are those of the 18S rDNA (small subunit of rDNA, SSU) and 28S rDNA (large subunit of rDNA, LSU), and those of the Internal Transcribed Spacer regions (ITS), as well as some mitochondrial and plastid genomes. The molecular markers, mostly located in the ribosomal DNA (rDNA) cluster, do not provide sufficient discriminatory power to distinguish among all *Prototheca* spp. Currently it has been recognized that mitochondrial *cytb* gene as a new and robust marker can be used for diagnostics and phylogenetic studies of the *Prototheca* algae. The *cytb* gene displayed important advantages over the rDNA markers; in fact, it had the highest discriminatory capacity for resolving all *Prototheca* species, but it also performed best in terms of technical feasibility, understood as ease of amplification, sequencing, and multiple alignment analysis.

Diagnosis of *Prototheca* spp. mastitis is typically based on morphological characteristics on conventional culture media, such as 5% sheep blood agar, and MacConkey and Sabouraud dextrose agars. On these media, the microorganism grows requiring two-three-day incubation period at 37 °C, showing small gray colonies of around 1 mm in diameter, without hemolysis (MacConkey) or visible, white to cream-colored colonies (Sabouraud dextrose agar). Specialized Prototheca Isolation Media (PIM) has been shown to improve diagnosis of *Prototheca* identification. Wet mounts and smears stained with Gram or methylene blue can quickly confirm the diagnosis.

Prototheca spp. is often ubiquitous in the farm environment making it difficult to demonstrate a causal environmental source. In many outbreak investigations no environmental source can be identified. There are no known effective or approved therapies for *Prototheca* mastitis. Since most infections become chronic with periodic shedding of infective organisms, recommended management of infected cows includes segregation and culling of culture positive animals. Clinical signs of *Prototheca* spp. infection range from watery appearance of milk to palpable swelling, edema and firmness of the affected quarters. Once the organisms have gained access to the mammary gland *Prototheca* spp. invade macrophages and udder tissue creating a chronic granulomatous lesion.

In veterinary diagnostics, *Prototheca* spp. isolates are currently characterized using molecular methods. The rDNA sequencing has been used for molecular species identification; more rapid methods, such as genotype-specific PCR or restriction fragment length polymorphism (RFLP) analysis, distinguish *P. blaschkeae* and *P. zopfii* but are unable to differentiate among the other *Prototheca* species. The 2-step real-time quantitative PCR (qPCR) reaction followed by DNA resolution melting analysis distinguishes, during the first step after 48 h of cell growth, *P. zopfii* genotype 1, *P. zopfii* genotype 2, and *P. blaschkeae*, while at the second step *P. ulmea*, *P. stagnora*, and *P. wickerhamii*. Moreover, single-stranded conformation polymorphism (SSCP) PCR is also discriminative and highly suitable for the identification of *P. zopfii* genotype 2 in field isolates directly in milk, if preceded by a specific DNA extraction method. Finally, some PCR assays, based on ITS1

and ITS2 amplification resulting in amplicons differing in size species-dependent manner, are useful for differentiation within *Prototheca* spp. but focused on *P. wickerhamii*.

Streptococcus spp.

The streptococci comprise an important group of pathogens and some of them are important etiological agents in dairy cows IMIs. Of those, the more prevalent are *Streptococcus uberis*, *Str. dysgalactiae* subsp. *dysgalactiae*, and *Str. agalactiae*. Due to the application of mastitis control programs, a dramatic decrease in clinical mastitis incidence has been achieved, but this has been accompanied by a change in the relative and absolute importance of different pathogens, particularly pathogens that predominate in the farm's environment.

Str. uberis and *Str. dysgalactiae* were classified as environmental, referring to pathogens that live in the cows' environment, and infection occurs mainly from environment sources. However, modern molecular epidemiology studies suggested that such classification is not strict, and infections can occur in a cow-to-cow fashion. *Str. uberis* is an important bovine mastitis pathogen, and because of the ample distribution of their virulence attributes in the cow environment and cow's body, its control has become extremely difficult. The pathogenesis of this bacteria follows the model adherence/internalization, avoidance of phagocytosis, and persistence. For this, *Str. uberis* expresses ligands for milk and cellular matrix proteins, and the binding of these host factors are exploited to enhance adherence and internalization into host epithelial cells. Through this mechanism, *Str. uberis* reaches an environment (i.e., intracellular milieu) where phagocytic defenses are not effective allowing the persistence of the infection. Besides, about 50% of *Str. uberis* strains produce hyaluronic acid capsules, which have been regarded as an antiphagocytic factor. This pathogen produces biofilm, and this virulence attribute has been linked to persistent infections and development of antibiotic resistance.

Str. dysgalactiae has also been classified as an environmental pathogen, but as with other environmental mastitis-causing bacteria, this microorganism was described as contagious and environmental pathogen as well. The pathogenesis of this bacterium resembles the description for *Str. uberis* that adheres/invades mammary epithelial cells, avoid phagocytosis and achieves persistence in the mammary gland. Differently than *Str. uberis*, transmission from cow-to-cow, in addition to the usual contagious ways of transmission, was described to be through flies, particularly during summertime. *Str. dysgalactiae* expresses ligands that bind host factors, and through this, a molecular bridge is formed with the host protein receptor on the epithelial cell surface, which is followed by the internalization into the mammary epithelial cells and persistence. *Str. agalactiae* belongs to group B streptococci (GBS), and it has been considered a strict contagious mastitis pathogen for many years. However, recent reports indicated that *Str. agalactiae* was isolated from bovine feces as well as vaginal mucosa, and the presence of this pathogen in bulk tank milk can be caused through contamination from sources other than milk from *Str. agalactiae* cows. Besides, *Str. agalactiae* expresses biofilms, which have been related to increased environment survival and increased antibiotic resistance. GBS are important human pathogens since are the most common cause of newborn infections leading to complications like pneumonia, meningitis, or a blood infection. It was reported that clonal complexes of *Str. agalactiae* were shared between cows and farm personnel, indicating the lack of genuine species barriers.

In general, the identification of *Streptococcus* species isolated from mastitis milk samples is based on their traditional division into α-, β- and γ-streptococci depending on their capacity to hemolyze erythrocytes in the blood agar medium. The Lancefield agglutination test is typically used in microbiology laboratories to determine the Lancefield grouping of large-colony β-hemolytic streptococci, including slightly beta-hemolytic *Str. agalactiae*; the extraction of polysaccharide antigen from the streptococcal cell walls allows the identification of the specific group antigen. Identification of *Str. uberis* is usually based on morphological characteristics, biochemical tests, and enzyme activities.

Currently, molecular tools, such as PCR-based protocols, have been developed for an accurate identification of *Str. uberis* isolates. Besides RFLP analysis of 16S rDNA as a general method for bacterial identification and typing, multiplex PCR assays (mPCR) have been recently used for the simultaneous detection of *Str. uberis* housekeeping genes (*tuf, sodA*, and *cpn60*) and virulence factors (*gapC, oppF, sua, pauA, hasA, hasB, hasC, lbp, ermB, linB*). Additionally, different molecular techniques, such as random amplified polymorphic DNA (RAPD), pulsed-field gel electrophoresis (PFGE) and MLST, have been designed to subtype individual strains of *Str. uberis*, while MLST, based on the sequencing of seven housekeeping genes, is useful for their clonal classification.

Pathogens Responsible of Foodborne Zoonotic Diseases

Enterotoxigenic Staphylococcus aureus

Staphylococcus aureus is the best known of the coagulase-positive staphylococci (CPS) belonging to the genus *Staphylococcus*. Among them, *S. aureus* is one of the most important opportunistic pathogens, able to colonize and infect a wide host range, including humans and food producing animals. This microorganism is also identified as one of the most common zoonotic agents, involved in foodborne disease. Staphylococcal Food Poisoning (SFP) is worldwide recognized as the most prevalent foodborne intoxication; in the USA, almost 240,000 cases occur per year, resulting in 1000 hospitalizations and 6 deaths, while in Europe 434 outbreaks, representing 10% of all instances reported there, were registered in 2015.

S. aureus can enter the food chain during farming process but also throughout preparation, transportation and storage of animal food products, including meat and milk. Human carriers, especially food handlers, are considered one of the main sources of SFP, as their bodies or gloves are often contaminated with *S. aureus*. The largest reservoir of *S. aureus* strains causing foodborne disease attributed to milk is bovine intramammary infection (IMI); SFP can be caused by the

consumption of milk from affected cows and of dairy products, particularly those made from raw milk, if they contain enterotoxigenic *S. aureus* at numbers $\geq 10^5$ CFU g^{-1}.

In fact, outbreaks of foodborne illness are linked to the ingestion of an adequate level of staphylococcal enterotoxins (SEs), produced in milk by *S. aureus*. SEs are water-soluble and heat-stable, and they are not completely destroyed at low pH; therefore, they are able to retain their biological activity even after heat treatment (e.g., pasteurization) and after digestion in gastrointestinal tract, despite the presence of proteolytic enzymes, such as pepsin and trypsin. Their oral uptake can cause nausea and violent vomiting accompanied by abdominal cramps, diarrhea or moderate fever; these symptoms, appearing after a short incubation time, are usually self-limited and recede completely within 24 h, but may get worse especially in children and the elderly. However, enterotoxins act not only as potent gastrointestinal toxins but also as superantigens, eliciting an effective neutralizing antibody response. The staphylococcal superantigens (SAgs), comprising enterotoxins, are potent immunogens triggering T-cells proliferation by a non- specific interaction of the class II major histocompatibility complex (MHC II) with T cell receptors; the consequent production of massive amounts of pro-inflammatory cytokines results in the symptoms.

Most *S. aureus* strains express at least one enterotoxin and many of them are positive for a combination of two or more toxin genes. Currently, 24 serologically different staphylococcal enterotoxins and staphylococcal enterotoxin-like proteins (SE*ls*) have been discovered and designated with alphabetical letters. The genes encoding the five classical SEs (SEA to SEE), responsible for the majority of the SFP outbreaks, are located on mobile genetic elements (MGEs). Among them, *sea*, the most frequently observed in enterotoxigenic *S. aureus* strains, and *see* are carried by prophages, while *seb* and *sec* are encoded in pathogenicity islands. The *sed* gene, producing the most common toxin associated with SFP after SEA, is located on a plasmid. The newly described SEs (SEG to SEU) and SE-like toxins (SE*l*U, SE*l*V, SE*l*W, SE*l*X, SE*l*Y) whose emetic activity has not yet been proven, have recently been reported as possibly involved in food poisoning. At least four new types of SE genes (*sej, ser, ses* and *set*) are known to be encoded by the same plasmid carrying *sed*, while the

enterotoxin gene cluster (*egc*) operon, located on a staphylococcal genomic island, comprises *seg, sei, sem, seo, sen* and sometimes *seu*. Other SEs genes are included in the methicillin resistance chromosomal cassette (SCC*mec*) in combination with the methicillin resistance gene (*mecA*), also carried by SCC*mec*. The enterotoxigenic methicillin-resistant *S. aureus* (MRSA) strains, entering the food chain, can represent a serious threat for public health; poultry meat rather than bovine milk has been considered as a probable source of MRSA infections in humans. Although MRSA isolates present in food products may not necessarily be transferred to consumers, the EFSA has suggested to monitor their occurrence in primary production. Moreover, MRSA, as well as methicillin-sensitive *S. aureus* (MSSA), can form biofilm on various surfaces and they are able to adhere to materials commonly used in the food industry, comprising stainless steel and polystyrene. Biofilm formation is mediated by the intercellular adhesion operon (*ica*), while biofilm accumulation seems to be linked to PBP2a, an alternative penicillin-binding protein encoded by SCC*mec* and characterized by low affinity for beta-lactam antibiotics. In conclusion, the biofilm-forming ability of MRSA that are potentially SEs producers and, generally, the enterotoxigenicity of *S. aureus* should be of concern for food safety because of the frequent occurrence of SFP worldwide; control of foodborne diseases should be based on hygiene measures to avoid food contamination.

Serologic tests are the earliest methods applied for the detection of enterotoxins; the gel diffusion test and agglutination test are *in vitro* antigen/antibody reactions based on the precipitation and agglutination in serum, respectively. However, diagnostic methods based on phenotypic analysis have been shown to be less efficient to detect enterotoxigenic *S. aureus* in food matrices than molecular methods, which are characterized by higher sensitivity and lower variability. The highly purified DNA template extracted from *S. aureus* isolates can be amplified by PCR to verify the occurrence of enterotoxin genes. Lately, several PCR variants have been developed, comprising mPCR, qPCR, reverse-transcriptase PCR (RT-PCR), and loop-mediated isothermal amplification (LAMP). Among them, mPCR-based assay has the advantage to include multiple pairs of primers specific for different SE genes in the same reaction, even if their detection does not necessarily implicate their expression.

The PCR-based techniques can also be combined with other techniques, such as most probable number (MPN-PCR) and PCR-enzyme linked immunosorbent assay (PCR-ELISA), which can provide sensitive results. The latter uses a fundamental colorimetric method determining analytes by comparing or measuring the absorbance of a colorful substance. This immunoassay, based on immobilizing artificial antigens or capturing antibodies on plastic supports, is commonly performed to immunologically detect SEs because it is efficient, specific and sensitive, but it is difficult to apply in multi-SEs detection. Other optical-based detection methods comprise fluorescent, chemiluminescence, electrochemiluminescence, surface plasmon resonance (SPR) and surface-enhanced raman scattering (SERS)-based immunoassays, that have been widely applied for SE detection in foods, included milk. New technologies, such as electrochemical immunoassays for the quantification of SEs based on the change in an electric signal and mass-based immunoassays for transduction based on small changes in mass, can represent alternative valuable options. Finally, new bioassays have been recently developed using aptamers or molecularly imprinted polymers (MIPs) with high affinity and selectivity for the target molecules.

Salmonella spp.

Salmonella is a genus of Gram-negative, facultative anaerobe bacillus belonging to the Enterobacteriaceae family, consisting of only two species, *Salmonella bongori* and *Salmonella enterica*. The latter is divided into six subspecies: *enterica, salamae, arizonae, diarizonae, houtenae,* and *indica.* All bacteria belonging to *Salmonella* genus contain a mosaic of somatic (O), flagellar (H), and capsular (Vi) antigens, which, combined according to the Kauffmann-White scheme, allow the differentiation of more than 2600 serotypes of *Salmonella*. The 99% of serotype causing infections in humans and warm-blooded animals belongs to *Salmonella enterica* subsp. *enterica*. Among these serotypes, the most common ones hosted by dairy ruminants are *Salmonella* Anatum, Dublin, Montevideo, Newport, Typhimurium and Abortus ovis.

In humans, there are three major diseases caused by *Salmonella*: typhoid fever (caused by *Salmonella* Typhi and related strains), gastroenteritis (caused by most *Salmonella* serovars, such as *Salmonella* Typhimurium and *Salmonella* enteritidis), and an invasive disease (caused by *Salmonella choleraesuis*). Non thyphoidal *Salmonella* is a worldwide foodborne pathogen and the second most responsible for gastrointestinal human infections after *Campylobacter* spp. The percentage of human cases due to milk and dairy consumption is low respect to other source of infection: only the 3.4% of *Salmonella* foodborne outbreaks reported in EU in 2017 were connected with dairy products consumption, while 36.4% were due to consumption of eggs and egg products.

Salmonella in domestic livestock is an important pathogen not only for risk of transmission to human, but also for the severity of the disease. Signs of clinical salmonellosis in dairy cattle can include septicemia, diarrhea and abortion. Salmonellosis can affect cattle of every age, although the clinical disease is more common in calves. The main risk for introduction of *Salmonella* infection in a farm is linked to the purchasing of asymptomatic shedders; consequently, all the new cattle introduced in the farm should be isolated for 3 weeks and tested with a bacteriological culture. Another important risk factor, associated with *Salmonella* entrance in dairy farm, is the environmental contamination by synanthropic animals. The control measures to be applied include minimizing the possibility of contacts between all these animals and the cattle, and using rodent proof and bird proof feed storage, to avoid fecal contamination of feed. When the infection is recognized in a farm, control measures can be summarized in the following points: test all the cattle of the farm, clean and sanitize all the barns and the equipment, improve housing and management of cattle, initiate control programs for rodents, flies, and feral cats.

Laboratory diagnosis can be performed with bacteriological culture, PCR or serology. Bacteriological culture can be performed on fecal samples or bowel content in dead animals, applying the specific analytical method codified by ISO (6579:2002) and relying on four basic steps. The pre-enrichment in non-selective media usually uses buffered peptone water (BPW) or lactose broth, while the selective media for successive enrichment are represented by Rappaport-Vassiliades soy (RVS) broth,

selenite cysteine (SC) broth or tetrahionate (TT) broth; then, *Salmonella* is isolated on selective agar plates constituted of BG, xylose lysine Tergitol-4 (XLT4), bismuth sulfide (BS), Hektoen enteric (HE) and xylose lysine deoxychocolate (XLD) agars. Finally, *Salmonella* is reliably identified by biochemical tests (i.e., Triple sugar iron agar, mannitol, urea, ornithin decarboxylase and lysine decarboxylase). Moreover, bacteriological culture allows to perform serotyping and genotyping of *Salmonella* isolates, and antimicrobial susceptibility test that provides several important information about the type of *Salmonella* isolated. Unfortunately, bacteriological culture has a good sensitivity (>60%) only if the sample bacterial load is >100 CFU g^{-1}, while when the shedding of *Salmonella* is lower, the sensitivity drops to an unacceptable level (20%). Only for the detection of cattle infected by *Salmonella* Dublin a specific serological test is available; using this test as screening tool and performing the bacteriological culture only in positive animals has been proved to improve the sensitivity and the specificity of the test. Several PCR methods have been used for *Salmonella* detection; qPCR allows also to estimate the bacterial load of the samples. All PCR based methods show a higher sensitivity when performed starting from an enrichment broth than directly from the sample. Recent advances in whole genome sequencing (WGS) technology have provided a wide range of *Salmonella* serovar genome sequences, increasing the potential for designing specific primers for genes as optimal targets to enhance *Salmonella* detection sensitivity and specificity. Among the new PCR-typing targets identified, the *flhB* gene enables to differentiate *Salmonella* Dublin, Enteritidis and Pullorum/Gallinarum by adding primers for *tcpS* and *lygD* genes in a mPCR assay.

Vero Toxin Producing Escherichia coli

Bovine mastitis is the most costly disease of dairy cows, caused largely by infection with bacterial pathogens. These had been categorized as contagious, including those pathogens that mainly live in the mammary gland. Their transmission is cow-to-cow and environmental, which comprises bacterial

pathogens that survive in the cows' environment, and transmission occurs through the direct contact of the teat ends with contaminated sources in the environment. *Escherichia coli* is among the environmental mastitis pathogens.

When infecting bovine mammary glands, *E. coli* induces a variety of clinical symptoms that could range from death, acute mastitis to spontaneous elimination of the infection. Dairy cattle have different clinical signs from acute coliform mastitis, a wide range of systemic disease severity, from mild, with only local inflammatory changes of the mammary gland, to severe, with significant systemic signs including rumen stasis, dehydration, shock, and even death. However, there are other *E. coli* strains causing mild intramammary infections (IMIs) that can persist in the infected gland during several lactation cycles. *Escherichia coli* strains can further be classified according to the presence of virulence factors or induced pathology into groups, which include enterohemorrhagic *E. coli* (EHEC), enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), attaching and effacing *E. coli* (AEEC), and Vero toxin-producing *E. coli* (VTEC). The VTEC, which are also commonly referred to as Shiga-toxin producing *E. coli* (STEC), comprise over 400 serotypes of *E. coli*. The common feature, which defines this group, is its ability to produce a distinct range of toxins termed Verocytotoxins (VT), which show potent cytotoxicity against Vero cells.

STEC strains are important pathogens causing IMI and that Shiga toxins (*stx1, stx2*) and *eae* (intimin) are the most important virulence genes in *E. coli* strains isolated from bovine mastitic milk. The serotype prevalence may vary according to the region where these strains are isolated, but there is a consensus that no-O157 STEC strains are the most prevalent in animals and food contaminants. It was described that cytotoxic necrotizing factor (*CNF*) toxins (*CNF1* and *CNF2* genes) are associated with damage of vascular endothelial cells and thrombotic microangiopathy.

Even though their role as mastitis pathogens is important, the position of these strains as foodborne pathogens has far more relevance in public health. This is particularly important in the case of consumption of raw dairy products. Raw milk can be a significant source of foodborne pathogens, and there have been numerous food-poisoning outbreaks associated with direct consumption of raw

milk, e.g., milk inadequately heat-treated, or re-contaminated after heat treatment. Cows are reservoir of these pathogens and therefore the presence of pathogens in milk arises from contamination by fecal material during the milking or milk storage process. Since this pathogen is always present in fecal material, milking equipment contaminated with feces and poor hygiene in the milking parlor can facilitate the spread of these pathogens to the udders. Additionally, milking equipment including teat cups, milk pipelines, and bulk tank milk can be colonized, creating additional reservoirs of these pathogens.

Procedures implemented in the farm preventing fecal contamination of the milk supply will very likely reduce the risk of raw milk being contaminated with VTEC. Since there are cows that may become removal of STEC super-shedders from a herd, this is a cumbersome and expensive procedure requiring veterinary supervision and surveillance of individual animals in a herd. Cattle vaccines are available in the USA and Canada for *E. coli* O157, but its value on controlling this pathogen on-farm conditions is still under study.

E. coli O157 can be isolated from food samples, included milk, by using conventional diagnostic techniques. The medium of choice to culture is represented by sorbitol-MacConkey (SMAC) agar on which sorbitol-negative colonies of *E. coli* O157 appear colorless; they differ from other *E. coli* strains that rapidly ferment sorbitol and produce β-glucuronidase. The detection of *E. coli* O157 can be improved by using CR-SMAC agar or CT-SMAC agar added with cefixime and rhamnose or cefixime and potassium tellurite, respectively. Then, potential colonies of O157 should be confirmed with latex agglutination test composed of latex particles coated with antiserum against O157 and H7 antigens; isolates agglutinating in antiserum should be definitely identified biochemically as *E. coli*. Several immunological methods like ELISA and molecular techniques, such as reversed passive latex agglutination (RPLA), PCR and qPCR, have been developed to detect O157 or H7 antigens, shiga toxins or their respective genes from multiple sample matrices, included milk and dairy products. Standard and mPCR assays have revealed to be less time-consuming and more sensitive than cell culture because they enable to detect low levels of *E. coli* O157 in food samples by amplification of

several genes (*rfbE, fliC, eaeA, hlyA, uidA, stx1 and stx2*); however, none of these genes by themselves can reliably identify *E. coli* O157 because of its high similarity to other *E. coli* strains. On the other hand, qPCR assay based on the TaqMan PCR detection system provides the simultaneous detection and quantification of low amounts of the target organism in food samples; despite high specificity and sensitivity, the use of expensive fluorescent material is needed. The PCR-ELISA is an appropriate alternative approach being more convenient for rapid and reliable detection and quantification of pathogen-specific gene sequences encoding shiga toxins.

Campylobacter spp.

Campylobacter species are small (0.2–0.9 µm wide and 0.2–5.0 µm long), Gram-negative, non-spore forming bacteria that have a curved or spiral shape with polar flagellum at one or both ends of the cells. Their ability to multiply in an atmosphere that contains nearly 10% CO_2 and 5% O_2, with a temperature range of 30–46 °C, distinguishes them from other foodborne pathogens. *Campylobacter* spp. can form biofilms on abiotic surfaces, which ensure a supply of nutrients and mechanical protection even though they cannot grow. At present, the genus *Campylobacter* consists of 32 species and 13 subspecies of the genus of which *C. jejuni* subsp. *jejuni*, *C. jejuni* subsp. *doley, C. coli, C. lari, C. upsaliensis* and *C. helveticus* form a genetically closed group of species categorized as thermophilic with optimal growth at 42 °C.

Campylobacter spp. are commensal organisms routinely found in cattle, sheep, swine, and avian species, especially broilers, which represent the most common hosts for this genus probably because of their higher body temperature. Thus, the major sources of infection are by consumption of contaminated poultry, but also unpasteurized milk and water. The presence of this microorganism in milk is due to the fecal cross-contamination during milking or as a result of udder infection. *Campylobacter* can survive in water or milk for many weeks at low temperatures. Among *Campylobacter, C. jejuni* and *C. coli* are recognized as the main causes of bacterial foodborne disease

in many developed countries with about nine million cases of campylobacteriosis each year and a cost estimated to be around € 2.4 billion a year. Campylobacteriosis presents a wide range of symptoms from watery diarrhea to dysentery, often accompanied by fever and severe abdominal cramps, urinary tract infections and septicemia. In a minority of individuals, this infection is a precursor of some neuropathies including immunoreactive complications, such as Guillain-Barre Syndrome (GBS) and Miller-Fisher Syndrome (MFS).

The culture media used for isolating *Campylobacter* spp. from foods derive from those originally designed for isolating the pathogen from human stool samples. However, due to the predicted low numbers present in foods, selective enrichment broths (Bolton broth, *Campylobacter* Enrichment broth, Preston broth) are also required for the isolation of this microorganism from foods. A standard procedure includes a pre-enrichment period in a selective broth, for 4–6 h at 37 °C, followed by an enrichment period in the same medium for 42–44 h at 42 °C to promote specific growth. After that, an incubation for 48 h period at 42 °C onto a selective agar plate gives a confirmation of *Campylobacter* spp. isolation, based on colony morphology, Gram stain, motility and oxidase text.

A reasonable understanding of the clinical, microbiological and epidemiological aspects of *Campylobacter* infection has been achieved. However, the molecular mechanisms involved in pathogenesis are still poorly understood. Not all the virulence determinants implicated in *Campylobacter* pathogenesis are known or well characterized. When present in food or water, *Campylobacter* spp. enter the host intestine via the stomach acid barrier and colonize the mucus of distal ileum and colon perturbing the absorptive capacity of the intestine by damaging epithelial cell function. Flagella-mediated motility (*flaA, flaB, fliF, fliM & fliY, flgI, flgH, flgE & fliK, fliA, rpoN* genes), bacterial adherence to the intestinal mucosa (*cadF, capA, pldA, jlpA, peb1A, peb3, peb4, flpA, virB11, jlpA* genes), and invasive capability (*flaC, ciaB* genes) have been identified as virulence factors. Chemotaxis is also a mechanism used by this genus to move toward more favorable conditions or invade the host (*cheA, cheB, cheR, cheV, cheW & cheZ, tlp1, tlp4, tlp10, cheY, cetA & cetB, luxS* and *acfB* genes). *Campylobacter* produce also several different cytotoxins of which only

cytolethal distending toxin (CDT) has been characterized in detail. It is composed of three subunits encoded by the *cdtA, cdtB* and *cdtC* genes (*cdtABC* operon), causes eukaryotic cells to arrest in the G2/M phase of the cell cycle, preventing them from entering mitosis and consequently leading to cell death. CDT induces also IL-8 secretion from epithelial cells, contributing to inflammatory diarrhea. This gene cluster is ubiquitously distributed in *C. jejuni, C. coli,* and *C. fetus* in a species-specific manner. *Campylobacter* spp. has complex multifactorial systems for multiplication and survival during food processing and virulence in humans, also with an increase in antimicrobial resistance to fluoroquinolones and erythromycin, especially for *C. jejuni* and *C. coli*.

DNA-based methods use PCR as the most versatile and widely used amplification technique. For detection of this microorganism, lots of PCR and multiplex-PCR assays have been developed based on the genes previously described, but also RT-PCR and nucleic acid sequence-based amplification (NASBA) techniques for RNA amplification (i.e., *cadF, 16S* rRNA, *gyrA* as target genes).

Listeria monocytogenes

Listeria monocytogenes is a rod-shaped Gram-positive bacterium that belongs to the *Listeria* genus, of which 17 species have been described (*L. monocytogenes, L. ivanovii, L. innocua, L. seeligeri, L. grayi, L. welshimerii, L. marthii, L. rocourtiae, L. fleishmannii, L. floridensis, L. aquatica, L. newyorkensis, L. cornellensis, L. weihestephanensis, L. grandensis, L. riparia,* and *L. booriae*). Each year, *L. monocytogenes* is responsible for numerous outbreaks and listeriosis cases in humans around the world. These outbreaks are characterized by high hospitalization rates and mortality, particularly among pregnant women, children, the elderly, and other individuals with impaired immunity or underlying health conditions. In these high-risk groups, the manifestations of the disease can be severe, leading to stillbirth, neurological ailments, or even death. Listeriosis has also been reported among healthy individuals in the form of meningitis or as a non-severe febrile illness. The route of infection is primarily foodborne, although other routes, such as intrauterine transmission, infection of

neonates during delivery, or nosocomial transmission, have also been suggested. Virulence factors, such as *lap, ami, fbpA, lapB, inlJ* (for cellular adhesion), *inlA* and *inlB* (for cellular invasion) as well as listeriolysin O, phosphatidylinositol-PLC and phosphatidylcholine-PLC (for survival, multiplication, and cell to cell extension) are involved in the pathogenesis of listeriosis. The presence of hypervirulent strains, which seem better adapted to the mammalian gut, have been associated to dairy products. Moreover, clonal complexes and virulence types of *L. monocytogenes* obtained from bulk tank milk and milk filters from dairies in the US have also been identified in cases of human listeriosis, including *L. monocytogenes* strains capable of causing severe disease.

L. monocytogenes has been the cause of numerous recalls of dairy products, as well as the cause of outbreaks in humans due to consumption of contaminated foods, such as raw milk, pasteurized milk, ice cream, butter, and cheese. As *Listeria* does not survive the pasteurization process, outbreaks are commonly caused by dairy products manufactured with raw or improperly pasteurized milk, or dairy products that become contaminated during post-pasteurization steps. *L. monocytogenes* is ubiquitous in nature, including dairy farms and the dairy environment, where *Listeria* has been isolated from several sources including water, silage, feedstuffs, ruminant feces, milking parlor, milking equipment, milk filters, and bulk tank milk. In addition, a high genetic diversity among *L. monocytogenes* strains from dairies has been reported. Some of these strains are apparently adapted to the farm environment or persist in the form of biofilms. Cows can be healthy carriers of *L. monocytogenes*, and fecal shedding may help spreading the microorganism throughout the dairy farm environment. Farm personnel may act as vehicle of *L. monocytogenes*, contributing to the on-farm dispersion of the pathogen, including personnel households. *L. monocytogenes* milk shedding in cows suffering from mastitis caused by *Listeria* seem to be uncommon. The high frequency of isolation of *L. monocytogenes* from multiple sources on dairy farms may be responsible for the contamination of raw milk and, subsequently, of dairy products manufactured with tainted unpasteurized or improperly pasteurized milk. Besides, *L. monocytogenes* strains can persist within the environment of the dairy processor and cause post-pasteurization contamination of dairy products. Certainly, *L.*

monocytogenes strains seem adapted to processor's environment, exhibit greater resistance to sub-lethal doses of commonly used disinfectants (e.g., belzalkonium chloride), and show increased biofilm production under stressful environmental conditions. Currently, unlike other microorganisms, bacteria from the genus *Listeria* are still susceptible to multiple antimicrobial drugs. Although, both the natural intrinsic resistance to certain antimicrobial drugs and acquired antimicrobial resistance have also been reported.

Molecular methods have been developed for identification of *Listeria* spp. with enhanced discriminatory power in recent years. Among them, DNA hybridization tests and PCR assays enable to identify *Listeria* and to differentiate *L. monocytogenes* from other *Listeria* species by targeting probes to virulence factor genes and by using primers for genes of virulence factors or RNA subunit genes. The most specific gene targets for *Listeria* comprise rRNA coding genes (small subunit 16S and large subunit 23S rRNA genes or region 16S-23S intergenic spacer), housekeeping genes (*groESL, rpoB, recA, gyrB, prs*), genes for invasion associated protein (*iap*) or flagellin A (*flaA*) or fibronectin-binding protein (*fbp*), species-specific genes (*hly, plcA, plcB, actA, mpl, inlA, inlB, lmo0733*) and group-specific genes (*lmo2821, lmo1134* and *lmo2672*); a mPCR including primers for a species-specific gene (*inlA*) and for two different virulence genes (*inlJ* or *lmo281* and *inlC*) has been developed to differentiate *L. monocytogenes* from other *Listeria* species.

Additionally, the mostly used molecular methods to type *Listeria* strains comprise ribotyping, macro restriction digests, PFGE, RFLP or RAPD. Other techniques based on DNA sequencing have revealed to be more accurate but also more expensive and time-consuming; particularly, MLST has been developed for typing of *L. monocytogenes* strains targeting several housekeeping genes but also some genes coding for virulence factors (*fla, hly, actA, iap, inl, mpl, prfA*).

Bacillus cereus

The genus *Bacillus* includes Gram-positive spore forming rod-shaped bacteria that are very diverse from physiology to the ecological niche, from DNA sequence to gene regulation. The *Bacillus cereus* group, also known as *B. cereus sensu lato*, is a subdivision of the *Bacillus* genus that consists of eight formally recognized species: *B. cereus sensu stricto* (or *B. cereus* as it is usually called), *B. anthracis, B. thuringiensis, B. weihenstephanensis, B. mycoides, B. pseudomycoides, B. cytotoxicus* and the more recently validated new species *B. toyonensis*.

B. cereus is a motile, Gram-positive, aerobic or facultative anaerobic, spore-forming rod of the family Bacillaceae (presently consisting of at least 226 species), that is widely distributed in air, dust, and water. *Bacillus* genus constitutes part of the microflora in various raw food and foodstuffs including cereals grain and cereals products, milk and dairy products, fruits, vegetables. *B. cereus* bacteria were also isolated from commercial ground roasted coffee and ready-to-eat meals and products. Their capacity to sporulate allows these bacteria to resist the common cleaning procedures used in the food industry (e.g., during milk processing) and in hospitals. Especially in products of dairy origin, *B. cereus* produces lipases and thermostable proteases, which remain active after pasteurization leading to defects in the final dairy products and to food poisoning outbreaks. As a human pathogen, the organism is known for its role as a mediator of self-limited foodborne illnesses and its association with severe local and systemic infections in immunosuppressed hospitalized patients and neonates. *B. cereus* generally causes two types of gastrointestinal illness, including emesis and diarrhea, after consumption of a contaminated food, which contains more than 10^4–10^5 spores or vegetative cells of *B. cereus* per gram.

Standard isolation and enumeration of *B. cereus* from foods, the environment and clinical settings, are usually performed by conventional selective plating media, such as polymyxin egg yolk-mannitol-bromothymol blue or mannitol-egg yolk-polymyxin agar. Recently, other selective media, which contain a chromogenic substrate for the detection of phosphatidylinositol-specific phospholipase C

have been used, even if these procedures are time consuming and unable to indicate the toxin production capability of the isolates.

The pathogenicity of *B. cereus* is caused by different toxins produced by this bacterium. Diarrhea is associated with a series of enterotoxins including hemolysin BL (Hbl), non-hemolytic enterotoxin (Nhe), and three enterotoxin proteins: cytotoxin K (CytK), enterotoxin FM, as well as potential enterotoxins hemolysin II (HlyII) and enterotoxin T (BceT). HBL and NHE are tripartite toxins, in which all three components are necessary for maximal cytotoxic activity. Emetic syndrome is caused by the toxin cereulide, which is synthesized by non-ribosomal peptide synthetases encoded by the *ces* gene cluster, coding for seven proteins involved in the synthesis of cereulide toxin. Unlike enterotoxins, cereulide is a heat and acidic-stable depsipeptide that is pre-formed in contaminated foods. Moreover, *B. cereus* is involved in many serious and potentially fatal non-gastrointestinal-tract infections, such as severe eye infections, osteomyelitis, hepatitis, inflammatory responses and even death. Antibiotic therapy, in general by using gentamicin, chloramphenicol, vancomycin, and ciprofloxacin, is still the primary treatment method for the infections of *B. cereus*. However, emergence of antibiotic resistant *B. cereus* strains, mainly due to antibiotic mis-usage or acquisition of resistance genes through horizontal gene transfer, results in the failure of antibiotic treatment.

Molecular-based quantification and identification of the *B. cereus* group directly in the sample has been successfully explored by qPCR in different food matrices, with the advantage that results can be obtained in a few hours rather than days, as in the case of conventional methods. PCR has been used extensively to amplify all the diarrheal toxin-producing genes, and genes encoding the emetic toxin cereulide, after their identification and sequencing. However, the presence of a toxin gene does not necessarily indicate that the bacterium is capable of producing the protein in concentrations high enough to determine the disease.

Overview of the Molecular Techniques Applied for the Detection of Pathogens in Milk

The identification and the detailed characterization of pathogens in milk and dairy are critical for the investigation of common outbreak sources in order to identify the source, implement control measures and/or take steps to remove the implicated food from the market place.

Table 1. Molecular approaches for the main pathogen detection in dairy products

Bacteria	*Approach*	*Detection limit*	*Dairy product*
Listeria monocytogenes	qPCR	CFU g^{-1} 4.9×10^3 CFU g^{-1}	Raw milk, milk, pasteurized milk, kulfi, ice cream, paneer, and infant foods
	PCR	8×10^0 CFU mL^{-1}	Milk
	Multiplex PCR	1 CFU g^{-1}	Quargel cheese
	Digital PCR	10 CFU g^{-1}	Alpine cheese, Quargel cheese
Salmonella spp.	qPCR	3 log CFU mL^{-1} 25 to 500 cells	Raw milk, pasteurized milk, kulfi, ice cream, paneer, and infant foods Milk
	PCR	5 bacteria mL^{-1} 10^3 bacteria mL^{-1}	Milk Ice-cream
	Multiplex PCR	$N \times 10^4$ CFU mL^{-1}	Milk
	Digital PCR	4×10^3 CFU g^{-1} 10^2 CFU mL^{-1}	Soft cheese Milk
Staphylococcus aureus	qPCR	10^2 to 10^3 CFU mL^{-1}	Raw milk
	PCR	10^2 CFU mL^{-1}	Raw milk
	Multiplex PCR	$N \times 10^4$ CFU mL^{-1}	Milk
	Digital PCR	10^2 CFU mL^{-1}	Culture
***E. coli* O157: H7**	qPCR	4×10^6 to 40 CFU mL^{-1}	Milk
	PCR	2,5% of samples	Raw milk
	Multiplex PCR	$N \times 10^4$ CFU mL^{-1}	Milk
	Digital PCR	10^3 CFU g^{-1}	Soft cheese
Campylobacter spp.	qPCR	50 CFU mL^{-1}	Milk
	PCR	10^3 CFU mL^{-1}	Bulk tank milk
	Multiplex PCR	10^3 CFU g^{-1}	Brie cheese, Cheddar cheese
	Digital PCR	1 CFU mL^{-1}	Milk
Bacillus cereus	qPCR	N x 10^2 CFU mL^{-1}	Milk
	PCR	10^3 CFU mL^{-1}	Milk
	Multiplex PCR	10^3 CFU mL^{-1} or CFU g^{-1}	Milk and cheese
	Digital PCR	0.5 cell mL^{-1}	Raw milk, pasteurized milk

Traditional methods for the detection of bacterial pathogens in foods have been widely used because they are sensitive and inexpensive, and can give both qualitative and quantitative information on the number and the nature of the microorganisms present in the food sample. Due to their limitations, conventional methods are now giving way to molecular diagnostic methods based on DNA analysis, such as polymerase chain reaction (PCR), multiplex PCR and qPCR, which have been used for rapid

and reliable detection of foodborne pathogens. In addition, typifying methods are also largely used for accurate genetic characterization in outbreak investigations.

Extraction of DNA is a crucial step for successful pathogen detection by a molecular method, since the reproducibility and sensitivity of detection directly depend on the purity and integrity of the DNA. As a matter of fact, chemical and thermal treatments of food result in fragmentation and random breaks of long DNA strands, consequently making their use in PCR very difficult. Moreover, various food matrix compositions require specific treatments/reagents to exclude inhibitors of amplification enzymes. Finally, in the case of quantitative PCRs, the extraction procedure has a strong impact on the limit of detection (LoD). To overcome most of these issues a general guidance for extraction and quantification of genomic DNA is provided by the ISO standard 21571 annexes (ISO 21571:2005).

The various molecular methods employed for identification of pathogens in milk and dairy are discussed below and showed in Table 1.

Polymerase Chain Reaction (PCR)

The emergence of PCR has changed the way of performing microbiological analyses toward the detection of specific microbial DNA as a target. PCR was invented about 30 years ago and it allows the detection of a single bacterial pathogen present in food by detecting a specific target DNA sequence. PCR is now a common and an extremely powerful tool that enables exponential amplification of a specific target sequence in a short time, and, hence, greatly reduces dependence on cultural enrichment step. PCR operates by amplifying a specific target DNA sequence in a cyclic three steps process. Firstly, the target double-stranded DNA is denatured into single-stranded DNA at high temperature. Then, two single-stranded synthetic oligonucleotides or specific primers, which are the forward and reverse primer, will anneal to the DNA strands. This is followed by the polymerization process whereby the primers complementary to the single-stranded DNA are extended with the presence of deoxyribonucleotides and a thermostable DNA polymerase. PCR

amplification products are visualized on electrophoresis gel as bands by staining with ethidium bromide. This method can detect a single copy of a target DNA sequence with respect to single pathogen in food. For this reason, PCR has distinct advantages over culture and other standard methods for the detection of microbial pathogens and offers the advantages of sensitivity, specificity, accuracy, rapidity, and capacity to detect small amounts of target nucleic acids in a sample. Moreover, some pathogens present in milk and dairy products, like many *Salmonella* and *Campylobacter* strains, may be viable but non-culturable (VBNC). Using culturing methods for their detection leads to a false negative result and a failure in pathogen detection. Molecular PCR-based methods that detect pathogen-derived nucleic acid (DNA or RNA) prevent this risk.

PCR has been used in the detection of numerous milk and dairy pathogens like *L. monocytogenes, E. coli* O157:H7, *S. aureus, C. jejuni, Salmonella* spp. and *Shigella* spp. The usual detection limit of conventional PCR, which uses gel electrophoresis for the detection of the amplicon, is 10^3–10^5 CFU ml^{-1}. For several bacterial pathogens, PCR-based methods are dependent on pre-enrichment step to increase the number of cells for detection and to eliminate the risks of detecting DNA from dead bacteria. New protocols tackle the problem by adding cell membrane-impermeable dye to PCR reagents that can penetrate only into dead cells (such as propidium monoazide). The dye can bind only to the extracellular DNA (e.g., passively released from dying cells), hence, selectively preventing PCR amplification of DNA from the dead cells. This ensures that only foods contaminated with living bacterial cells produce an amplicon.

Some bacteria, such as *S. aureus, B. cereus*, Shiga-toxin-Producing *E. coli* (STEC) may produce toxins, which cause foodborne illnesses. PCR is also used for toxins detection by amplifying specific genes that encode bacterial toxins. Given the variability among bacterial strains and their toxins, as well as their adaptability, the field of toxin detection is required to continually evolve.

Multiplex PCR (mPCR)

Multiplex PCR involves the simultaneous detection or amplification of multiple target sequences in a single reaction by using different primers for each target. mPCR has the potential to produce considerable saving of time and effort within the laboratory without compromising test utility. The basic principle of mPCR is similar to conventional PCR. However, several sets of specific primers are used in mPCR assay whereas only one set of specific primers are used in conventional PCR assay. Primer design is very important for the development of mPCR, as the primer sets should have similar annealing temperature in order to produce a successful mPCR assay. Besides, the concentration of primers is also important in mPCR. This is because interaction may occur between the multiple primer sets in mPCR that results in primer dimers, thus, the concentration of primers may need to be adjusted to ensure the production of reliable PCR products. Other important factors for a successful mPCR assay include the PCR buffer concentrations, the balance between magnesium chloride and deoxynucleotide concentrations, the quantities of DNA template, cycling temperatures and Taq DNA polymerase. The main advantage of the mPCR as compared to the conventional method is lower cost. The primary advantage is less reagent and enzyme (Taq DNA polymerase) utilization. The only limitation is that the amplified fragments of same length cannot be detected and lower quantity of amplified product may not be visible on agarose gel. This could be overcome by designing the primers longer than those used in conventional PCR having a higher melting temperature (Tm).

Multiplex PCR has been successfully applied in the detection of foodborne pathogens. At first, mPCR was used to detect around two to three pathogens only. Now, mPCR is more advanced and it can detect up to five or more pathogens simultaneously. For example, a mPCR assay was developed for the simultaneous detection of *S. enteritidis, S. aureus, Shigella flexneri, L. monocytogenes*, and *E. coli* O157:H7 using five pairs of primers targeting invasion protein (*invA*), 16S rDNA, invasion plasmid antigen H (*ipaH*), listeriolysin O (*hlyA*) and intimin (*eaeA*) gene, respectively.

Real-Time or Quantitative PCR (qPCR)

Real-time PCR, also called quantitative real-time PCR, is a technique used to amplify and simultaneously quantify a targeted DNA molecule. It enables both detection and quantification. The quantity can be either an absolute number of copies or a relative amount when normalized to DNA input or additional normalizing genes. The amplified DNA is detected as the reaction progresses in real time.

Two common methods for the detection of products in qPCR are: (1) non-specific fluorescent dyes that intercalate with any double-stranded DNA, such as SYBR-green I and EtBr, and (2) sequence-specific DNA probes comprising oligonucleotides that are labeled with a fluorescent reporter, such as TaqMan, Molecular Beacons, Scorpions, etc., which permits detection only after hybridization of probe with its complementary DNA target. SYBR green is simple and less costly as compared to TaqMan probes or molecular beacons, whereas some studies have shown that TaqMan-based qPCR is more sensitive compared to SYBR green or molecular beacons-based qPCR.

Apart from that, the sensitivity of the method is mainly affected by primer specificity, primer sequence and annealing temperature, rather than the choice of detection probe.

The key features of this technique are its sensitivity and speed due to which it has become a very attractive method for the detection of foodborne pathogens. Overall, qPCR is more sensitive than conventional PCR and it minimizes the risk of cross contamination. Moreover, conventional PCR and multiplex PCR that require agarose gel analysis for the detection of PCR products are laborious and time-consuming, thus, not suitable for high-throughput analysis, and difficult to automate. The advantages of qPCR have led to the development of various commercial qPCR kits for the detection of milk and dairy pathogens, such as *Salmonella* spp., *L. monocytogenes, E. coli* O157:H7 and *Campylobacter* spp.

Furthermore, multiplex qPCR assay has been also developed for the detection and quantification of multiple foodborne pathogens. Notably, multiplex qPCR can be used for multiple pathogen detection

in the presence of high numbers of contaminating microorganisms. For example, a mPCR for the simultaneous detection of six pathogens: *S. enterica, L. monocytogenes, S. aureus, E. coli* O157:H7, *Shigella* spp. and *Campylobacter jejuni* was developed. The LoD of these methods, which also depends on the DNA extraction methods used, was similar to those obtained by traditional culturing methods or slightly lower (approximately 10 CFU per 25 g). Nevertheless, in the case of samples analyzed with qPCR prior to the enrichment step, the detection limit was in the range of 10^2–10^3 CFU g^{-1} (or mL^{-1}).

Digital PCR

The ability to quantify foodborne pathogens with accuracy and precision is important for several applications, such as tracing pathogens in food processing environments or in tenacity studies monitoring survival of micro-organisms in food matrices. Although qPCR has found widespread use for one-step nucleic acid quantification, it has been found vulnerable to some bias caused by comparatively low precision of the standard curve that is required to quantify unknown samples. Digital PCR (dPCR) has been introduced to provide absolute quantification. The technique is based on endpoint PCR with a series of diluted template DNA solutions, which are carried out as 15,000–20,000 parallel low-volume PCR reactions. The reactions take place either in a microdroplet format or in a microvolume chip, and the amplification product is detected fluorometrically in endpoint mode in a microfluidic device and by a scanner, respectively. Calculation of the absolute quantities is based upon counting positive versus negative amplification results at an appropriate dilution level. Studies targeting low-copy-number genes, typically in the field of molecular oncology, demonstrated high sensitivity and precision of dPCR compared with qPCR.

Application of dPCR technology is very similar to traditional qPCR and has been implemented to quantify biomass in a variety of microbial systems. As the latest generation of PCR and one of the most robust methods in molecular quantification, several articles in recent years have reported ddPCR

in the field of food testing and bacteria monitoring. Commercial ddPCR platforms developed droplets or silicon substrate approaches running tens of thousands of individual reactions in parallel. After PCR amplification and fluorescence signal collection, initial concentration of the target is calculated according to the pattern of Poisson distribution. After a comparison with qPCR, ddPCR exhibited more sensitive (10–4 ng μL^{-1} or 10^2 CFU mL^{-1}) and less pre-culturing time (saving 2 h). Moreover, dPCR had stronger resistance to inhibitors than qPCR, yet absolute quantification is hardly performed when target's concentration is over 1 ng μL^{-1} or 10^6 CFU mL^{-1}. Moreover, in detecting foodborne pathogens in milk ddPCR is a suitable analytical tool especially for zero-tolerance bacteria in food control. In addition, high resistant to PCR inhibitors makes ddPCR deal with DNA samples deriving from food sources as dairy more stable and reliable. For example, an accurate quantitative protocol based on dPCR was developed to detect simultaneously, without selective enrichment, *Listeria* spp., *L. monocytogenes, Salmonella* spp., verocytotoxin-producing *E. coli* and *Campylobacter* spp. in milk and cheese.

Whole Genome Sequencing

Recently, WGS has offered discriminatory power with the potential to enhance epidemiological investigations and elucidate transmission pathways. WGS has been a very useful and powerful tool for establishing potential links between clinical, food and environmental isolates of pathogens, which could allow the identification of the source of contamination and remove contaminated foods from markets. For example, WGS has been recently used to understand outbreak sources and the transmission patterns of bacteria, including *Escherichia coli*, *Campylobacter* spp., *Listeria* spp. and *Salmonella* spp.

To date, WGS technology is used to evaluate and identify new species based on the entire DNA sequence of a bacterium thereby making it as a good surveillance tool. WGS can be used also to characterize individual microbes which include the full complement of resistance determinants,

providing definite genotype information. Furthermore, WGS has the potential to discriminate between sporadic and outbreak isolates which may be indistinguishable by current methods of subtyping. WGS usefulness in food safety is undeniable; however, this approach is expensive and is not currently in place in the majority of public health laboratories. Additionally, analysis of WGS data can be difficult due to the extensive computational capacity and bioinformatics skills needed for genomic comparisons and to determine a threshold to establish relatedness.

Conclusion

The identification of causative bacteria of outbreaks associated with the consumption of dairy products is very complex. Novel molecular techniques have been crucial for accuracy in the detection of foodborne bacteria in diverse types of dairy products (including pasteurized milk), and it is probable that without these molecular approaches, the outbreaks' etiological agents would not have been correctly identified. In this regard, one analytical challenge that still remains in food safety is to present reliable results with respect to official guidelines as fast as possible without impairing method properties, such as recovery. There are still many problems waiting to be solved, such as sample preparation, elimination of the effects caused by the unspecific binding and cross-hybridization, and achievement of highest sensitivity in the methods. However, the potential of molecular-biology techniques is almost revolutionary. Technological advancements are taking place at a great pace, and the next generation assays have already been developed, which potentially have the capability for near real-time and on-line monitoring of multiple pathogens in foods.

Further Reading

Chlebicz, A., and K Slizewska. 2018. Campylobacteriosis, salmonellosis, yersiniosis, and listeriosis as zoonotic foodborne diseases: a review. Int. J. Environ. Res. Public Health 15(5):863. https://doi.org/10.3390/ijerph15050863.

European Food Safety Authority. 2016. Risks for public health related to the presence of *Bacillus cereus* and other *Bacillu*s spp. including *Bacillus thuringiensis* in foodstuffs. EFSA J. 14(7): 4524. https://doi.org/10.2903/j.efsa.2016.4524.

European Food Safety Authority. 2018. The European Union summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2017. EFSA J. 16 (12):5500. https://doi.org/10.2903/j.efsa.2018.5500.

Farrokh, C., K. Jordan, F. Auvray, K. Glass, H. Oppegaard, S. Raynaud, D. Thevenot, R. Condron, K. De Reu, A. Govaris, K. Heggum, M. Heyndrickx, J. Hummerjohann, D. Lindsay, S. Miszczycha, S. Moussiegt, K. Verstraete, and O. Cerf 2013. Review of Shiga-toxin-producing *Escherichia coli* (STEC) and their significance in dairy production. Int. J. Food Microbiol. 162:190–212. https://doi.org/10.1016/j.ijfoodmicro.2012.08.008.

Fetsch, A., and S. Johler. 2018. *Staphylococcus aureus* as a foodborne pathogen. Curr. Clin. Microbiol. Rep. 5:88–96. https://doi.org/10.1007/s40588-018-0094-x.

Grumann, D., U. Nübel, and B.M. Bröker. 2014. *Staphylococcus aureus* toxins – their functions and genetics. Infect. Genet. Evol. 21:583–592. https://doi.org/10.1016/j.meegid.2013.03.013.

Janosi, S., Rats, G., Szigeti, G., Kulcsar, M., Kerènyi, J., Laukò, T., Katona, F., Huszenicza, G., 2001. Pathophysiology: review of the microbiological, pathological, and clinical aspects of bovine mastitis caused by the alga *Prototheca zopfii*. Vet. Q. 23, 58–61.

Lamas, A., J.M. Miranda, P. Regal, B. Vázquez, C.M. Franco, and A. Cepeda. 2018. A comprehensive review of non-enterica subspecies of *Salmonella enterica*. Microbiol. Res. 206:60–73. https://doi.org/10.1016/j.micres.2017.09.010.

Liu, D. (Ed) 2009. Molecular detection of human bacterial pathogens. First ed. Taylor & Francis CRC Press, Boca Raton, Florida, USA. https://doi.org/10.1201/9781420076448.

Maury, M.M., H. Bracq-Dieye, L. Huang, G. Vales, M. Lavina, P. Thouvenot, O. Disson, A. Leclercq, S. Brisse, and M. Lecruit. 2019. Hypervirulent *Listeria monocytogenes* clone's adaption to

mammalian gut account for their association with dairy products. Nat. Commun. 10:2488. https://doi.org/10.1038/s41467-019-10380-0.

Nielsen, L.R. 2012. Review of pathogenesis and diagnostic methods of immediate relevance for epidemiology and control of *Salmonella* Dublin in cattle. Vet. Microbiol. 162:1–9. https://doi.org/10.1016/j.vetmic.2012.08.003.

Peton, V., and Y. Le Loir. 2014. *Staphylococcus aureus* in veterinary medicine. Infect. Genet. Evol. 21:602–615. https://doi.org/10.1016/j.meegid.2013.08.011.

Ruegg, P.L. 2017. A 100-year review: mastitis detection, management, and prevention. J. Dairy Sci. 100:10381–10397. https://doi.org/10.3168/jds.2017-13023.

Scheutz, F., and N.A. Strockbine. 2005. *Escherichia*. Pages 607-624 in Bergey's Manual of Systematic Bacteriology. G.M. Garrity, D.J. Brenner, N.R. Krieg, and J.T. Staley, ed. Springer, New York, NY, pp. 607–624. https://doi.org/10.1007/0-387-28022-7.

Tomasz, J., J. Gawor, Z. Bakuła, P. Decewicz, K. Maciszewski, and A. Karnkowskad. 2018. *cytb* as a new genetic marker for differentiation of *Prototheca* species. J. Clin. Microbiol. 56:e00584-18. https://doi.org/10.1128/JCM.00584-18.

Conclusions and Future Perspectives

The significance of this project laid in establishing the diffusion of antimicrobial resistance in bovine mastitis pathogens and in determining their molecular characteristics. Although the overuse of drugs to treat animal diseases has become a matter of common concern with the worldwide emergence of AMR, the MDR levels found in bacteria from bovine IMI and analyzed in this study, did not warrant a strong public health response. The confirmation of our hypothesis that only few strains, with specific genotypic patterns, could be responsible for the intramammary infection spreading could be of great clinical importance. The monitoring of the harmful strains dissemination within bovine population might help to formulate strategies for focused control and treatment.

General Conclusions

This research confirmed the genetic variety of *S. aureus* and *Strep. uberis* involved in the development of IMI. Our studies proved the large diffusion of few genotypic clusters characterized by a specific combination of virulence factors related to host adhesion and invasion. The close genetic relationship of bacterial strains within the same species might suggest their contagious behavior within dairy herds. Their spread may be linked to a greater ability of bacterial survival and colonization of the mammary gland, and to a higher risk of mastitis.

The ongoing emergence of MDR notwithstanding, the majority of the *S. aureus* and *Strep. uberis* strains analyzed in our studies were susceptible to most antimicrobials tested, demonstrating the low incidence of highly multidrug-resistant isolates, including MRSA strains. These results strengthened our knowledge on the AMR in *S. aureus* and *Strep. uberis* mastitis and supported the presence but not prevalence of MDR globally.

Recommendations

The screening of the genetically related strains of *S. aureus* and S*trep. uberis* could be useful in avoiding their further spread by strategies aimed at controlling contagious mastitis and at reducing the rate of *S. aureus* and *Strep. uberis* IMIs at farm-level.

The greatest challenge in treating staphylococcal and streptococcal infections is the identification of the most appropriate approach. When treating mild or moderate clinical cases, the therapeutic intervention can be postponed until the results of bacterial culture testing are available. The selection of the antimicrobial agent should be based on the herd-level antimicrobial susceptibility data obtained through the analysis of the strains isolated from previous mastitis cases. Our works provided useful information to develop a herd-level plan for the control and treatment of staphylococcal and streptococcal infections. Surveillance data could be meaningful to keep track of resistance levels in dairy cattle populations and beyond. The comprehension that bacterial resistance predates the era of antibiotic use validates the necessity to monitor AMR of mastitis causative agents. The understanding of the negative effects of their resistance to HPCIAs supports the need to apply adequate control and treatment protocols, that can be based on their epidemiological and pathogenic properties and can help to limit the rise of MDR worldwide.

Limitations

Some problems of our studies could be related to the limited number of *S. aureus* and *Strep. uberis* strains analyzed. To date, the present project aimed to be a descriptive study using a simple random sample of *S. aureus* isolates from cases of bovine mastitis occurring in different countries, and of *Strep. uberis* isolates from Italy. We selected only dairy farms able to contribute for a sufficient amount of data and isolates, that could be compared to reach representative conclusions.

Further Research

We intend to investigate the phenotypic and genotypic characteristics of other pathogens involved in development of IMI and are interested in their comparison with the *S. aureus* and *Strep. uberis* profiles previously studied. Our findings to date, limited to a few bacteria from a restricted number of farms and geographical regions, could prospectively be used for research in order to understand if our conclusions can be universally applicable. The collaboration with a greater number of countries to exchange information and to standardize the sample collection, and the analysis of more comprehensive data could help to increase the strengths of this study and to solve some potential pitfalls. The next steps of this project could include the high-throughput sequencing of different bacterial isolates from clinical and subclinical cases of bovine mastitis occurring in globally distributed herds. The phylogenetic analysis of bacterial strains within the same species could be useful to explore their genetic relationships in association with their virulence and antimicrobial resistance patterns, and the genotypic variability among countries. The obtained results could be further combined with the evaluation of the samples collected from the farm environment via metagenomic approach. Representative conclusions could be reached by comparing bacterial populations within and among countries, particularly with regard to the link of antibiotic usage and management strategies with the molecular characteristics of mastitis causative agents.

Bibliography

Introduction

Aghamohammadi, M., D. Haine, D.F. Kelton, H.W. Barkema, H. Hogeveen, G.P. Keefe, and S. Dufour. 2018. Herd-level mastitis-associated costs on Canadian dairy farms. Front Vet Sci. 5:100. https://doi.org/10.3389/fvets.2018.00100.

Ajiboye, R.M., O.D. Solberg, B.M. Lee, E. Raphael, C. Debroy, and L.W. Riley. 2009. Global spread of mobile antimicrobial drug resistance determinants in human and animal *Escherichia coli* and *Salmonella* strains causing community-acquired infections. Clin Infect Dis. 49(3):365–71. https://doi.org/10.1086/600301.

Alhussien, M.F., and A.K. Dang. 2018. Milk somatic cells, factors influencing their release, future prospects, and practical utility in dairy animals: An overview. Vet World. 11(5):562–577. https://doi.org/10.14202/vetworld.2018.562-577.

Ali, T., S.U. Rahman, L. Zhang, M. Shahid, D. Han, J. Gao, S. Zhang, P.L. Ruegg, U. Saddique, and B. Han. 2017. Characteristics and genetic diversity of multi-drug resistant extended-spectrum beta-lactamase (ESBL)-producing *Escherichia coli* isolated from bovine mastitis. Oncotarget. 8(52):90144-90163. https://doi.org/10.18632/oncotarget.21496.

Amoako, D.G., A.M. Somboro, A.L.K. Abia, M. Allam, A. Ismail, L. Bester, and S.Y. Essack. 2019. Genomic analysis of methicillin-resistant *Staphylococcus aureus* isolated from poultry and occupational farmworkers in Umgungundlovu District, South Africa. Sci Total Environ. 670:704-716. https://doi.org/10.1016/j.scitotenv.2019.03.110.

Barkema, H.W., Y.H. Schukken, and R.N. Zadoks. 2006. Invited review: The role of cow, pathogen, and treatment regimen in the therapeutic success of bovine *Staphylococcus aureus* mastitis. J Dairy Sci. 89:1877–1895. https://doi.org/10.3168/jds.S0022-0302(06)72256-1.

Barkema, H.W., M.A. von Keyserlingk, J.P. Kastelic, T.J. Lam, C. Luby, J.P. Roy, S.J. LeBlanc, G.P. Keefe, and D.F. Kelton. 2015. Invited review: Changes in the dairy industry affecting dairy cattle health and welfare. J Dairy Sci. 98:7426–7445. https://doi.org/10.3168/jds.2015-9377.

Barreiro, J.R., C.R. Ferreira, G.B. Sanvido, M. Kostrzewa, T. Maier, B. Wegemann, V. Böttcher, M.N. Eberlin, and M.V. dos Santos. 2010. Short communication: Identification of subclinical cow mastitis pathogens in milk by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. J Dairy Sci. 93(12):5661–7. https://doi.org/10.3168/jds.2010-3614.

Benites, N.R., J.L. Guerra, P.A. Melville, and E.O. da Costa. 2002. Aetiology and histopathology of bovine mastitis of espontaneous occurrence. J Vet Med B Infect Dis Vet Public Health. 49(8):366-370. https://doi.org/10.1046/j.1439-0450.2002.00566.x.

Bradley, A.J. 2002. Bovine mastitis: an evolving disease. The Veterinary Journal, 164:116-128. https://doi.org/10.1053/tvjl.2002.0724.

Bradley, A.J., and M.J. Green. 2004. The importance of the nonlactating period in the epidemiology of intramammary infection and strategies for prevention. Vet Clin Food Anim. 20:547–568. https://doi.org/doi:10.1016/j.cvfa.2004.06.010.

Bradley, A.J., K.A. Leach, J.E. Breen, L.E. Green, and M.J. Green. 2007. Survey of the incidence and aetiology of mastitis on dairy farms in England and Wales. Veterinary Record. 160(8):253-257. http://doi.org/10.1136/vr.160.8.253.

Bronzo, V., V. Lopreiato, F. Riva, M. Amadori, G. Curone, M.F. Addis, P. Cremonesi, P. Moroni, E. Trevisi, and B. Castiglioni. 2020. The role of innate immune response and microbiome in resilience of dairy cattle to disease: the mastitis model. Animals (Basel). 10(8):1397. https://doi.org/10.3390/ani10081397.

Capra, E., P. Cremonesi, A. Pietrelli, S. Puccio, M. Luini, A. Stella, and B. Castiglioni. 2017. Genomic and transcriptomic comparison between *Staphylococcus aureus* strains associated with high and low within herd prevalence of intra-mammary infection. BMC Microbiol. 17:1–16. https://doi.org/10.1186/s12866-017-0931-8.

Cheng, J., W. Qu, H.W. Barkema, D.B. Nobrega, J. Gao, G. Liu, J. De Buck, J.P. Kastelic, H. Sun, and B. Han. 2019. Antimicrobial resistance profiles of 5 common bovine mastitis pathogens in large Chinese dairy herds. J. Dairy Sci. 102(3):2416-2426. https://doi.org/10.3168/jds .2018-15135.

Collado, R., C. Montbrau, M. Sitjà, and A. Prenafeta. 2018. Study of the efficacy of a *Streptococcus uberis* mastitis vaccine against an experimental intramammary infection with a heterologous strain in dairy cows. J. Dairy Sci. 101:10290–10302. https://doi.org/10.3168/jds.2018-14840.

Contreras, G.A., and J.M. Rodríguez. 2011. Mastitis: comparative etiology and epidemiology. J Mammary Gland Biol Neoplasia.16:339–356. https://doi.org/10.1007/s10911-011-9234-0.

Cordovana, M., A. Boniface Pranada, S. Ambretti, and M. Kostrzewa. 2019. MALDI-TOF bacterial subtyping to detect antibiotic resistance. Clinical Mass Spectrometry. 14:3–8. https://doi.org/10.1016/j.clinms.2019.06.002.

Curone, G., J. Filipe, P. Cremonesi, E. Trevisi, M. Amadori, C. Pollera, B. Castiglioni, L. Turin, V. Tedde, D. Vigo, P. Moroni, A. Minutic, V. Bronzo, M.F. Addis, and F. Riva. 2018. What we have lost: Mastitis resistance in Holstein Friesians and in a local cattle breed. Research in Veterinary Science. 116:88–98. https://doi.org/10.1016/j.rvsc.2017.11.020.

da Costa, L.B., P.J. Rajala-Schultz, A. Hoet, K.S. Seo, K. Fogt, and B.S. Moon. 2014. Genetic relatedness and virulence factors of bovine *Staphylococcus aureus* isolated from teat skin and milk. J Dairy Sci. 97:6907–6916. http://dx.doi.org/10.3168/jds.2014-7972.

Davies, J., and D. Davies. 2010. Origins and evolution of antibiotic resistance. Microbiol Mol Biol Rev. 74(3):417–433. https://doi.org/10.1128/MMBR.00016-10.

Davies, P.L., J.A. Leigh, A.J. Bradley, S.C. Archer, R.D. Emes, and M.J. Green. 2016. Molecular epidemiology of *Streptococcus uberis* clinical mastitis in dairy herds: strain heterogeneity and transmission. J Clin Microbiol. 54(1):68-74. https://doi.org/10.1128/JCM.01583-15.

Derakhshani, H., K.B. Fehr, S. Sepehri, D. Francoz, J. De Buck, H.W. Barkema, J.C. Plaizier, and E. Khafipour. 2018 Invited review: Microbiota of the bovine udder: contributing factors and

potential implications for udder health and mastitis susceptibility. J Dairy Sci. 101:10605–10625. https://doi.org/10.3168/jds.2018-14860.

De Vliegher, S., H. Laevens, L.A. Devriese, G. Opsomer, J.L.M. Leroy, H.W. Barkema, A. de Kruif. 2003. Prepartum teat apex colonization with *Staphylococcus chromogenes* in dairy heifers is associated with low somatic cell count in early lactation. Vet Microbiol. 92(3):245-52. https://doi.org/10.1016/s0378-1135(02)00363-2.

De Vliegher, S., L.K. Fox, S. Piepers, S. McDougall, and H.W. Barkema. 2012. Invited review: Mastitis in dairy heifers: nature of the disease, potential impact, prevention, and control. J Dairy Sci. 95:1025–1040. https://doi.org/10.3168/jds.2010-4074.

Duarte, C.M., P.P. Freitas, and R. Bexiga. 2015. Technological advances in bovine mastitis diagnosis: an overview. J Vet Diagn Invest. 27(6):665-72. https://doi.org/10.1177/1040638715603087.

Dufour, S., V. Wellemans, J.P. Roy, P. Lacasse, A. Ordonez-Iturriaga, and D. Francoz. 2019. Non-antimicrobial approaches at drying-off for treating and preventing intramammary infections in dairy cows. Part 1. Meta-analyses of efficacy of using an internal teat sealant without a concomitant antimicrobial treatment. Anim Health Res Rev. 20:86–97. https://doi.org/10.1017/S1466252319000070.

ECDC (European Centre for Disease Prevention and Control), EFSA (European Food Safety Authority) and EMA (European Medicines Agency). 2015. ECDC/EFSA/EMA first joint report on the integrated analysis of the consumption of antimicrobial agents and occurrence of antimicrobial resistance in bacteria from humans and food - producing animals. EFSA Journal. 13(1):4006. https://doi.org/10.2903/j.efsa.2015.4006.

EMA (European Medicines Agency). 2020. Categorisation of antibiotics used in animals promotes responsible use to protect public and animal health. Accessed Sep. 7, 2021. https://www.ema.europa.eu/en/news/categorisation-antibiotics-used-animals-promotes-responsible-use-protect-public-animal-health.

Entorf, M., A.T. Feßler, H. Kaspar, K. Kadlec, T. Peters, and S. Schwarz. 2016. Comparative erythromycin and tylosin susceptibility testing of streptococci from bovine mastitis. Vet Microbiol. 194:36-42. https://doi.org/10.1016/j.vetmic.2015.12.003.

Ericsson Unnerstad, H., A. Lindberg, K. Persson Waller K, T. Ekman, K. Artursson, M. Nilsson-Ost, and B. Bengtsson. 2009. Microbial aetiology of acute clinical mastitis and agent-specific risk factors. Vet Microbiol. 137:90–97. https://doi.org/10.1016/j.vetmic.2008.12.005.

European Commission. 2015. Commission Notice — Guidelines for the prudent use of antimicrobials in veterinary medicine (2015/C 299/04). Off J Eur Union. C299:7–26.

Foster, T.J. 2004. The *Staphylococcus aureus* "superbug". The Journal of clinical investigation. 114(12):1693–1696. https://doi.org/10.1172/JCI23825.

Foster, T.J. 2017. Antibiotic resistance in *Staphylococcus aureus*. Current status and future prospects. FEMS Microbiol. Rev. 41:430–449. https://doi.org/10.1093/femsre/fux007.

Gomes, F., and M. Henriques. 2016. Control of bovine mastitis: old and recent therapeutic approaches. Curr Microbiol. 72(4):377-82. https://doi.org/10.1007/s00284-015-0958-8.

Goodridge, L., A.R. Hill, and R.W. Lencki. 2004. A review of international standards and the scientific literature on farm milk bulk-tank sampling protocols. J Dairy Sci. 87(9):3099–104. https://doi.org/10.3168/jds.S0022-0302(04)73445-1.

Gorden, P.J., M.D. Kleinhenz, J.A. Ydstie, T.A. Brick, L.M. Slinden, M.P. Peterson, D.E. Straub, and D.T. Burkhardt. 2018. Efficacy of vaccination with a *Klebsiella pneumoniae* siderophore receptor protein vaccine for reduction of *Klebsiella* mastitis in lactating cattle. J Dairy Sci. 101(11):10398-10408. https://doi.org/10.3168/jds.2017-14267.

Gussmann, M., W. Steeneveld, C. Kirkeby, H. Hogeveen, M. Farred, and T. Halasa. 2019a. Economic and epidemiological impact of different intervention strategies for subclinical and clinical mastitis. Preventive Veterinary Medicine 166:78–85. https://doi.org/10.1016/j.prevetmed .2019.03.001.

Gussmann, M., W. Steeneveld, C. Kirkeby, H. Hogeveen, M. Nielen, M. Farre, and T. Halasa, 2019b. Economic and epidemiological impact of different intervention strategies for clinical contagious mastitis. J Dairy Sci. 102:1483–1493. https://doi.org/10.3168/jds.2018-14939.

Hagnestam-Nielsen, C., U. Emanuelson, B. Berglund, and E. Strandberg. 2009. Relationship between somatic cell count and milk yield in different stages of lactation. J Dairy Sci. 92:3124–3133. https://doi.org/10.3168/jds.2008-1719.

Halasa, T., K. Huijps, O. Østerås, and H. Hogeveen. 2007. Economic effects of bovine mastitis and mastitis management: a review. Veterinary Quarterly. 29(1):18-31. https://doi.org/10 .1080/01652176.2007.9695224.

Heikkilä, A.M., J.I. Nousiainen, and S. Pyörälä. 2012. Costs of clinical mastitis with special reference to premature culling. J. Dairy Sci. 95:139-150. http://dx.doi.org/10.3168/jds.2011-4321.

Herry, V., C. Gitton, G. Tabouret, M. Reperant, L. Forge, C. Tasca, F.B. Gilbert, E. Guitton, C. Barc, C. Staub, D.G.E. Smith, P. Germon, G. Foucras, and P. Rainard. 2017. Local immunization impacts the response of dairy cows to *Escherichia coli* mastitis. Sci. Rep. 7:3441. https://doi.org/10.1038/s41598-017-03724-7.

Hertl, J.A., Y.H. Schukken, F.L. Welcome, L.W. Tauer, and Y.T. Gröhn. 2014. Pathogen-specific effects on milk yield in repeated clinical mastitis episodes in Holstein dairy cows. J Dairy Sci. 97:1465–1480. http://dx.doi.org/10.3168/jds.2013-7266.

Hillerton, J.E., and J.M. Booth. 2018. The Five-point mastitis control plan - A revisory tutorial! in NMC 57th Annual Meeting Proceedings. National Mastitis Council, Tucson, Arizona.

Huijps, K., H. Hogeveen, T.J.G.M. Lam, and A.G.J.M. Oude Lansink. 2010. Costs and efficacy of management measures to improve udder health on Dutch dairy farms. J Dairy Sci. 93:115–124. https://doi.org/10.3168/jds.2009-2412.

Kabera, F., J.P. Roy, M. Afifi, S. Godden, H. Stryhn, J. Sanchez, and S. Dufour. 2021. Comparing blanket vs. selective dry cow treatment approaches for elimination and prevention of

intramammary infections during the dry period: a systematic review and meta-analysis. Front Vet Sci. 8:688450. https://doi.org/10.3389/fvets.2021.688450.

Kaczorek, E., J. Małaczewska, R. Wójcik, W. Rękawek, and A.K. Siwicki. 2017. Phenotypic and genotypic antimicrobial susceptibility pattern of *Streptococcus* spp. isolated from cases of clinical mastitis in dairy cattle in Poland. J Dairy Sci. 100(8):6442-6453. https://doi.org/10.3168/jds.2017-12660.

Keane, O.M., K.E. Budd, J. Flynn, and F. McCoy. 2013. Pathogen profile of clinical mastitis in Irish milk-recording herds reveals a complex aetiology. Vet Rec. 173(1):17. https://doi.org/10 .1136/vr.101308.

Keane, O.M. 2019. Symposium review: Intramammary infections—Major pathogens and strain-associated complexity. J Dairy Sci. 102:4713–4726. https://doi.org/10.3168/jds.2018-15326.

Klaas, I.C., and R.N. Zadoks. 2018. An update on environmental mastitis: challenging perceptions. Transbound Emerg. Dis. 65(Suppl 1):166–185. https://doi.org/10.1111/tbed.12704.

Krömker, V., F. Reinecke, J.H. Paduch, and N. Grabowski. 2014. Bovine *Streptococcus uberis* intramammary infections and mastitis. Clin Microbial. 3:157. https://doi.org/10.4172/2327-5073.1000157.

Krömker, V., and S. Leimbach. 2017. Mastitis treatment—Reduction in antibiotic usage in dairy cows. Reprod Domest Anim. 52(Suppl 3):21-29. https://doi.org/10.1111/rda.13032.

Ismail, Z.B. 2017. Mastitis vaccines in dairy cows: recent developments and recommendations of application. Vet World. 10(9):1057–62. https://doi.org/10.14202/vetworld.2017.1057-1062.

Lago, A., S.M. Godden, R. Bey, P.L. Ruegg, and K. Leslie. 2011a. The selective treatment of clinical mastitis based on on-farm culture results: I. Effects on antibiotic use, milk withholding time, and short-term clinical and bacteriological outcomes. J Dairy Sci. 94:4441–4456. https://doi.org/10.3168/jds.2010-4046.

Lago, A., S.M. Godden, R. Bey, P.L. Ruegg, and K. Leslie. 2011b. The selective treatment of clinical mastitis based on on-farm culture results: II. Effects on lactation performance, including

clinical mastitis recurrence, somatic cell count, milk production, and cow survival. J Dairy Sci. 94:4457–4467. https://doi.org/10.3168/jds.2010-4047.

Leigh, J.A., J.M. Finch, T.R. Field, N.C. Real, A. Winter, A.W. Walton, and S.M. Hodgkinson. 1999. Vaccination with the plasminogen activator from *Streptococcus uberis* induces an inhibitory response and protects against experimental infection in the dairy cow. Vaccine 17:851–857. https://doi.org/10.1016/s0264-410x(98)00270-9.

Liang, D., L.M. Arnold, C.J. Stowe, R.J. Harmon, and J.M. Bewley. 2017. Estimating US dairy clinical disease costs with a stochastic simulation model. J Dairy Sci. 100:1472–1486. http://doi.org/10.3168/jds.2016-11565.

Makovec, J.A., and P.L. Ruegg. 2003. Results of milk samples submitted for microbiological examination in Wisconsin from 1994 to 2001. J Dairy Sci. 86:3466–3472. https://doi.org/10 .3168/jds.S0022-0302(03)73951-4.

Marshall, B.M., and S.B. Levy. 2011. Food animals and antimicrobials: impacts on human health. Clin Microbiol Rev. 24(4):718–33. https://doi.org/10.1128/CMR.00002-11.

McDougall, S., H. Hussein, and K. Petrovski. 2014. Antimicrobial resistance in *Staphylococcus aureus*, *Streptococcus uberis* and *Streptococcus dysgalactiae* from dairy cows with mastitis. N. Z. Vet. J. 62:68–76. https://doi.org/10.1080/00480169.2013.843135.

Moroni, P., G. Pisoni, P. Cremonesi, and B. Castiglioni. 2011. *Staphylococcus*. Pages 307–317 in Molecular Detection of Human Bacterial Pathogens. Dongyou Liu, ed. CRC Press, Boca Raton, FL.

Neave, F. K., F.H. Dodd, and E. Henriques. 1950. Udder infections in the dry period. J. Dairy Res. 17:37–49.

Neave, F.K., F.H. Dodd, R.G. Kingwill, and D.R. Westgarth. 1969. Control of mastitis in the dairy herd by hygiene and management. J Dairy Sci. 52:696–707. https://doi.org/10 .3168/jds.S0022-0302(69)86632-4.

Nickerson, S.C., W.E. Owens, and R.L. Boddie. 1995. Mastitis in dairy heifers: initial studies on prevalence and control. J Dairy Sci. 78:1607–1618. https://doi.org/10.3168/jds.S0022-0302(95)76785-6.

Nickerson, S.C. and V. Ryman. 2019.Vaccination as a tool to control mastitis in dairy cows. UGA Cooperative Extension Bulletin 1501. Pages 1-6.

NMC. 2016. NMC Current concepts of bovine mastitis. – Fifth edition. National Mastitis Council, New Prague, Minnesota, USA.

NMC. 2017. NMC Laboratory handbook on bovine mastitis – Third edition. National Mastitis Council, New Prague, Minnesota, USA.

Olde Riekerink, R.G., H.W. Barkema, D.F. Kelton, and D.T. Scholl. 2008 Incidence rate of clinical mastitis on Canadian dairy farms. J Dairy Sci. 91(4):1366–77. https://doi.org/10.3168/jds.2007-0757.

Oliveira, L., C. Hulland, C., and P.L. Ruegg. 2013. Characterization of clinical mastitis occurring in cows on 50 large dairy herds in Wisconsin. J Dairy Sci. 96(12):7538–7549. https://doi.org/10.3168/jds.2012-6078.

Oliveira, L., and P.L. Ruegg. 2014. Treatments of clinical mastitis occurring in cows on 51 large dairy herds in Wisconsin. J Dairy Sci. 97(9):5426–36. https://doi.org/10.3168/jds.2013-7756.

Pankey, J.W., N.T. Boddie, J.L. Watts, and S.C. Nickerson. 1985. Evaluation of protein A and a commercial bacterin as vaccines against *Staphylococcus aureus* mastitis by experimental challenge. J Dairy Sci. 68:726–731. https://doi.org/10.3168/jds.S0022-0302(85)80879-1.

Paterson G.K., E.M. Harrison, and M.A. Holmes. 2014. The emergence of *mecC* methicillin-resistant *Staphylococcus aureus*. Trends Microbiol. 22:42–47. https://doi.org/10.1016/j.tim.2013.11.003.

Peton, V., and Y. Le Loir. 2014. *Staphylococcus aureus* in veterinary medicine. Infect Genet Evol. 21:602-15. https://doi.org/10.1016/j.meegid.2013.08.011.

Pol, M., and P.L. Ruegg. 2007. Treatment practices and quantification of antimicrobial drug usage in conventional and organic dairy farms in Wisconsin. J Dairy Sci. 90: 249–261. https://doi.org/10.3168/jds.S0022-0302(07)72626-7.

Prado, M.E., R.A. Almeida, C. Ozen, D.A. Luther, M.J. Lewis, S.J. Headrick, and S.P. Oliver. 2011. Vaccination of dairy cows with recombinant *Streptococcus uberis* adhesion molecule induces antibodies that reduce adherence to and internalization of *S. uberis* into bovine mammary epithelial cells. Vet Immunol Immunopathol. 141:201–208. https://doi.org/10.1016/j.vetimm .2011.02.023.

Prenafeta, A., R. March, A. Foix, I. Casals, and L. Costa. 2010. Study of the humoral immunological response after vaccination with a *Staphylococcus aureus* biofilm-embedded bacterin in dairy cows: possible role of the exopolysaccharide specific antibody production in the protection from *Staphylococcus aureus* induced mastitis. Vet. Immunol. Immunopathol. 134:208‑217. https://doi.org/10.1016/j.vetimm.2009.09.020

Pyörälä, S. 2002. New strategies to prevent mastitis. Reprod Domest Anim. 37(4):211-6. https://doi.org/10.1046/j.1439-0531.2002.00378.x.

Rabiee, A.R., and I.J. Lean. 2013. The effect of internal teat sealant products (Teatseal and Orbeseal) on intramammary infection, clinical mastitis, and somatic cell counts in lactating dairy cows: a meta-analysis. J Dairy Sci. 96(11):6915-6931. https://doi.org/10.3168/jds.2013-6544.

Rajala-Schultz, P.J., K.L. Smith, J.S. Hogan, and B.C. Love. 2004. Antimicrobial susceptibility of mastitis pathogens from first lactation and older cows. Vet Microbiol. 102(1-2):33-42. https://doi.org/10.1016/j.vetmic.2004.04.010.

Rollin, E., K.C. Dhuyvetterb, and M.W. Overtona. 2015. The cost of clinical mastitis in the first 30 days of lactation: an economic modeling tool. Preventive Veterinary Medicine. 122:257–264. https://doi.org/10.1016/j.prevetmed.2015.11.006.

Ruegg, P.L. 2003. Practical food safety interventions for dairy production. J Dairy Sci. 86(E. Suppl.):E1–E9. https://doi.org/10.3168/jds.S0022-0302(03)74034-X.

Ruegg, P.L., 2011. Managing mastitis and producing quality milk. Pages 207-232 in Dairy Production Medicine. C.A. Risco, and P. Melendez Retamal, ed. Wiley-Blackwell, John Wiley & Sons, Inc. Chichester, West Sussex.

Ruegg, P.L., L. Oliveira, W. Jin, and O. Okwumabua. 2015. Phenotypic antimicrobial susceptibility and occurrence of selected resistance genes in gram-positive mastitis pathogens isolated from Wisconsin dairy cows. J. Dairy Sci. 98:4521–4534. https://doi.org/10.3168/jds.2014-9137.

Ruegg, P.L. 2017. A 100-Year Review: mastitis detection, management, and prevention. J Dairy Sci. 100:10381–10397. https://doi.org/10.3168/jds.2017-13023.

Ruegg, P.L. 2018. Making antibiotic treatment decisions for clinical mastitis. Vet Clin North Am Food Anim Pract. 34(3):413-425. https://doi.org/10.1016/j.cvfa.2018.06.002.

Sawant, A.A., B.E Gillespie, and S.P. Oliver. 2009. Antimicrobial susceptibility of coagulase-negative *Staphylococcus* species isolated from bovine milk. Vet. Microbiol. 134:73–81. https://doi.org/10.1016/j.vetmic.2008.09.006.

Schroeder, J.W. 2012. Bovine mastitis and milking management. Extension Dairy Specialist. AS1129 (Revised):1-16.

Schukken, Y.H., K.E. Leslie, D.A. Barnum, B.A. Mallard, J.H. Lumsden, P.C. Dick, G.H. Vessie, and M.E. Kehrli. 1999. Experimental *Staphylococcus aureus* intramammary challenge in late lactation dairy cows: quarter and cow effects determining the probability of infection. J Dairy Sci. 82:2393-401. https://doi.org/10.3168/jds.S0022-0302(99)75490-1.

Schukken, Y.H., R.N González, L.L. Tikofsky, H.F. Schulte, C.G. Santisteban, F.L. Welcome, G.J. Bennett, M.J. Zurakowski, and R.N. Zadoks. 2009. CNS mastitis: nothing to worry about? Vet Microbiol. 134(1-2):9–14. https://doi.org/10.1016/j.vetmic.2008.09.014.

Schukken, Y.H., J. Gunther, J. Fitzpatrick, M.C. Fontaine, L. Goetze, O. Holst, J. Leigh, W. Petzl, H.J. Schuberth, A. Sipka, D.G.E. Smith, R. Quesnell, J. Watts, R. Yancey, H. Zerbe, A. Gurjar, R.N. Zadoks, H.M. Seyfert., and members of the Pfizer mastitis research consortium.

2011. Host-response patterns of intramammary infections in dairy cows. Vet. Immunol Immunopathol. 144(3-4):270–289. https://doi.org/10.1016/j.vetimm.2011.08.022.

Schukken, Y.H., E. Schmitt-Van De Leemput, P. Moroni, F. Welcome, A. Gurjar, M. Zurakowski, and C. Gutierrez. 2012. Contagious or environmental - A herd diagnosis. Pages 145-148 in Proceedings of World Buiatrics Congress. World Association for Buiatrics, Lisbon, Portugal.

Shkreta, L., B.G. Talbot, M.S. Diarra, and P. Lacasse. 2004. Immune responses to a DNA/protein vaccination strategy against *Staphylococcus aureus* induced mastitis in dairy cows. Vaccine. 23:114–126. https://doi.org/10.1016/j.vaccine.2004.05.002.

Sol, J., O. C. Sampimon, J. J. Snoep, and Y. H. Schukken. 1997. Factors associated with bacteriological cure during lactation after therapy for subclinical mastitis caused by *Staphylococcus aureus*. J Dairy Sci. 80:2803–2808. https://doi.org/10.3168/jds.S0022-0302(97)76243-X.

Sommer, F., J. Anderson, R. Bharti, J. Raes, and P. Rosenstiel. 2017. The resilience of the intestinal microbiota influences health and disease. Nat. Rev. Microbiol. 15:630–638. https://doi.org/10.1038/nrmicro.2017.58.

Sommerhauser, J., B. Kloppert, W. Wolter, M. Zschock, A. Sobiraj, and K. Failing. 2003. The epidemiology of *Staphylococcus aureus* infections from subclinical mastitis in dairy cows during a control programme. Vet Microbiol. 96:91–102. https://doi.org/10.1016/s0378-1135(03)00204-9.

Song, B., X. Yang, H. Sun, L. Yu, J. Ma, Z. Wu, and Y. Cui. 2017. Immunogenicity of amino acids 1–150 of *Streptococcus* GapC displayed on the surface of *Escherichia coli*. Microb Pathog. 105:288–297. https://doi.org/10.1016/j.micpath.2017.02.003.

Sordillo, L.M. 2018. Mammary gland immunobiology and resistance to mastitis. Vet Clin Food Anim. 34: 507–523. https://doi.org/10.1016/j.cvfa.2018.07.005.

Sørensen, L.P., T. Mark, M.K. Sørensen, and S. Østergaard. 2010. Economic values and expected effect of selection index for pathogen-specific mastitis under Danish conditions. J. Dairy Sci. 93:358-369. http://dx.doi.org/10.3168/jds.2009-2506.

Suojala, L., L. Kaartinen, and S. Pyörälä. 2013. Treatment for bovine *Escherichia coli* mastitis – An evidence-based approach. Journal of Veterinary Pharmacology and Therapeutics. 36(6):521–531. https://doi.org/10.1111/jvp.12057.

Taponen, S., and S. Pyörälä. 2009. Coagulase-negative staphylococci as cause of bovine mastitis-not so different from *Staphylococcus aureus*? Vet Microbiol. 134(1-2):29–36. https://doi.org/10.1016/j.vetmic.2008.09.011.

Tassi, R., T.N. McNeilly, J.L. Fitzpatrick, M.C. Fontaine, D. Reddick, C. Ramage, M. Lutton, Y.H. Schukken, and R.N. Zadoks. 2013. Strain-specific pathogenicity of putative host-adapted and nonadapted strains of *Streptococcus uberis* in dairy cattle. J Dairy Sci. 96:5129–5145. https://doi.org/10.3168/jds.2013-6741.

Timms, L. 2001. Field trial evaluations of a novel persistent barrier teat dip for preventing mastitis during the dry period and as a potential substitute for dry cow antibiotic therapy. In NMC 40th Annual Meeting Proceedings. National Mastitis Council, Reno, Nevada.

Tomazi, T., G. Freu, B.G. Alves, A.F. de Souza Filho, M.B. Heinemann, and M. Veiga Dos Santos. 2019. Genotyping and antimicrobial resistance of *Streptococcus uberis* isolated from bovine clinical mastitis. PLoS One. 14(10):e0223719. https://doi.org/10.1371/journal.pone.0223719.

Torres, A.H., P.J. Rajala-Schultz, F.J. DeGraves, and K.H. Hoblet. 2008. Using dairy herd improvement records and clinical mastitis history to identify subclinical mastitis infections at dry-off. J Dairy Res. 75:240–247. https://doi.org/10.1017/S0022029908003257.

Trevisi, E., A. Zecconi, S. Cogrossi, E. Razzuoli, P. Grossi, and M. Amadori. 2014. Strategies for reduced antibiotic usage in dairy cattle farms. Res Vet Sci. 96(2):229-33. https://doi.org/10.1016/j.rvsc.2014.01.001.

USDA (United States Department of Agriculture), APHIS (Animal and Plant Health Inspection Service), and VS (Veterinary Services). 1996. Part III: Reference of 1996 dairy health and health management. NAHMS (National Animal Health Monitoring System), Fort Collins, CO.

van Soest, F.J.S., I. Santman-Berends, T. Lam, and H. Hogeveen. 2016. Failure and preventive costs of mastitis on Dutch dairy farms. J Dairy Sci. 99(10):8365–74. https://doi.org/10 .3168/jds.2015-10561.

Wald, R., M. Baumgartner, J. Gutschireiter, B. Bazzanella, K. Lichtmannsperger, M. Wagner, T. Wittek, and B. Stessl. 2020. Comparison of the population structure of *Streptococcus uberis* mastitis isolates from Austrian small-scale dairy farms and a Slovakian large-scale farm. J Dairy Sci. 103(2):1820-1830. https://doi.org/10.3168/jds.2019-16930.

Walker, R.D. 2006. Antimicrobial susceptibility testing methods and interpretation of results. Pages 11-26 in Antimicrobial Therapy in Veterinary Medicine. S. Guiguere, J.F. Prescott, J.D. Baggot, R.D. Walker, P.M. Dowling, ed. Blackwell Publishing, Ames, IA.

WHO. 2014. Antimicrobial resistance: global report on surveillance. World Health Organization, Geneva, Switzerland.

WHO. 2015. Global action plan on antimicrobial resistance. World Health Organization, Geneva, Switzerland.

WHO. 2017. Global priority list of antibiotic resistant bacteria to guide research, discoveries and development of new antibiotics. World Health Organization, Geneva, Switzerland.

Williams, W.L. 1937. The detection of shedders of the *Streptococcus* of mastitis in composite control milk samples. J Dairy Sci. 20:711–717.

Wilson, D.J., B.A. Mallard, J.L. Burton, Y.H. Schukken, and Y.T. Grohn. 2009. Association of *Escherichia coli* J5-specific serum antibody responses with clinical mastitis outcome for J5 vaccinate and control dairy cattle. Clin Vaccine Immunol. 16(2):209–217. https://doi.org/10 .1128/CVI.00324-08.

Zadoks, R.N., W.B. van Leeuwen, D. Kreft, L.K. Fox, H.W. Barkema, Y.H. Schukken, and A. van Belkum. 2002. Comparison of *Staphylococcus aureus* isolates from bovine and human skin, milking equipment, and bovine milk by phage typing, pulsed-field gel electrophoresis, and binary typing. J Clin Microbiol. 40:3894–3902. https://doi.org/10.1128/JCM.40.11.3894-3902.2002.

Zadoks, R.N., B.E. Gillespie, H.W. Barkema, O.C. Sampimon, S.P. Oliver, and Y.H. Schukken. 2003. Clinical, epidemiological and molecular characteristics of *Streptococcus uberis* infections in dairy herds. Epidemiol Infect. 130(2):335–349. https://doi.org/10.1017/s0950268802008221.

Zadoks, R.N., L.L. Tikofsky, and K.J. Boor KJ. 2005. Ribotyping of *Streptococcus uberis* from a dairy's environment, bovine feces and milk. Vet Microbiol. 109:257–265. https://doi.org/10.1016/j.vetmic.2005.05.008

Zadoks, R.N., and J.L. Fitzpatrick. 2009. Changing trends in mastitis. Ir Vet J. 62:S59. https://doi.org/10.1186/2046-0481-62-S4-S59.

Zadoks, R.N., J.R. Middleton, S. McDougall, J. Katholm, and Y.H. Schukken. 2011. Molecular epidemiology of mastitis pathogens of dairy cattle and comparative relevance to humans. J Mammary Gland Biol Neoplasia. 16:357–372. https://doi.org/10.1007/s10911-011-9236-y.

Zhao, X., and P. Lacasse. 2008. Mammary tissue damage during bovine mastitis: Causes and control. J Anim. Sci. 86:57–65. https://doi.org/10.2527/jas.2007-0302.

Part I: *Staphylococcus aureus*

Alonzo, F. 3rd, M.A. Benson, J. Chen, R.P. Novick, B. Shopsin, and V.J. Torres. 2012. *Staphylococcus aureus* leucocidin ED contributes to systemic infection by targeting neutrophils and promoting bacterial growth in vivo. Mol. Microbiol. 83:423–435. https://doi.org/10.1111/j.1365-2958.2011.07942.x.

Antók, F.I., R. Mayrhofer, H. Marbach, J.C. Masengesho, H. Keinprecht, V. Nyirimbuga, O. Fischer, S. Lepuschitz, W. Ruppitsch, M. Ehling-Schulz, A.T. Feßler, S. Schwarz, S. Monecke, R. Ehricht, T. Grunert, J. Spergser, and I. Loncaric. 2019. Characterization of antibiotic and biocide resistance genes and virulence factors of *Staphylococcus* species associated with bovine mastitis in Rwanda. Antibiotics. 9(1):1. https://doi.org/10.3390/antibiotics9010001.

Barkema, H.W., Y.H. Schukken, and R.N. Zadoks. 2006. Invited review: The role of cow, pathogen, and treatment regimen in the therapeutic success of bovine *Staphylococcus aureus* mastitis. J Dairy Sci. 89:1877–1895. https://doi.org/10.3168/jds.S0022-0302(06)72256-1.

Basset, P., E.J. Feil, G. Zanetti, and D.S. Blanc. 2011. The evolution and dynamics of methicillin-resistant *Staphylococcus aureus*. Pages 669-688 in Genetics and Evolution of Infectious Disease. M. Tibayrenc, ed. Elsevier, London. https://doi.org/10.1016/B978-0-12-384890-1.00025-X.

Bitrus, A.A, Z. Zunita, S.K. Bejo, S. Othman, and N.A. Nadzir. 2017. In vitro transfer of methicillin resistance determinants *mecA* from methicillin resistant *Staphylococcus aureus* (MRSA) to methicillin susceptible *Staphylococcus aureus* (MSSA). BMC microbiology. 17(1):83. https://doi.org/10.1186/s12866-017-0994-6.

Bloemendaal, A.L.A., E.C. Brouwer, and A.C. Fluit. 2010. Methicillin resistance transfer from *Staphylocccus epidermidis* to methicillin-susceptible *Staphylococcus aureus* in a patient during antibiotic therapy. PLoS One. 5(7):e11841. https://doi.org/10.1371/journal.pone.0011841.

Clarke, S.R., and S. J. Foster. 2006. Surface adhesins of *Staphylococcus aureus*. Adv Microb Physiol. 51:187-224. https://doi.org/10.1016/S0065-2911(06)51004-5.

Cockerill, F.R. 1999. Genetic methods for assessing antimicrobial resistance. Antimicrob. Agents Chemother. 43:199–212. https://doi.org/10.1128/AAC.43.2.199.

Connell, S.R., C.A. Trieber, G.P. Dinos, E. Einfeldt, D.E. Taylor, K.H. Nierhaus. 2003. Mechanism of Tet(O)-mediated tetracycline resistance. EMBO J. 22:945–953. https://doi.org/10 .1093/emboj/cdg093.

Cosandey, A., R. Boss, M. Luini, K. Artursson, M. Bardiau, F. Breitenwieser, E. Hehenberger, T. Lam, M. Mansfeld, A. Michel, G. Mösslacher, J. Naskova, S. Nelson, O. Podpečan, A. Raemy, E. Ryan, O. Salat, P. Zangerl, A. Steiner, and H.U. Graber. 2016. *Staphylococcus aureus* genotype B and other genotypes isolated from cow milk in European countries. J Dairy Sci. 99(1):529-540. https://doi.org/10.3168/jds.2015-9587.

Cremonesi, P., F. Pozzi, M. Raschetti, G. Bignoli, E. Capra, H.U. Graber, F. Vezzoli, R. Piccinini, B. Bertasi, S. Biffani, B. Castiglioni, and M. Luini. 2015. Genomic characteristics of *Staphylococcus aureus* strains associated with high within-herd prevalence of intramammary infections in dairy cows. J Dairy Sci. 98:6828-6838. https://doi.org/10.3168/jds.2014-9074.

Cuny, C., M. Abdelbary, F. Layer, G. Werner, and W. Witte. 2015. Prevalence of the immune evasion gene cluster in *Staphylococcus aureus* CC398. Veterinary Microbiology. 177:219–223. https://doi.org/10.1016/j.vetmic.2015.02.031.

Dumont, A.L., T.K. Nygaard, R.L. Watkins, A. Smith, L. Kozhaya, B.N. Kreiswirth, B. Shopsin, D. Unutmaz, J.M. Voyich, and V.J. Torres. 2011. Characterization of a new cytotoxin that contributes to *Staphylococcus aureus* pathogenesis. Mol. Microbiol. 79:814–825. https://doi.org/10.1111/j.1365-2958.2010.07490.x.

Enright, M.C., N.P. Day, C.E. Davies, S.J. Peacock, and B.G. Spratt. 2000. Multilocus sequence typing for characterization of methicillin-resistant and methicillin-susceptible clones of *Staphylococcus aureus*. J. Clin. Microbiol. 38:1008. https://doi.org/10.1128/JCM.38.3.1008-1015.2000.

Entorf, M., A.T. Feßler, H. Kaspar, K. Kadlec, T. Peters, and S. Schwarz. 2016. Comparative erythromycin and tylosin susceptibility testing of streptococci from bovine mastitis. Vet Microbiol. 194:36-42. https://doi.org/10.1016/j.vetmic.2015.12.003.

Foster, T.J. 2005. Immune evasion by staphylococci. Nat Rev Microbiol. 3(12):948-58. https://doi.org/10.1038/nrmicro1289.

Foster, T.J., 2017. Antibiotic resistance in *Staphylococcus aureus*. Current status and future prospects. FEMS Microbiol. Rev. 41:430–449. https://doi.org/10.1093/femsre/fux007.

Founou, L.L., R.C. Founou, S.Y. Essack, and C.F. Djoko. 2018. Mannitol-fermenting methicillin-resistant staphylococci (MRS) in pig abattoirs in Cameroon and South Africa: a serious food safety threat. Int J Food Microbiol. 285:50-60. https://doi.org/10.1016/j.ijfoodmicro .2018.07.006.

Fournier, C., P. Kuhnert, J. Frey, R. Miserez, M. Kirchhofer, T. Kaufmann, A. Steiner, and H.U. Graber. 2008. Bovine *Staphylococcus aureus*: association of virulence genes, genotypes and clinical outcome. Res. Vet. Sci. 85:439–448. https://doi.org/10.1016/j.rvsc.2008.01.010.

Gao, J., M. Ferreri, F. Yu, X. Liu, L. Chen, J. Su, and B. Han. 2012. Molecular types and antibiotic resistance of *Staphylococcus aureus* isolates from bovine mastitis in a single herd in China. Vet. J. 192:550–552. https://doi.org/10.1016/j.tvjl.2011.08.030.

Gatermann, S.G., T. Koschinski, and S. Friedrich. 2007. Distribution and expression of macrolide resistance genes in coagulase-negative staphylococci. Clinical Microbiology and Infection. 13:777–781. https://doi.org/10.1111/j.1469-0691.2007.01749.x.

Graber, H.U., J. Naskova E. Studer, T. Kaufmann, M. Kirchhofer, M. Brechbuhl, W. Schaeren, A. Steiner, and C. Fournier. 2009. Mastitis-related subtypes of bovine *Staphylococcus aureus* are characterized by different clinical properties. J. Dairy Sci. 92(4):1442–1451. https://doi.org/10.3168/jds.2008-1430.

Graveland, H., B. Duim, E. van Duijkeren, D. Heederik, and J.A. Wagenaar. 2011. Livestock-associated methicillin-resistant *Staphylococcus aureus* in animals and humans. Int J Med Microbiol. 301(8):630-4. https://doi.org/10.1016/j.ijmm.2011.09.004.

Grumann, D., U. Nubel, and B.M. Barbara. 2014. *Staphylococcus aureus* toxins – Their functions and genetics. Infect Genet Evol. 21:583-92. https://doi.org/10.1016/j.meegid.2013.03.013.

Haim, M., A. Trost, C.J. Maier, G. Achatz, S. Feichtner, H. Hintner, J.W. Bauer, and K. Onder. 2010. Cytokeratin 8 interacts with clumping factor B: a new possible virulence factor target. Microbiology. 156:3710–21. https://doi.org/10.1099/mic.0.034413-0.

Harmsen, D., H. Claus, W. Witte, J. Rothganger J., H. Claus, D. Turnwald, and U. Vogel. 2003. Typing of methicillin-resistant *Staphylococcus aureus* in a university hospital setting by using novel software for *spa* repeat determination and database management. J. Clin. Microbiol. 41:5442–5448. https://doi.org/10.1128/JCM.41.12.5442-5448.2003.

Jensen, M.A., J.A. Webster, and N. Straus. 1993. Rapid identification of bacteria on the basis of polymerase chain reaction-amplified ribosomal DNA spacer polymorphisms. Appl. Environ. Microbiol. 59:945–952. https://doi.org/10.1128/aem.59.4.945-952.1993.

Jevons, M.P., A.W. Coe, and M.T. Parker.1963. Methicillin resistance in staphylococci. Lancet 1:904-907.

Kaczorek, E., J. Małaczewska, R. Wójcik, W. Rękawek, and A.K. Siwicki. 2017. Phenotypic and genotypic antimicrobial susceptibility pattern of *Streptococcus* spp. isolated from cases of clinical mastitis in dairy cattle in Poland. J Dairy Sci. 100(8):6442-6453. https://doi.org/10 .3168/jds.2017-12660.

Kirby, W.M. 1944. Extraction of a highly potent penicillin inactivator from penicillin resistant staphylococci. Science. 99:452. https://doi.org/10.1126/science.99.2579.452.

Leuenberger, A., C. Sartori, R. Boss, G. Resch, F. Oechslin, A. Steiner, P. Moreillon, and H.U. Graber. 2019. Genotypes of *Staphylococcus aureus*: on-farm epidemiology and the consequences for prevention of intramammary infections. J. Dairy Sci. 102:1–15. https://doi.org/10.3168/jds.2018-15181.

Levy, S.B., L.M. McMurry, T.M. Barbosa, V. Burdett, P. Courvalin, W. Hillen, M.C. Roberts, J.I. Rood, and D.E. Taylor. 1999. Nomenclature for new tetracycline resistance determinants. Antimicrob. Agents Chemother. 43:1523–1524. https://doi.org/10.1128/AAC.43.6.1523.

Magro, G., S. Biffani, G. Minozzi, R. Ehricht, S. Monecke, M. Luini, and R. Piccinini. 2017. Virulence genes of *S. aureus* from dairy cow mastitis and contagiousness risk. Toxins (Basel) 9:195. https://doi.org/10.3390/toxins9060195.

Makovec, J.A., and P.L. Ruegg. 2003. Antimicrobial resistance of bacteria isolated from dairy cow milk samples submitted for bacterial culture: 8,905 samples (1994–2001). J. Am. Vet. Med. Assoc. 222(1):1582–1589. https://doi.org/10.2460/javma.2003.222.1582.

McCallum, N., B. Berger-Bächi, and M.M. Senn. 2010. Regulation of antibiotic resistance in *Staphylococcus aureus*. Int J Med Microbiol. 300(2-3):118-29. https://doi.org/10 .1016/j.ijmm.2009.08.015

McCarthy, A.J., J.A. Lindsay, and A. Loeffler. 2012. Are all methicillin-resistant *Staphylococcus aureus* (MRSA) equal in all hosts? Epidemiological and genetic comparison between animal and human MRSA. Vet. Dermatol. 23:267–274. https://doi.org/10.1111/j.1365-3164.2012.01072.x.

Mekonnen, H., S. Workineh, M. Bayleyegne, A. Moges, and K. Tadele. 2005. Antimicrobial susceptibility profile of mastitis isolates from the cows in three major Ethiopian dairies. Vet. Med. 7:391-394.

Mekonnen, S.A., T.J.G.M. Lam, J. Hoekstra, V.P.M.G. Rutten, T.S. Tessema, E.M. Broens, A.E. Riesebos, M.P. Spaninks and G. Koop. 2018. Characterization of *Staphylococcus aureus* isolated from milk samples of dairy cows in small holder farms of North-Western Ethiopia. BMC Vet Res. 14(1):246. https://doi.org/10.1186/s12917-018-1558-1.

Monistero, V., H.U. Graber, C. Pollera, P. Cremonesi, B. Castiglioni, E. Bottini, A. Ceballos-Marquez, L. Lasso-Rojas, V. Kroemker, N. Wente, I.M. Petzer, C. Santisteban, J. Runyan, M. Veiga Dos Santos, B.G. Alves, R. Piccinini, V. Bronzo, M.S. Abbassi, M.B. Said, and P. Moroni. 2018. *Staphylococcus aureus* isolates from bovine mastitis in eight countries: genotypes, detection of genes encoding different toxins and other virulence genes. Toxins. 10(6):247. https://doi.org/10.3390/toxins10060247.

Monistero, V., A. Barberio, F. Biscarini, P. Cremonesi, B. Castiglioni, H.U. Graber, E. Bottini, A. Ceballos-Marquez, V. Kroemker, I.M. Petzer, C. Pollera, C. Santisteban, M. Veiga Dos Santos, V. Bronzo, R. Piccinini, G. Re, M. Cocchi, and P. Moroni. 2020. Different distribution of antimicrobial resistance genes and virulence profiles of *Staphylococcus aureus* strains isolated from clinical mastitis in six countries. J Dairy Sci. 103(4):3431-3446. https://doi.org/10.3168/jds.2019-17141.

Ndahetuye, J.B., Y. Persson, A.K. Nyman, M. Tukei, M.P Ongol, and R. Båge. 2019. Aetiology and prevalence of subclinical mastitis in dairy herds in peri-urban areas of Kigali in Rwanda. Trop Anim Health Prod. 51(7):2037-2044. https://doi.org/10.1007/s11250-019-01905-2.

Olsen, J.E., H. Christensen, and F.M. Aarestrup. 2006. Diversity and evolution of *blaZ* from *Staphylococcus aureus* and coagulase-negative staphylococci. Journal of Antimicrobial Chemotherapy. 57:450–460. https://doi.org/10.1093/jac/dki492.

Paterson G.K., E.M. Harrison, and M.A. Holmes. 2014. The emergence of *mecC* methicillin-resistant *Staphylococcus aureus*. Trends Microbiol. 22:42–47. https://doi.org/10.1016/j.tim.2013 .11.003.

Peton, V., and Y. Le Loir. 2014. *Staphylococcus aureus* in veterinary medicine. Infect Genet Evol. 21:602-15. https://doi.org/10.1016/j.meegid.2013.08.011.

Pol, M., and P.L. Ruegg. 2007. Treatment practices and quantification of antimicrobial drug usage in conventional and organic dairy farms in Wisconsin. J Dairy Sci. 90: 249–261. https://doi.org/10.3168/jds.S0022-0302(07)72626-7.

Ruegg, P.L., L. Oliveira, W. Jin, and O. Okwumabua. 2015. Phenotypic antimicrobial susceptibility and occurrence of selected resistance genes in gram-positive mastitis pathogens isolated from Wisconsin dairy cows. J. Dairy Sci. 98:4521–4534. https://doi.org/10.3168/jds.2014-9137.

Schlotter, K., R. Ehricht, H. Hotzel, S. Monecke, M. Pfeffer, and K. Donat. 2012. Leukocidin genes *lukF-P83* and *lukM* are associated with *Staphylococcus aureus* clonal complexes 151, 479 and

133 isolated from bovine udder infections in Thuringia, Germany. Vet Res. 43:42. https://doi.org/10.1186/1297-9716-43-42.

Schroeder, J. W. 2012. Bovine mastitis and milking management. Extension Dairy Specialist. AS1129 (Revised):1-16.

Silva, V., J.L. Capelo, G. Igrejas, and P. Poeta. 2020. Molecular epidemiology of *Staphylococcus aureus* lineages in wild animals in Europe: a review. Antibiotics. 9(3):122. https://doi.org/10.3390/antibiotics9030122.

Speziale, P., G. Pietrocola, S. Rindi, M. Provenzano, G. Provenza, A. Di Poto, L. Visai, and C.R. Arciola. 2009. Structural and functional role of *Staphylococcus aureus* surface components recognizing adhesive matrix molecules of the host. Future Microbiol. 4(10):1337-1352. https://doi.org/10.2217/fmb.09.102.

USDA (United States Department of Agriculture), APHIS (Animal and Plant Health Inspection Service), and VS (Veterinary Services). 2007. Part V: Changes in dairy cattle health and management practices in the United States, 1996–2007. NAHMS (National Animal Health Monitoring Service), Washington, DC.

van den Borne, B.H.P., H.U. Graber, V. Voelk, C. Sartori, A. Steiner, M.C. Haerdi-Landerer, and M. Bodmer. 2017. A longitudinal study on transmission of *Staphylococcus aureus* genotype B in Swiss communal dairy herds. Prev. Vet. Med. 136:65–68. https://doi.org/10.1016/j.prevetmed.2016.11.008.

Vandenesch, F., G. Lina, and T. Henry. 2012. *Staphylococcus aureus* hemolysins, bicomponent leukocidins, and cytolytic peptides: a redundant arsenal of membrane-damaging virulence factors? Frontiers in cellular and infection microbiology 2:12. https://doi.org/10.3389/fcimb.2012.00012.

Ventura, C.L., N. Malachowa, C.H. Hammer, G.A. Nardone, M.A. Robinson, S.D. Kobayashi, and F.R. DeLeo. 2010. Identification of a novel *Staphylococcus aureus* two-component

leukotoxin using cell surface proteomics. PLoS ONE. 5(7):e11634. https://doi.org/10 .1371/journal.pone .0011634.

Verkaik, N.J., M. Benard, H.A. Boelens, C.P. de Vogel, J.L. Nouwen, H.A. Verbrugh, D.C. Melles, A. van Belkum, and W.J. van Wamel. 2011. Immune evasion cluster-positive bacteriophages are highly prevalent among human *Staphylococcus aureus* strains, but they are not essential in the first stages of nasal colonization. Clin Microbiol Infect. 17(3):343-8. https://doi.org/10 .1111/j.1469-0691.2010.03227.x.

Voss, A., F. Loeffen, J. Bakker, C. Klaassen, and M. Wulf. 2005. Methicillin-resistant *Staphylococcus aureus* in pig farming. Emerging infectious diseases. 11(12):1965–1966. https://doi.org/10.3201/eid1112.050428.

Walker, R.D. 2006. Antimicrobial susceptibility testing methods and interpretation of results. Pages 11-26 in Antimicrobial Therapy in Veterinary Medicine. Guiguere S., Prescott J.F., Baggot J.D., Walker R.D., Dowling P.M. ed. Blackwell Publishing, Ames, IA.

Part II: *Streptococcus uberis*

Almeida, R.A., O.K. Dego, S.I. Headrick, M.J. Lewis, and S.P. Oliver. 2015. Role of *Streptococcus uberis* adhesion molecule in the pathogenesis of *Streptococcus uberis* mastitis. Vet Microbiol. 179(3-4):332-5. https://doi.org/10.1016/j.vetmic.2015.07.005.

Boonyayatra, S., P. Tharavichitkul, and S.P. Oliver. 2018. Virulence-associated genes and molecular typing of *Streptococcus uberis* associated with bovine mastitis in northern Thailand. Turk. J. Vet. Anim. Sci. 42:73–81. https://doi.org/10.3906/vet-1704-75.

Davies, P.L., J.A. Leigh, A.J. Bradley, S.C. Archer, R.D. Emes, and M.J. Green. 2016. Molecular epidemiology of *Streptococcus uberis* clinical mastitis in dairy herds: strain heterogeneity and transmission. J Clin Microbiol. 54(1):68-74. https://doi.org/10.1128/JCM.01583-15.

de Jong, A., F.E. Garch, S. Simjee, H. Moyaert, M. Rose, M. Youala, E. Siegwart, and VetPath Study Group. 2018. Monitoring of antimicrobial susceptibility of udder pathogens recovered from cases of clinical mastitis in dairy cows across Europe: VetPath results. Vet Microbiol. 213:73-81. https://doi.org/10.1016/j.vetmic.2017.11.021.

Entorf, M., A.T. Feßler, H. Kaspar, K. Kadlec, T. Peters, and S. Schwarz. 2016. Comparative erythromycin and tylosin susceptibility testing of streptococci from bovine mastitis. Vet Microbiol. 194:36-42. https://doi.org/10.1016/j.vetmic.2015.12.003.

Ericsson Unnerstad, H., A. Lindberg, K. Persson Waller, T. Ekman, K. Artursson, M. Nilsson-Ost, and B. Bengtsson. 2009. Microbial aetiology of acute clinical mastitis and agent-specific risk factors. Vet Microbiol. 137:90–97. https://doi.org/10.1016/j.vetmic.2008.12.005.

Fang, W., and S.P. Oliver. 1999. Identification of lactoferrin-binding proteins in bovine mastitis-causing *Streptococcus uberis*. FEMS Microbiol. Lett. 176(1):91–96. https://doi.org/10.1111/j .1574-6968.1999.tb13647.x.

Feßler, A., C. Scott, K. Kadlec, R. Ehricht, S. Monecke, and S. Schwarz. 2010. Characterization of methicillin-resistant *Staphylococcus aureus* ST398 from cases of bovine mastitis. J Antimicrob Chemother. 65:619–625. https://doi.org/10.1093/jac/dkq021.

Haenni, M., L. Galofaro, M. Ythier, M. Giddey, P. Majcherczyk, P. Moreillon, and J.Y. Madec. 2010. Penicillin-binding protein gene alterations in *Streptococcus uberis* isolates presenting decreased susceptibility to penicillin. Antimicrob Agents Chemother. 54(3):1140–1145. https://doi.org/10.1128/AAC.00915-09.

Haenni, M., E. Saras, S. Chaussière, M. Treilles, and J.Y. Madec. 2011. *ermB*-mediated erythromycin resistance in *Streptococcus uberis* from bovine mastitis. Vet J. 189(3):356-8. https://doi.org/10.1016/j.tvjl.2010.06.021.

Hossain, M., S.A. Egan, T. Coffey, P.N. Ward, R. Wilson, J.A. Leigh, and R.D. Emes. 2015. Virulence related sequences; insights provided by comparative genomics of *Streptococcus*

uberis of differing virulence. BMC Genomics. 16:334. https://doi.org/10.1186/s12864-015-1512-6.

Kaczorek, E., J. Małaczewska, R. Wójcik, and A.K. Siwicki. 2017. Biofilm production and other virulence factors in *Streptococcus* spp. isolated from clinical cases of bovine mastitis in Poland. BMC Veterinary Research. 13:398. https://doi.org/10.1186/s12917-017-1322-y.

Käppeli, N., M. Morach, K. Zurfluh, S. Corti, M. Nüesch-Inderbinen, and R. Stephan. 2019. Sequence types and antimicrobial resistance profiles of *Streptococcus uberis* isolated from bovine mastitis. Front Vet Sci. 6:234. https://doi.org/10.3389/fvets.2019.00234.

Krömker, V., F. Reinecke, J.H. Paduch, and N. Grabowski. 2014. Bovine *Streptococcus uberis* intramammary infections and mastitis. Clin Microbial. 3:157. https://doi.org/10.4172/2327-5073.1000157.

Lasagno, M.C., E.B. Reinoso, S.A. Dieser, L.F. Calvinho, F. Buzzola, C. Vissio, C.I. Bogni, and L.M. Odierno. 2011. Phenotypic and genotypic characterization of *Streptococcus uberis* isolated from bovine sub-clinical mastitis in Argentinean dairy farms. Rev Argent Microbiol. 43(3):212-7. https://doi.org/10.1590/S0325-75412011000300009.

Leelahapongsathon, K., Y.H. Schukken, A. Srithanasuwan, and W. Suriyasathaporn. 2020. Molecular epidemiology of *Streptococcus uberis* intramammary infections: persistent and transient patterns of infection in a dairy herd. J Dairy Sci. 103(4):3565-3576. https://doi.org/10 .3168/jds.2019-17281.

Lopez-Benavides, M.G., J.H. Williamson, G.D. Pullinger, S.J. Lacy-Hulbert, R.T. Cursons, and J.A. Leigh. 2007. Field observations on the variation of *Streptococcus uberis* populations in a pasture-based dairy farm. J Dairy Sci. 90:5558–5566. https://doi.org/10.3168/jds.2007-0194.

Lüthje, P., and S. Schwarz. 2007. Molecular basis of resistance to macrolides and lincosamides among staphylococci and streptococci from various animal sources collected in the resistance monitoring program BfT-GermVet. Int J Antimicrob Agents 29(5):528–535. https://doi.org/10.1016/j.ijantimicag.2006.12.016.

McDougall, S., L. Clausen, H.J. Ha, I. Gibson, M. Bryan, N. Hadjirin, E. Lay, C. Raisen, X. Ba, O. Restif, J. Parkhill, and M.A. Holmes. 2020. Mechanisms of β-lactam resistance of *Streptococcus uberis* isolated from bovine mastitis cases. Vet Microbiol. 242:108592. https://doi.org/10.1016/j.vetmic.2020.108592.

Parin, U., S. Kirkan, E. Cicek, and H. T. Yuksel. 2017. Detection of virulence genes in *Streptococcus uberis* isolated from bovine mastitis in Aydin province by multiplex polymerase chain reaction. F.Ü. Sağ. Bil. Vet. Derg. 31(3):213–219.

Petinaki, E., V. Guerin-Faublee, V. Pichereau, C. Villers, A. Achard, B. Malbruny, and R. Leclercq. 2008. Lincomycin resistance gene *lnu*(D) in *Streptococcus uberis*. Antimicrobial Agents and Chemotherapy. 52(2):626–630. https://doi.org/10.1128/AAC.01126-07.

Pol, M., and P.L. Ruegg. 2007. Treatment practices and quantification of antimicrobial drug usage in conventional and organic dairy farms in Wisconsin. J Dairy Sci. 90: 249–261. https://doi.org/10.3168/jds.S0022-0302(07)72626-7.

Pyatov, V., I. Vrtková, and A. Knoll. 2017. Detection of selected antibiotic resistance genes using multiplex PCR assay in mastitis pathogens in the Czech Republic. Acta Vet. Brno. 86:167–174. https://doi.org/10.2754/avb201786020167.

Reinoso, E.B., M.C. Lasagno, S.A. Dieser, and L.M. Odierno. 2011. Distribution of virulence-associated genes in *Streptococcus uberis* isolated from bovine mastitis. FEMS Microbiol. Lett. 318:183–188. https://doi.org/10.1111/j.1574-6968.2011.02258.x.

Reinoso, E.B., M.C. Lasagno, and L.M. Odierno. 2015. Genetic patterns of *Streptococcus uberis* isolated from bovine mastitis. Rev Argent Microbiol. 47(2):108-111. https://doi.org/10.1016/j.ram.2015.02.001.

Reyes, J., J.C. Rodriguez-Lecompte, A. Blanchard, J.T. McClure, and J. Sánchez. 2019. Molecular variability of *Streptococcus uberis* isolates from intramammary infections in Canadian dairy farms from the Maritime region. Can J Vet Res. 83(3):168-176.

Saini, V., J.T. McClure, D. Léger, S. Dufour, A.G. Sheldon, D.T. Scholl, and H.W. Barkema. 2012. Antimicrobial use on Canadian dairy farms. J Dairy Sci. 95: 1209–1221. https://doi.org/10.3168/jds.2011-4527.

Schmitt-Van de Leemput, E., and R.N. Zadoks. 2007. Genotypic and phenotypic detection of macrolide and lincosamide resistance in *Streptococcus uberis*. Journal of Dairy Science 90:5089–5096. https://doi.org/10.3168/jds.2007-0101.

Shome, B.R., M. Bhuvana, S.D. Mitra, N. Krithiga, R. Shome, D. Velu, A. Banerjee, S.B. Barbuddhe, K. Prabhudas, and H. Rahman. 2012. Molecular characterization of *Streptococcus agalactiae* and *Streptococcus uberis* isolates from bovine milk. Trop Anim Health Prod. 44(8):1981-92. https://doi.org/10.1007/s11250-012-0167-4.

Smith, A.J., A.J. Kitt, P.N. Ward, and J.A. Leigh. 2002. Isolation and characterization of a mutant strain of *Streptococcus uberis*, which fails to utilize a plasmin derived beta-casein peptide for the acquisition of methionine. J Appl Microbiol. 93(4):631–639. https://doi.org/10.1046/j.1365-2672.2002.01723.x.

Tabit, F.T. 2016. Advantages and limitations of potential methods for the analysis of bacteria in milk: a review. J Food Sci Technol. 53:42–49. https://doi.org/10.1007/s13197-015-1993-y.

Tian, X.Y., N. Zheng, R.W. Han, H. Ho, J. Wang, Y.T. Wang, S.Q. Wang, H.G. Li, H.W. Liu, and Z.N. Yu. 2019. Antimicrobial resistance and virulence genes of *Streptococcus* isolated from dairy cows with mastitis in China. Microb Pathog. 131:33-39. https://doi.org/10.1016/j.micpath.2019.03.035.

Tomazi, T., G. Freu, B.G. Alves, A.F. de Souza Filho, M.B. Heinemann, and M. Veiga Dos Santos. 2019. Genotyping and antimicrobial resistance of *Streptococcus uberis* isolated from bovine clinical mastitis. PLoS One.14(10):e0223719. https://doi.org/10.1371/journal.pone.0223719.

Wald, R., M. Baumgartner, J. Gutschireiter, B. Bazzanella, K. Lichtmannsperger, M. Wagner, T. Wittek, and B. Stessl. 2020. Comparison of the population structure of *Streptococcus uberis*

mastitis isolates from Austrian small-scale dairy farms and a Slovakian large-scale farm. J Dairy Sci. 103(2):1820-1830. https://doi.org/10.3168/jds.2019-16930.

Ward, P.N., T.R. Field, C.D. Rapier, and J.A. Leigh. 2003. The activation of bovine plasminogen by PauA is not required for virulence of *Streptococcus uberis*. Infect Immun 71(12):7193–7196. https://doi.org/10.1128/iai.71.12.7193-7196.2003.

Ward, P.N., and J.A. Leigh. 2004. Genetic analysis of *Streptococcus uberis* plasminogen activators. Indian J Med Res. 119(suppl):136–140.

Wente, N., D. Klocke, J.H. Paduch, Y. Zhang, M.T. Seeth, V. Zoche-Golob, F. Reinecke, E. Mohr, and V. Krömker. 2019. Associations between *Streptococcus uberis* strains from the animal environment and clinical bovine mastitis cases. J Dairy Sci. 102(10):9360-9369. https://doi.org/10.3168/jds.2019-16669.

Zadoks, R.N., B.E. Gillespie, H.W. Barkema, O.C. Sampimon, S.P. Oliver, and Y.H. Schukken. 2003. Clinical, epidemiological and molecular characteristics of *Streptococcus uberis* infections in dairy herds. Epidemiol Infect. 130:335–349. https://doi.org/10.1017/s0950268802008221.

Zadoks, R.N., L.L. Tikofsky, and K.J. Boor KJ. 2005a. Ribotyping of *Streptococcus uberis* from a dairy's environment, bovine feces and milk. Vet Microbiol. 109:257–265. https://doi.org/10 .1016/j.vetmic.2005.05.008

Zadoks, R.N., Y.H. Schukken, and M. Wiedmann. 2005b. Multilocus sequence typing of *Streptococcus uberis* provides sensitive and epidemiologically relevant subtype information and reveals positive selection in the virulence gene *pauA*. J Clin Microbiol. 43(5): 2407–2417. https://doi.org/10.1128/JCM.43.5.2407-2417.2005.

Part III: Other Pathogens and Foodborne Diseases

Ayele, Y., F.D. Gutema, B.M. Edao, R. Girma, T.B. Tufa, T.J. Beyene, F. Tadesse, M. Geloye, and A.F. Beyi, 2017. Assessment of *Staphylococcus aureus* along milk value chain and its public

health importance in Sebeta, central Oromia, Ethiopia. BMC Microbiol. 17(1):141. https://doi.org/10.1186/s12866-017-1048-9.

Bottieau, E., J. Clerinx, E. Vlieghe, M. Van Esbroeck, J. Jacobs, A. Van Gompel, and J. Van Den Ende. 2011. Epidemiology and outcome of *Shigella*, *Salmonella* and *Campylobacter* infections in travellers returning from the tropics with fever and diarrhoea. Acta Clin Belg. 66(3):191-5. https://doi.org/10.2143/ACB.66.3.2062545.

Moroni, P., G. Pisoni, P. Cremonesi, and B. Castiglioni. 2010. *Staphylococcus*. Pages 245-255 in Molecular Detection of Foodborne Pathogens. Dong Liu, ed. CRC Press, Boca Raton, FL.

Paswan, R. and Y.W. Park. 2020. Survivability of *Salmonella* and *Escherichia coli* O157:H7 pathogens and food safety concerns on commercial powder milk products. Dairy. 1(3):189-20. https://doi.org/10.3390/dairy1030014.

Regasa, S., S. Mengistu, and A. Abraha. 2019. Milk safety assessment, isolation, and antimicrobial susceptibility profile of *Staphylococcus aureus* in selected dairy farms of Mukaturi and Sululta Town, Oromia Region, Ethiopia. Vet Med Int. 2019:3063185. https://doi.org/10 .1155/2019/3063185.

Ruegg, P.L. 2003. Practical food safety interventions for dairy production. J Dairy Sci. 86(E. Suppl.):E1–E9. https://doi.org/10.3168/jds.S0022-0302(03)74034-X.

Scallan, E., R.M. Hoekstra, F.J. Angulo, R.V. Tauxe, M-A. Widdowson, S.L. Roy, J.L. Jones, and P.M. Griffin. 2011. Foodborne illness acquired in the United States − Major pathogens. Emerg Infect Dis. 17:7‑15. https://doi.org/10.3201/eid1701.P11101.

Thomas, K.M., W.A. de Glanville, G.C. Barker, J. Benschop, J.J. Buza, S. Cleaveland, M.A. Davis, N.P. French, B.T. Mmbaga, G. Prinsen, E.S. Swai, R.N. Zadoks, and J.A. Crump. 2020. Prevalence of *Campylobacter* and *Salmonella* in African food animals and meat: a systematic review and meta-analysis. Int J Food Microbiol. 315:108382. https://doi.org/10.1016/j .ijfoodmicro.2019.108382.

Scientific Contributions

List of Publications

Peer Reviewed Journals

Gioia, G., J. Freeman, A. Sipka, C. Santisteban, M. Wieland, V. Alanis Gallardo, **V. Monistero**, J. Scott, and P. Moroni. Pathogens associated with houseflies from different areas within a New York State Dairy. *JDS Communications.* -accepted-

Addis, M.F., S. Pisanu, **V. Monistero**, A. Gazzola, M. Penati, J. Filipe, S. de Mauro, P. Cremonesi, B. Castiglioni, P. Moroni, D. Pagnozzi, S. Tola, and R. Piccinini. Comparative secretome analysis of *Staphylococcus aureus* strains with different within-herd intramammary infection prevalence. *Virulence*. **2022**. 13(1):174-190. https://doi.org/10.1080/21505594.2021.2024014.

Monistero, V., A. Barberio, P. Cremonesi, B. Castiglioni, S. Morandi, D.C.K Lassen, L.B. Astrup, C. Locatelli, R. Piccinini, M.F. Addis, V. Bronzo, and P. Moroni. Genotyping and antimicrobial susceptibility profiling of *Streptococcus uberis* isolated from a clinical bovine mastitis outbreak in a dairy farm. *Antibiotics*. **2021**. 10(6):644. https://doi.org/10.3390/antibiotics10060644.

Monistero, V., A. Barberio, F. Biscarini, P. Cremonesi, B. Castiglioni, H.U. Graber, E. Bottini, A. Ceballos-Marquez, V. Kroemker, I.M. Petzer, C. Pollera, C. Santisteban, M. Veiga Dos Santos, V. Bronzo, R. Piccinini, G. Re, M. Cocchi, and P. Moroni. Different distribution of antimicrobial resistance genes and virulence profiles of *Staphylococcus aureus* strains isolated from clinical mastitis in six countries**.** *Journal of Dairy Science*. **2020.** 103:3431–3446. https://doi.org/10.3168/jds.2019-17141.

Calonzi, D., A. Romanò, **V. Monistero**, P. Moroni, M.V. Luini, F. Biscarini, B. Castiglioni, and P. Cremonesi. Technical note: Development of multiplex PCR assays for the molecular

characterization of *Streptococcus uberis* strains isolated from bovine mastitis. *Journal of Dairy Science.* **2020.** 103(1):915-921. https://doi.org/10.3168/jds.2019-16823.

Book Chapters

Cremonesi, P., **V. Monistero**, P. Moroni, A. Barberio, R. Almeida, A.A. Latorre, and B. Castiglioni. Detection methods. *Encyclopedia of Dairy Sciences (Third edition).* Paul L.H. McSweeney, John P. McNamara, editors, Academic Press. **2022**. 457–468. https://doi.org/10.1016/B978-0-08-100596-5.22977-6.

Conference Presentations

Oral Presentations

Monistero, V., P. Cremonesi, S. Morandi, A. Barberio, C. Locatelli, R. Piccinini, M.F. Addis, and P. Moroni. AMR patterns and RAPD profiles of *Streptococcus uberis* strains isolated from a clinical bovine mastitis outbreak. *2021 Annual Conference of the AABP (American Association of Bovine Practitioners).* **October 7-9, 2021;** Salt Lake City, Utah.

Monistero, V., P. Cremonesi, S. Morandi, A. Barberio, B. Castiglioni, C. Locatelli, R. Piccinini and, P. Moroni. Molecular characterization of *Streptococcus uberis* strains isolated from a clinical bovine mastitis outbreak in an Italian dairy farm. *National Mastitis Council 60th Annual meeting.* **January 26-28, 2021**; virtual format.

Monistero V. Relationship between antibiotic resistance gene and virulence gene profiles in *Staphylococcus aureus* isolates from bovine clinical mastitis. *Mastitis Research Workers.* **October 24-26, 2018**; Chicago, Illinois, USA.

Poster Presentations

Penati, M. & **V. Monistero**, S. Fusar Poli, C. Locatelli, R. Piccinini, P. Moroni, and M.F. Addis. MALDI-TOF Bacterial subtyping for the rapid detection of methicillin-resistant *Staphylococcus aureus* in milk bacteriology. *National Mastitis Council 61st Annual meeting.* **February 1-3, 2022**; San Diego, California, USA.

Addis, M.F., S. Pisanu, M. Penati, **V. Monistero,** A. Gazzola, B. Castiglioni, P. Cremonesi, P. Moroni, D. Pagnozzi, and R. Piccinini. Comparative secretome analysis of *Staphylococcus aureus* strains belonging to sequence types with different within-herd mastitis prevalence. *National Mastitis Council 60th Annual meeting.* **January 26-28, 2021**; virtual format.

Monistero, V., P. Cremonesi, B. Castiglioni, R. Piccinini, and P. Moroni. Molecular characterization of *Staphylococcus aureus* strains isolated from bovine subclinical and clinical mastitis in Italy. *National Mastitis Council 59th Annual meeting*. **January 28-31, 2020;** Orlando, Florida, USA.

Monistero, V. & P. Cremonesi, B. Castiglioni, S. Morandi, and P. Moroni. Molecular typing and toxins characterization of *Streptococcus uberis* strains isolated from bovine subclinical mastitis. *National Mastitis Counci 59th Annual meeting*. **January 28-31, 2020**; Orlando, Florida, USA.

Monistero, V., F. Biscarini, A. Barberio, S. Bonamico, P. Cremonesi, B. Castiglioni, and P. Moroni. *S. aureus* antibiotic-resistance: phenotypic and genotypic analysis of bovine mastitis isolates. *IDF mastitis conference.* **May 14-16, 2019**; Copenhagen, Denmark.

Monistero, V., P. Cremonesi, B. Castiglioni, M. Penati, C. Santisteban, and P. Moroni. *Staphylococcus aureus* strains isolated from bovine clinical mastitis in New York State and Italy: antibiotic-resistance genes and genotype characteristics. *National Mastitis Council 58th Annual meeting*. **January 29-February 1, 2019**; Savannah, Georgia, USA.

Pisanu, S., C. Cacciotto, G.M.G. Puggioni, D. Pagnozzi, S. Uzzau, C. Pollera, M. Penati, **V. Monistero,** J. Guccione, P. Ciaramella, G. Borriello, P. Moroni, V. Bronzo, and M.F. Addis. Proteomic changes occurring in water buffalo milk upon intramammary infection by *Staphylococcus aureus* and by non-aureus staphylococci. *National Mastitis Council 58th Annual meeting.* **January 29-February 1, 2019**; Savannah, Georgia, USA.

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