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Doctoral Thesis

EPIDEMIOLOGY AND MOLECULAR CHARACTERIZATION OF SELECTED PROTOZOA IN DOMESTIC RUMINANTS

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"We are the recipients of scientific method.
We can each be a creative and active part of it
if we so desire"

Kary Mullis

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ABSTRACT

Apicomplexa parasites are single-celled, obligate intracellular cyst-forming protozoa, infecting humans and animals, that pose major threats to world health and global economy.

Among the most relevant species for farm animals, *Besnoitia besnoiti*, *Neospora caninum* and *Toxoplasma gondii* are parasites of medical (*T. gondii*) and veterinary (*T. gondii*, *N. caninum*, *B. besnoiti*) importance in domestic ruminants.

Bovine besnoitiosis, caused by *Besnoitia besnoiti*, is a chronic and debilitating parasitic disease of cattle, characterized by both cutaneous and systemic manifestations, compromising animal welfare and responsible for economic losses on affected farms. In Europe, including Italy, bovine besnoitiosis is an emerging or re-emerging disease, with an increasing geographical distribution and the number of cases of infection.

Neospora caninum, a coccidian protozoan, represents an important cause of bovine abortion. It was suggested that the parasite may have adverse effects on fertility and milk production, but only few and contrasting data are available to date. Besides, while only a single genotype of *N. caninum* exists worldwide, available parasite strains show considerable variation *in vitro* and *in vivo*, including different virulence in cattle. Microsatellite markers allow to fingerprint *N. caninum* isolates or DNAs and undertake population studies.

Toxoplasmosis represents an important public health issue, with the consumption of raw or undercooked meat being a major way of human infection. The role of beef in the transmission of the parasite to humans is questioned due to lower quantity of tissue cysts compared with other meat-producing species. However, the habit of consuming raw beef is regionally diffused, and the risk posed by *Toxoplasma gondii* infection in cattle should not be overlooked.

The aim of my doctoral thesis project was to investigate on the epidemiology and molecular characterization of selected protozoa parasites of medical and veterinary importance in domestic ruminants, i.e., *B. besnoiti*, *N. caninum* and *T. gondii* in cattle. A multidisciplinary approach based on clinical features, laboratory tests including serological and molecular techniques, was applied throughout the research project, to achieve a multi-level comprehension of the epidemiology of these parasite infections.

Three main research lines were developed:

Research Line 1: Exploring bovine besnoitiosis: a multi method approach.

A case of bovine besnoitiosis in a dairy farm housing 217 cattle in Italy was reported. A serological screening was performed on the whole herd using the recommended approach of ELISA and confirmatory Western Blot. Seropositive animals were clinically examined to reveal symptoms and lesions of besnoitiosis. Risk factors and the effects of the parasite infection on reproductive and productive performances were evaluated. Histopathology and molecular analyses on tissues from a slaughtered cow affected by the chronic phase of the disease were carried out. An overall seroprevalence of 23.5%, which increased up to 43.5% considering only cows, was recorded. Clinical examination of 33 of the seropositive cows evidenced the presence of tissue cysts in at least one of the typical localizations (sclera, vulva, or skin) in 25 animals. Statistical analysis did not evidence any significative impact of the parasite infection on herd efficiency; however, a decrease of productive parameters was recorded in cows showing cutaneous cysts. Concerning the chronically affected cow, histopathology revealed *B. besnoiti* tissue cysts in the skin of the neck, rump, hind legs, eyelid and vulva, in the muzzle, in mucosal membranes of the upper respiratory tract, and in the lungs. Parasite DNA was detected also in masseter muscles, tonsils, mediastinal lymph nodes, liver, cardiac muscle, aorta wall, ovaries, uterus, and vulva.

Moreover, alterations of laboratory parameters, i.e., hematological and biochemical parameters, enzyme activities and serum cortisol levels in *Besnoitia besnoiti* naturally infected cows were deeply investigated. Laboratory parameters of 107 cows, 61 seronegative and 46 seropositive to *B. besnoiti*, including 27 with clinical signs of bovine besnoitiosis, were compared. Generalized Linear Models were used to evaluate the effect of *Besnoitia* infection on the considered laboratory parameters. Hematological analyses revealed that *B. besnoiti* infection determined a significant alteration of the leukocyte differential with a higher percentage of neutrophil granulocytes and a lower percentage of lymphocytes in seropositive animals; Erythrocyte and Platelet counts did not show any difference between the considered groups of cows. Biochemistry evidenced that the parasite infection influenced serum protein values in seropositive animals and GLDH in clinically affected cows. No or only slight differences were revealed for all the other biochemical and enzyme activity parameters in *B. besnoiti* infected animals. Seropositive and clinically affected cows evidenced mild higher concentrations of serum cortisol values if compared to seronegative animals, indicating that bovine besnoitiosis could be related to stress in infected animals and that the parasite could compromise animal welfare and also influence disease onset and progression.

Furthermore, a form of generalized demodectic mange in two dairy cows infected with *Besnoitia besnoiti* was described. Two out of the cows seropositive to *B. besnoiti*, at clinical examination presented skin nodules, widespread all over the body, and in particular in anterior regions. Skin biopsies from the region of the neck were collected and the nodules were microscopically examined through compression method. *B. besnoiti* tissue cysts were not revealed but a semi-solid yellowish content was evidenced with the presence of several mites, morphologically identified as *Demodex bovis*. Histological examination of skin biopsies evidenced slight acanthosis and hyperkeratosis of the epidermis and superficial dermatitis with oedema and macrophagic and eosinophilic infiltration. Cystic formations located in the deep dermis were lined by metaplastic squamous epithelium and severe cellular infiltration. A treatment with eprinomectin was attempted and clinical improvement of both cows was observed, particularly at the fifteenth day after treatment, with nodules reduced in size and mites in there degenerated.

Moreover, an additional sub research line from this one was implemented to investigate on these Apicomplexa protozoa, particularly *Besnoitia* spp. infection, in Italian equids:

Research Line 1 bis: Exposure of Italian equids to selected protozoa infections and investigation on clinical besnoitiosis in donkeys.

A serosurvey was planned to estimate the prevalence of Sarcocystidae species (*Besnoitia* spp., *Toxoplasma gondii* and *Neospora* spp.) in Italian equids. Serum samples from 268 horses and 18 donkeys raised in Italy were collected and serologically analyzed to detect anti-*Besnoitia* spp., anti-*T. gondii* and anti-*Neospora* spp. antibodies: an approach based on an initial screening by in-house ELISA followed by a confirmatory Western Blot was used. Two horses (0.7%) and four donkeys (22.2%), showed antibodies anti-*Besnoitia* spp. Ten horses (3.7%) resulted positive to *T. gondii* and one of these (0.4%) was seropositive also to *Neospora* spp. This is the first detection of anti-*Besnoitia* spp. specific antibodies in Italian horses and donkeys. Low prevalence of *T. gondii* and *Neospora* spp. in horses raised in Italy was reported.

A case of clinical besnoitiosis in two donkeys was reported. Two donkeys, one male and one female, reared in northern Italy, showed suspected skin lesions and poor body condition. The animals were clinically examined, and endoscopy of upper respiratory tract and of the vagina for the female donkey was performed. Blood samples and skin biopsies were collected. Serum samples were analyzed by Western Blot to detect anti-*Besnoitia* spp. antibodies; a PCR targeting ITS-1 region and sequencing were carried out on DNA extracted from skin biopsies. Moreover, blood samples were examined for hematology, biochemistry, and enzyme activity. Clinical examination revealed numerous scleral pearls in the eyes of both animals; besides, alopecia and

hyperkeratosis with skin nodules in the region of the neck, hind leg and on the pinnae were detected. No cysts were evidenced in the nares, in the upper respiratory tract and in the vagina and vulva in the female animal. Both animals resulted seropositive according to Western Blot results. Skin biopsies collected from the donkeys resulted positive for the presence of parasitic DNA. Sequencing demonstrated a homology of 100% with *Besnoitia* spp. sequences deposited in GenBank. Hematology evidenced light anemia, leukocytosis with eosinophilia, and lymphocytosis, whereas biochemistry and enzyme activity revealed hypoalbuminemia with decreased albumin/globulin ratio and elevated alkaline phosphatase values. This first clinical case of besnoitiosis in donkeys in Italy confirmed the circulation of *Besnoitia* spp. in Italian and European equids.

Research Line 2: Genetic characterization of *Neospora caninum* isolates in cattle and impact of neosporosis on herd performances.

With the aim of evaluating the spread of neosporosis and its effects on herd efficiency, an epidemiological study was designed in two dairy farms recruited as case-study. Both selected farms, located in Lombardy region, performed genetic improvement of Holstein Friesian, and reported cases of abortions ascribable to *N. caninum*. Blood samples were collected from 540 animals, including cows and heifers above 24 months, and analyzed by indirect immunofluorescent antibody test. Epidemiological data (individual, reproductive and productive data) were noted. Overall, 94 animals (17.4%) resulted positive to *N. caninum* (15.5% and 18.5% in Farm 1 and Farm 2), with differences between the farms concerning the antibody titres (Chi-square, p-value = 0.04), particularly in cows (Chi-square, p-value = 0.018). Regarding the episodes of abortions, a different pattern was depicted in the two investigated farms. Data on fertility and production were considered. The number of insemination necessary to get an animal pregnant resulted higher in seropositive animals (2.4 and 2.9) than in seronegative animals (2.1 and 2.4 in Farm 1 and 2, respectively). Similarly, particularly in Farm 1, the number of days in lactation of not-pregnant cows resulted higher in seropositive (167.7) than seronegative animals (133.4). Moreover, although the association between *N. caninum* infection and milk production is still unclear, both the daily production and the mature equivalent milk yield were lower in seropositive (31.02 and 11838.94) than seronegative cows (33.59 and 12274.88) in Farms 1.

To genotype *N. caninum* in aborted bovine foetuses from Lombardy, the proportion of *N. caninum* PCR-positive aborted foetuses in this area was determined and the available isolates were characterized by multi-locus microsatellite genotyping. Aborted bovine foetuses were collected between 2015 and early 2019 from Italian Holstein Friesian dairy herds suffering from

reproductive problems. A total of 198 samples were collected from 165 intensive farms located in Lombardy, northern Italy. *N. caninum* positive samples were then subjected to multilocus-microsatellite genotyping (MLMG) using ten previously established microsatellite markers. In addition to own data, those from a recent study providing data on five markers were included and analyzed. 55 aborted foetuses were positive for *N. caninum* by RT-PCR, yielding a prevalence of 27.8%; 43 farms recorded at least one positive fetus (26.1%). Of the 55 samples finally subjected to MLMG, 35 were typed at all or 9 out of 10 loci. Linear regression revealed a statistically significant association between the spatial distance of the sampling sites with the genetic distance of *N. caninum* MLMGs ($P< 0.001$). Including data from a previous north Italian study (eBURST analysis) revealed that part of *N. caninum* MLMGs from northern Italy separate into four groups; most of the samples from Lombardy clustered in one of these groups. Principle component analysis revealed similar clusters and confirmed MLMG groups identified by eBURST. These findings confirm the concept of local *N. caninum* subpopulations. The geographic distance of sampling was associated with the genetic distance as determined by MLMGs.

Research Line 3: Evaluation of *Toxoplasma gondii* infection in beef cattle raised in Italy.

To update information on *T. gondii* in cattle reared in Italy, a multicentric seroepidemiological survey was designed and implemented in four northern regions (Liguria, Lombardy, Piedmont, and Trentino Alto Adige) and Sardinia. A convenience sampling was performed, collecting 1444 serum samples from 57 beef cattle herds. Thirteen beef breeds were sampled, besides crossbreed; bovines age varied from 3 months to over 12 years. Sera were tested with a commercial ELISA for the detection of anti-*T. gondii* antibodies. Individual and herd data were analyzed by binary logistic regression analysis. A *T. gondii* seroprevalence of 10.2% was recorded, with differences among regions and values ranging from 5.3% in Liguria to 18.6% in the Piedmont region (p value = 0.0001). Both young and adult animals and males and females tested positive, without any significant difference (age and gender: p value >0.05). Lower seroprevalence values were recorded in cattle born in Italy (8.7%) if compared with animals imported from abroad (13.4%) (p value = 0.046).

To obtain epidemiological and molecular data on *T. gondii* infection in cattle slaughtered in Italy, 80 animals were sampled from one of the biggest Italian slaughterhouses. Dairy (Holstein Friesian) or dual purpose (Alpine Brown and Pezzata Rossa Italiana) breeds or crossbreeds from 15 farms located in northern Italy were sampled; age of animals varied from six months to three years. Approximately 50 g of diaphragm was collected to obtain meat juice and muscle homogenate samples. Individual data were noted. Meat juice samples were analyzed with a commercial ELISA

to detect anti-*T. gondii* antibodies. DNA extracted from muscle homogenate samples were subjected to B1 real-time PCR. Anti-*T. gondii* antibodies were found in 10 (12.5%) out of 80 examined animals, whereas parasitic DNA was detected in 26 diaphragm muscle samples (32.5%). Only seven samples scored positive in both test: a fair agreement between ELISA and B1 real-time PCR results was achieved (κ value = 0.254). Nevertheless, higher ELISA S/P% values were recorded in diaphragm samples scoring positive to PCR. Higher number of positive samples were found in younger than older animals considering both ELISA and B1 real-time PCR results. Similarly, considering the provenience, animals that have been acquired from other holdings scored more frequently positive to both ELISA and B1 real-time PCR compared to animals that have never left the holding of origin until slaughter. Statistical analysis showed an effect of ELISA S/P% values on B1 real-time PCR results, increasing the risk of parasitic DNA detection when increasing the S/P% values. The other considered variables (age and provenience) did not show any effect on neither ELISA nor B1 real-time PCR.

In conclusion, obtained results from the studies of my PhD project allowed to update information on protozoa of medical and veterinary importance both in domestic ruminants and in equids.
It was demonstrated that bovine besnoitiosis continues to spread in Italy: both clinical and laboratory tests are needed for an accurate diagnosis, and thus to implement plans for the control of the disease. Moreover, *Besnoitia* spp. infection circulates in Italian equids and besnoitiosis may be considered an emerging disease of donkeys in Italy and also in Europe.
Serological screening of cows and molecular analysis of aborted foetuses confirmed the role of *N. caninum* in abortion and reproductive failure in dairy herds in northern Italy; besides, multilocus microsatellite genotyping confirmed the concept of local *N. caninum* subpopulations.
The zoonotic importance of *T. gondii* should not be underestimated in animal species destined to human consumption, including cattle and horses. Serological data are useful to give an indication of the population exposure to the parasite, whereas molecular methods allow to detect tissue cysts in the edible parts reflecting the risk for human infection.

GENERAL INTRODUCTION

Apicomplexa parasites are single-celled, obligate intracellular cyst-forming protozoa, infecting humans and animals, that pose major threats to world health and global economy.
Indeed, these parasites include species infecting humans, which are estimated to kill over 1 million people every year, and species infecting animals causing agricultural losses of over US $1 billion per year.
The genera *Toxoplasma*, *Cryptosporidia*, *Isospora* and *Plasmodium*, include species with high human medical importance. High veterinary importance is instead attributed to *Neospora*, *Eimeria*, *Babesia* and *Theileria*.
Molecular tools to characterize and monitor parasite populations, in relation to genetic diversity, infection dynamics and population structure, were established as an integral part of field studies and intervention trials: the key question is to understand how genetic diversity influences epidemiology and pathogenicity, as well as its implication in therapy, vaccination and thus disease control (Beck et al., 2009; Tomley, 2009).

Among the most relevant species for farm animals, *Besnoitia besnoiti, Neospora caninum* and *Toxoplasma gondii* are parasites with importance for human health (*T. gondii*) and animal health, welfare, and productivity (*T. gondii*, *N. caninum*, *B. besnoiti*) importance in domestic ruminants.
B. besnoiti causes besnoitiosis, an (re)emerging disease of cattle in Europe, compromising animal health and welfare, and responsible for economic losses on affected farms. *N. caninum* is a major cause of abortion in cattle, whereas *T. gondii*, in addition to being one of the major causes of infectious reproductive failure in small ruminants, and one of the more common parasitic zoonoses worldwide.

Besnoitia besnoiti

Closely related to *T. gondii* and *N. caninum*, *Besnoitia besnoiti* a protozoan parasite belonging to the group of cyst-forming coccidians. Among 10 recognized species, the genus *Besnoitia* includes four closely related species (*B. besnoiti*, *B. caprae*, *B. bennetti* and *B. tarandi*) infecting domestic and wild ungulates (cattle, goats, equids and deers, respectively) (Olias et al., 2011).

The life cycle of the parasite species infecting ungulates is in part unknown: in fact, the definitive host is not yet identified. Currently, the only form of transmission known for *Besnoitia* is mechanical transmission by hematophagous insects (*Glossina*, *Stomoxys* and tabanids) (Lienard et al., 2011). Moreover, a close contact between animals or an incorrect medical procedure were suggested as potential ways of transmission of the infection (Alvarez-Garcia et al., 2013; Cortes et al., 2014). Besides, animal trade and movement throughout countries were identified as major risk factors for establishment of new bovine besnoitiosis foci in naive areas and countries (Alvarez-Garcia et al. 2013).

B. besnoiti is the causative agent of bovine besnoitiosis, a chronic and debilitating disease of cattle, characterized by both cutaneous and systemic manifestations, that compromise animal health and welfare. The disease progresses in two sequential clinical phases. The acute phase is characterized by hyperthermia, edemas, and other non-specific clinical signs, including depression, swelling of the superficial lymph nodes, the arrest of rumination, weight loss, anorexia, photophobia, epiphora, ocular and nasal discharge, tachycardia and tachypnea. In the subsequent chronic the skin becomes progressively thickened and wrinkled with alopecia, seborrhea, and hyperkeratosis, with the presence of pathognomonic subcutaneous thick-walled tissue cysts. Chronic infected cattle show loss of weight and progressive deterioration of the body condition (Jacquiet et al., 2010). Field studies evidenced that in *B. besnoiti*-infected herds, only a small part of the animals shows the clinical form of the disease, with the majority showing only mild clinical signs or being subclinically infected. However, the seroprevalence of *B. besnoiti* infection could rapidly increase in recently infected herds, after the detection of the first clinical case of the disease (Jacquiet et al. 2010; Liénard et al. 2011; Gutiérrez-Expósito et al. 2017a; Gollnick et al. 2015, 2018). The disease is responsible for economic losses on affected farms including mortality, weight loss, prolonged convalescence, definitive or transient sterility in males, decline in milk production, and a poor value of the hides for leather production (Jacquiet et al., 2010).

Native of Sub-Saharan Africa, in Europe bovine besnoitiosis is an emerging or re-emerging disease, with an increasing geographical distribution and the number of cases of infection (EFSA, 2010). Bovine besnoitiosis is endemic in France, Spain, and Portugal (Alvarez-Garcia et al., 2013),

and cases of infection were also recorded in other European countries, including Germany, Switzerland, Hungary, Croatia, Belgium, and Ireland (Cortes et al., 2014; Vanhoudt et al., 2015; Ryan et al., 2016). In Italy, outbreaks of bovine besnoitiosis were diagnosed in the northern and central regions (Agosti et al., 1994; Gollnick et al., 2010; Manuali et al., 2011; Mutinelli et al., 2011; Gentile et al., 2012; Gazzonis et al., 2017) and serological surveys on the spread of *B. besnoiti* in cattle were carried out both in northern and southern Italy (Rinaldi et al., 2013; Gazzonis et al., 2014).

To date, the control of bovine besnoitiosis is only based on standardized diagnostic procedures and management measures, since chemotherapeutics are not available and no vaccines are licensed in Europe (Gutierrez-Exposito et al., 2017b).

The outcome of the *B. besnoiti* infection may be influenced by parasite and host-related factors. Recently, it was evidenced that the parasite stage (bradyzoite) and the inoculation route influenced the outcome of parasite infection (Diezma-Diaz et al., 2020), whereas no effect was determined by route, parasite dose and host age in the case of tachyzoite infection (Diezma-Diaz et al., 2018; Diezma-Diaz et al., 2019).

Besides, in analogy to other closely related Apicomplexan parasites, i.e., *T. gondii* and *N. caninum*, parasite invasion and proliferation, as well as clinical outcome, were demonstrated to be significantly influenced by parasite strain, associated with virulence (Saeij et al., 2005; Regidor-Cerrillo et al., 2014). Frey et al. (2013) evidenced different invasion efficiency and proliferation rates comparing *in vitro* characteristics of different *Besnoitia* spp. isolates; however, it is currently not possible to address virulence in *B. besnoiti* isolates. Regarding genetic heterogenicity, to date data concerning *Besnoitia* spp. molecular characterization are scarce. One study analyzing four *B. besnoiti* isolates of different countries found genetic differences in at least four microsatellites (Madubata et al., 2012); another study reported that out of 11 identical *B. besnoiti* isolates one differed at one microsatellite (Gutierrez-Exposito et al., 2016).

Neospora caninum

Neospora caninum, an obligate intracellular protozoan parasite, is the causative agent of neosporosis, primarily a clinical disease of cattle and dogs, causing bovine abortion and stillbirth, or neuromuscular disorder in dogs. Serological evidence in domestic and wild animals indicates that many species were exposed to this parasite (Almería and López-Gatius, 2013); to date, there is no evidence that *N. caninum* infects humans (Calero-Bernal et al., 2019).

Domestic dogs and in wild canids (gray wolves, coyotes, and dingoes) are the definitive hosts; various species were reported as intermediate hosts of the parasite, among which the primary hosts are cattle (Dubey et al., 2017).

Ruminants get infected via the ingestion of oocysts (horizontal transmission) and transplacentally (vertical transmission) because of a primary infection by oocysts (exogenous transplacental transmission) or by recrudescence of a chronic infection (endogenous transplacental transmission) during pregnancy.

N. caninum is one of the most efficiently transplacentally transmitted organisms in cattle: indeed, up to 95% of calves are born infected (Dubey et al., 2007). *N. caninum* causes abortion in both beef and dairy cattle: infection in pregnant ruminants can induce damage to the fetus in the uterus and abortion or produce a still-born calf, a new-born calf with clinical signs or a clinically healthy but infected calf (Dubey et al., 2006).

N. caninum is a major cause of abortion, the main clinical manifestation of bovine neosporosis: worldwide, these abortions are a cause of economic loss (about 2-5% but also up to 20% annually) to both the dairy and beef industries (Goodswen et al., 2013). Cows of any age may abort from 3 months of gestation to term, with most abortions occurring at 5–7 months of gestation (Dubey et al., 2007). Moreover, the parasite may also cause reproductive problems, such as stillbirths, early fetal death, and resorption, manifested as return to service, increased time to conception or infertility.

Abortion associated to *N. caninum* in bovine herds may have an epidemic or an endemic (sporadic) pattern. Abortion outbreaks are defined as epidemic (abortion storm) if the outbreak is temporary and if more than 10% of cows at risk abort within 6–8 weeks, normally due to an exogenous transplacental transmission. In contrast, an abortion problem is regarded as endemic if it persists in the herd for several months or years being related to endogenous transplacental transmission. However, intermediate abortion patterns (mixed patterns), possibly caused by both endogenous and exogenous transmission, can be observed in the field (Dubey et al., 2017).

Serologic prevalences of *N. caninum* vary among countries, within countries, between regions, and between beef and dairy cattle. *N. caninum* antibodies in cattle have been reported worldwide (Dubey et al., 2017); in Europe, a pooled prevalence of 13% was reported (Ribeiro et al., 2019). In Italy, only few epidemiological studies were conducted in cattle. Seroprevalence values between 16 (Otranto et al., 2003) and 24.3% (Magnino et al., 1999) and between 8.7 (Otranto et al., 2003) and 30% (Rinaldi et al., 2005) were reported in northern and southern Italy, respectively; besides, a serological screening on tank bulk milk reported a farm prevalence of 55% in Sardinia (Varcasia et al., 2006).

To date, no safe and effective chemotherapy for bovine neosporosis, and no vaccine against abortion or *N. caninum* transmission, are available (Sánchez-Sánchez et al., 2018). To identify new methods to control neosporosis, it is necessary to determine its population genetic structure and to understand host-pathogen interactions (Khan et al, 2020).

Regarding the population genetic structure of *N. caninum*, the parasite was isolated from different host species and consists of heterogeneous populations around the world; the genetic diversity among isolates may influences pathogenicity and virulence (Al-Qassab et al., 2009). The pioneering work of Regidor-Cerrillo et al. (2006) contributed a number of microsatellite markers that allow to fingerprint *N. caninum* isolates or DNAs and undertake population studies: microsatellite marker analysis was suggested as a suitable tool for the genetic analysis of *N. caninum* isolates, enabling detailed studies of the genetic complexity of parasite infections. Obtaining and characterizing isolates of the parasite from animals is necessary to evaluate correlations among parasite strains, genotypes, clinical manifestations, and pathogenicity (Regidor-Cerrillo et al., 2014). Indeed, since differences in virulence within *N. caninum* isolates were evidenced, the population diversity circulating in Europe may be implicated on the observed differences in the prevalence, transmission, and occurrence of disease in different countries (Dubey and Schares, 2011). To date, only one study investigated on *N. caninum* molecular characterization in ruminants in Italy (Regidor-Cerrillo et al., 2020): this study demonstrated a high genetic diversity of different parasite subpopulations throughout northern Italy.

Toxoplasma gondii

T. gondii is a well-known ubiquitous protozoan parasite infecting a wide range of hosts and different host cells. Intermediate hosts are almost all warm-blooded animals including mammals, birds, and humans, while definitive hosts are members of the family Felidae, including domestic cats (Dubey, 2010)

T. gondii is a parasite of both medical and veterinary importance. Indeed, toxoplasmosis is one of the most common parasitic zoonoses worldwide, affecting one third of the world human population. In the European Union 1.31 cases of congenital toxoplasmosis out of 100,000 inhabitants were reported (EFSA and ECDC, 2018). However, even if the disease burden of toxoplasmosis is comparable to that of other foodborne diseases, toxoplasmosis received little attention from policy makers in past years (Kijlstra and Jongert, 2008), and despite EFSA recommendations classified *T. gondii* as a high priority for meat inspection (EFSA, 2007), toxoplasmosis is not included within diseases subjected to research.

In humans *T. gondii* may be transmitted vertically from the mother to the foetus via the placenta. The horizontal transmission may occur through the ingestion of oocysts from environmental contamination of water, soil, or food, but also the consumption of raw or undercooked meat containing tissue cysts is an important source of parasite infection for humans. Food-producing animals have a different significance in the transmission of *T. gondii* to humans, due to species-related differences in prevalence and intensity of tissue cysts in the edible parts (Stelzer et al., 2019). Indeed, tissue cysts of *T. gondii* are most frequently observed in infected pigs, sheep, and goats; tissue cysts are found only rarely in beef meat. Additional source of human infection is through the consumption of unpasteurized dairy products, since tachyzoites of *T. gondii* were found in milk of cows, sheep, and goats. Human transmission may also occur due to blood transfusion or organ transplantation from infected hosts (Cook et al., 2000; Tenter et al., 2000).

From a veterinary viewpoint, concerning cattle, reports on clinical toxoplasmosis in naturally infected cattle are rare; in fact, before the discovery of *N. caninum* as a cause of abortion, this parasite in cattle was misdiagnosed as *T. gondii* (Dubey, 2010). However, *T. gondii* is recognized worldwide as one of major causes of infectious reproductive failure in small ruminants; the parasite causes fetal reabsorption, abortion at any stage of pregnancy, fetal mummification, stillbirth or birth of alive but weak offspring (Ortega-Mora et al., 2007).

Even if cattle are considered a poor host for *T. gondii*, since the parasite is eliminated or reduced to within a few time, perhaps due to innate resistance (Dubey and Jonas, 2008), a systematic review and meta-analysis of case-controlled studies demonstrated that consumption of raw/undercooked

beef is an important risk factors for *T. gondii* infection (Belluco et al., 2018). *T. gondii* prevalence in bovines was shown to be 2.6% worldwide (2.2% in Europe) (Belluco et al., 2016). In Italy, *T. gondii* infection is largely spread among humans and animals. Serological surveys reported prevalence values of 92% in Northern Italy (Avezza et al., 1993) and 11.3% in Sicily (Vesco et al., 2005). Moreover, recently, a quantitative risk assessment model, considering parasite prevalence and concentration in meat, consumption data, eating habits and the dose-response relationship, estimated that the yearly probability of acquiring toxoplasmosis due to consumption of bovine meat in Italy was higher compared to pork meat (0.034% and 0.019%, respectively), confirming that bovine meat cannot be considered a negligible source of *T. gondii* infection in Italy (Belluco et al., 2018).

To correctly evaluate the risk for humans from consumption of meat of infected animals, determination of *T. gondii* genotype involved is crucial: in fact, illness severity and clinical aspects of toxoplasmosis vary based on virulence of different *T. gondii* strains (Dubey and Su, 2009). For what concerns *T. gondii* genotypes, in Europe and USA the genetic population of the parasite is highly clonal with three predominant Types (I, II and III), whereas in other parts of the world (South America and Africa) atypical or different genotypes seem to be predominant (Ajzenberg et al., 2004). In Europe, strains belonging to the Type II lineage are predominant both in humans and in domestic and wild animals; however, recent data also reported the circulation of Type I, Type III and recombinant and atypical strains (Shwab et al., 2014). In Italy, studies conducted both in livestock and wildlife reported the circulation of all three clonal genotypes and also atypical ones (Cenci-Goga et al., 2013; Mancianti et al., 2014; Bacci et al., 2015; Formenti et al., 2016; Gazzonis et al., 2018a; Gazzonis et al., 2018b). Regarding ruminant livestock, both in Europe and Italy, among clonal genotypes Type II is predominant, but also findings of novel genotypes, non-clonal isolates, and mixed infections were reported (Mancianti et al., 2013; Chessa et al., 2014; Vismarra et al., 2017; Battisti et al., 2018; Fernández-Escobar et al., 2020; Gazzonis et al., 2020).

AIMS

The aim of my doctoral thesis project was to investigate on protozoa parasites of relevance for human health and animal health, welfare, and productivity: *B. besnoiti*, *N. caninum* and *T. gondii* in cattle. A multidisciplinary approach based on clinical features, laboratory tests including serological and molecular techniques, was applied throughout the research project, to achieve a multi-level comprehension of the epidemiology of these parasites.

Three main research lines were developed:

Research Line 1. Exploring bovine besnoitiosis: a multi method approach.

The aim was to deeply investigate on bovine besnoitiosis in an endemically affected dairy cattle herd: a multi method approach was applied, including serological and clinical observations, the evaluation of the effects of parasite infection on reproductive and productive performances, and its risk factors. Besides, alterations of hematological and biochemical parameters, enzyme activities and serum cortisol in infected cows were explored, to figure out the role of *B. besnoiti* in alterations of those laboratory parameters, that may aid in the diagnosis of bovine besnoitiosis.

Moreover, since these parasites could infect equids, an additional sub research line was implemented from this one to investigate on the same Apicomplexa protozoa, and particularly *Besnoitia* spp., in Italian equids; indeed, besnoitiosis is an emerging disease of donkeys.

Research Line 1 bis: Exposure of Italian equids to selected protozoa infections and investigation on clinical besnoitiosis in donkeys.

A serosurvey was performed to estimate the exposure of Italian horses and donkeys to *Besnoitia* spp., *Neospora* spp. and *T. gondii*. To achieve an accurate diagnosis, a suspected clinical case of besnoitiosis in two donkeys were deeply investigated through a multidisciplinary approach.

Research Line 2: Genetic characterization of *Neospora caninum* isolates in cattle and impact of neosporosis on herd performances.

To evaluate the spread of *N. caninum* and its effects on reproductive and productive parameters, the dynamics of the parasite infection were studied in two study herds. Furthermore, the study aimed to determine the proportion of *N. caninum* PCR-positive aborted foetuses in Lombardy and to genetically characterize the isolates by multi-locus microsatellite genotyping.

Research Line 3: Evaluation of *Toxoplasma gondii* infection in beef cattle raised in Italy.

Two aims were considered: 1) to obtain information on the exposure to *T. gondii* infection of beef cattle raised in northern Italy, and therefore a multicentric seroepidemiological survey was carried out; 2) to investigate on the risk of cattle meat to cause *Toxoplasma* infection in humans, then the protozoa in beef edible muscles was surveyed by molecular methods.

Research Line 1:

Exploring bovine besnoitiosis: a multi method approach

Bovine besnoitiosis in an endemically infected dairy cattle herd in Italy: serological and clinical observations, risk factors, and effects on reproductive and productive performances

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Abstract

Bovine besnoitiosis (*Besnoitia besnoiti*) is an emerging parasitic disease of cattle in Europe. This study reports a case of bovine besnoitiosis in a dairy farm housing 217 cattle in Italy. A serological screening was performed on the whole herd using the recommended approach of ELISA and confirmatory Western Blot. Seropositive animals were clinically examined to reveal symptoms and lesions of besnoitiosis. Risk factors and the effects of the parasite infection on reproductive and productive performances were evaluated. Histopathology and molecular analyses on tissues from a slaughtered cow affected by the chronic phase of the disease were carried out. An overall seroprevalence of 23.5%, which increased up to 43.5% considering only cows, was recorded. Clinical examination of 33 of the seropositive cows evidenced the presence of tissue cysts in at least one of the typical localizations (sclera, vulva, or skin) in 25 animals. Statistical analysis did not evidence any significative impact of the parasite infection on herd efficiency; however, a decrease of productive parameters was recorded in cows showing cutaneous cysts. Concerning the chronically affected cow, histopathology revealed *B. besnoiti* tissue cysts in the skin of the neck, rump, hind legs, eyelid and vulva, in the muzzle, in mucosal membranes of the upper respiratory tract, and in the lungs. Parasite DNA was detected also in masseter muscles, tonsils, mediastinal lymph nodes, liver, cardiac muscle, aorta wall, ovaries, uterus, and vulva. Bovine besnoitiosis continues to spread in the Italian cattle population. Breeders and veterinarians should be aware of this parasitic disease, and control programs should be developed based on surveillance through a diagnostic procedure including both clinical examination and laboratory tests.

Keywords: Besnoitia besnoiti; Dairy cows; Herd efficiency; Histology; Molecular biology; Serology.

Introduction

Bovine besnoitiosis is a parasitic disease caused by *Besnoitia besnoiti*, a cystogenic coccidia closely related to *Toxoplasma gondii* and *Neospora caninum*. The disease is chronic and debilitating, characterized by both cutaneous and systemic manifestations, compromising animal welfare and responsible for economic losses on affected farms, including mortality, weight loss, prolonged convalescence, definitive or transient sterility in males, a decline in milk production, and a poor value of the hides for leather production (Alvarez-Garcia et al., 2013; Cortes et al., 2014; Gazzonis et al., 2017). In Europe, bovine besnoitiosis is an emerging or re-emerging disease, with an increasing geographical distribution and the number of cases of infection (EFSA, 2010). Bovine besnoitiosis is endemic in France, Spain, and Portugal (Alvarez-Garcia et al., 2013), and cases of infection were also recorded in other European countries, including Germany, Switzerland, Hungary, Croatia, Belgium, and Ireland (Cortes et al., 2014; Vanhoudt et al., 2015; Ryan et al., 2016). In Italy, outbreaks of bovine besnoitiosis were diagnosed in the Northern and Central regions (Manuali et al., 2011; Mutinelli et al., 2011; Gentile et al., 2012; Gazzonis et al., 2017) and serological surveys on the spread of B. besnoiti in cattle were carried out both in Northern and Southern Italy (Rinaldi et al., 2013; Gazzonis et al., 2014). Furthermore, *Besnoitia* spp.-specific antibodies were recently detected for the first time in Italy also in horses and donkeys reared in the Northern regions (Villa et al., 2018).

Field studies evidenced that in *B. besnoiti*-infected herds, only a small part of the animals shows the clinical form of the disease, with the majority showing only mild clinical signs or being subclinically infected. However, the seroprevalence of *B. besnoiti* infection could rapidly increase in recently infected herds, after the detection of the first clinical case of the disease (Jacquiet et al., 2010; Liénard et al., 2011; Gutiérrez-Expósito et al., 2017a; Gollnick et al., 2015; 2018).

To characterize a case of *B. besnoiti* infection in a dairy cattle herd, a study was planned using a multidisciplinary approach. A serological screening on the whole herd was performed. Then, a part of seropositive animals was clinically examined to evidence any clinical signs of bovine besnoitiosis. Risk factors associated with the parasite infection and the impact of *B. besnoiti* on reproductive and productive performances in the herd were also evaluated. Furthermore, the study was aimed to report a case of chronic bovine besnoitiosis in a cow and explore by histological and molecular analyses the parasite distribution in organ samples collected at post-mortem examination.

Materials and Methods

Herd study

Background

In September 2017 in a dairy herd located in Northern Italy, suspicious abortions and clinical cases suggestive of bovine besnoitiosis were reported in 15 animals, 12 cows and three heifers. Placentas were collected from four cows and three of these resulted positive to *Coxiella burnetii* by molecular analysis (Pisoni et al., 2017). Serum samples from eight out of ten aborting cows that were referred to our laboratory resulted positive to *B. besnoiti* antibodies by both ELISA and confirmatory Western Blot.

Herd description and study area

A dairy cattle herd with 217 Holstein Frisian was involved in the study. The herd is family-run under the intensive production system with animals stabled together in different groups according to the productive category. Male calves are sold at the age of 1 month for meat, while female ones are kept in the farm for replacement stock. Bulls are not present in the herd since only artificial insemination is performed. Animals are fed with hay supplemented with a unifeed ration. Concerning productive parameters, the herd had a mean of 2.1 lactations with a medium length of 178 days and a total daily production of 2931 kg of milk.

The farm is located in the area called "Bassa Bresciana" (Province of Brescia, Northern Italy) (45°33′51″N 9°59′59″E) included in the Po Valley, an area with a high density of dairy cattle farms and one of the largest milk-producing areas in Italy. The site has an altitude of about 165 m above sea level. The climate is the one typical of the Po Valley with hot muggy summer with a few thunderstorms and cold and foggy winter with some snow. The mean annual temperature is 10.9 °C, with a mean maximum temperature of 17.7 °C and a mean minimum temperature of 7.7 °C. Rainfall is well distributed throughout the year with an average total annual rainfall of approximately 888.2 mm.

Sampling and data collection

In November 2017, all the animals of the farm were sampled, including newborn calves under 3 weeks (n = 3), calves between 3 weeks and 6 months (n = 9), heifers above 6 months (n = 97) and cows (n = 108). All sampled animals were females except from one newborn male calf; besides, all of them were born in the farm.

Blood samples were collected in tubes without anticoagulants by puncturing of the tail vein using a Vacutainer® sterile collection system and preserved refrigerated during the transportation to the laboratory within a few hours. Once in the laboratory, sera were separated by centrifugation (2120 g, 15 min) and stored at − 20 °C until serological analysis.

Epidemiological data, including individual data and information regarding reproductive and productive parameters, were noted. Data were collected both by interviewing the farmer and directly from the farm managerial software. Individual data included breed, sex, productive category, age, and origin of the animals. Concerning reproductive performances, data on episodes of embryonal reabsorption and abortion, the number of parturitions and inseminations, and the interval between calving were recorded for each sampled animal. Productive parameters regarding daily kg of milk, % fat, % of protein, somatic cell count, and 305-mature equivalent milk yield were also noted. 305-mature equivalent milk yield adjusts all cows to the same age, season of calving and lactation length, and also to the different geographic area of the herd (Si@llEvA, Italian Breeder Association, www.sialleva.it).

Serology

A serological screening was performed on the whole herd. According to international recommendations (Gutiérrez-Expósito et al., 2017b), an ELISA test and a subsequent confirmatory Western Blot were employed to detect the presence of anti-*B. besnoiti* specific antibodies. Serum samples were tested for *B. besnoiti* antibodies using a commercial ELISA kit (ID Screen® Besnoitia Indirect 2.0, IDVET, Montpellier, France) according to the manufacturer's instruction. Positive and negative control sera provided with the kit were used as controls. For each sample, the resulting values were calculated, applying the formula supplied in the kit: $S/P\% = (\text{net OD}_{\text{sample}} / \text{net OD}_{\text{positive control}}) \times 100$. Both samples considered doubtful ($25\% < S/P\% < 30\%$) and positive ($S/P\% \geq 30\%$) were submitted to confirmatory Western Blot, performed and interpreted according to Fernandez-Garcia et al. (2009), to increase specificity and avoid cross-reactions with other Sarcocystidae (Garcia-Lunar et al., 2015).

Clinical examination

A part of the animals ($n = 33$) resulted seropositive to *B. besnoiti* was clinically examined to reveal symptoms and lesions ascribable to bovine besnoitiosis, according to Alvarez-Garcia et al. (2013). At first, body temperature (°C) was measured and the presence of ocular and nasal discharge was noticed; then the animals were carefully examined to reveal the presence of tissue cysts in skin, sclera, and vulva. Clinical examination was performed by a practitioner with animals restrained in

a cattle chute. The premises for visual examination were illuminated with spotlights for direct and indirect lightning and headlamps. Cattle with at least one cyst were defined as clinically positive. Besides, skin biopsies from three cows with lesions suggestive of the chronic phase of bovine besnoitiosis were collected, compressed between glasses of a trichinoscope and observed under a stereomicroscope.

Biological samples collection and clinical examination were performed by qualified veterinarians applying adequate procedures of handling and disinfection to minimize pain or distress in sampled animals. All these procedures were accomplished following good clinical practices in the respect of animal welfare according to all applicable international, national, and institutional guidelines for the care and use of animals.

Data analysis

The seroprevalence of *B. besnoiti* antibodies was calculated considering different productive categories (newborn calves, calves between 3 weeks and 6 months, heifers and cows), according to Bush et al. (1997). Cohen's kappa (k) was performed to evaluate the agreement between ELISA and Western Blot tests. Analysis of risk factors associated with the parasite infection was carried out. A generalized linear model (GLM) with binary logistic distribution was performed to verify the influence of age and reproductive (number of parturitions, number of inseminations, days between calving) and productive parameters (daily Kg milk, % fat, % protein, somatic cell count, mature equivalent milk yield) on *B. besnoiti* infection; the binary outcome (presence/absence of anti-*B. besnoiti* antibodies) on the basis of Western Blot results was used as dependent variable. Furthermore, a second model was run considering the same independent variables and as dependent variable the presence of *B. besnoiti* tissue cysts in the skin, i.e., affected by chronic besnoitiosis (binary outcome) demonstrated at the clinical examination in seropositive animals. In both models, among individual characteristics, only age was considered, since sex, breed, and origin were the same for all the sampled animals. Besides, GLMs were carried out considering only the productive category of cows. The models were developed through a backward selection procedure (significance level to remove variables from the model = 0.05), based on AIC values. Statistical analysis was performed using SPSS software (Statistical Package for Social Science, IBM SPSS Statistics for Windows, version 25.0., Chicago, IL, USA).

Case report

Among clinically examined animals, a form of chronic besnoitiosis was diagnosed in a cow that was regularly slaughtered being in poor conditions and with severe skin lesions. Tissue sample from several organs, including skin of neck, rump and hind legs, eyelid, muzzle, scleral conjunctiva, masseter muscles, mucous membranes of the upper respiratory tract, tonsils, mediastinal lymph nodes, lungs, liver, cardiac muscle, aorta wall, spleen, ovaries, uterus, and vulva, were collected at slaughterhouse and transported refrigerated to the laboratory. An aliquot of these tissues was fixed in 10% buffered formalin for histological examination; another part was mechanically homogenized and stored at − 20 °C for molecular analyses.

Histology and molecular analysis

Tissues samples submitted for histological analysis were embedded in paraffin wax, sectioned at 5 μm, stained with hematoxylin and eosin (HE), and microscopically examined.

Tissue sample homogenates were processed to extract genomic DNA using a commercial kit (NucleoSpin® Tissue, Macherey-Nagel, Germany), following the manufacturer's instructions. DNA samples were analyzed using a conventional PCR targeting a region of 231 bp of the ITS-1 region as described by Cortes et al. (2007). Positive (Gazzonis et al., 2017) and negative (non-template) controls were inserted in each run. PCR products were run on 1.5% agarose gel containing 0.05% ethidium bromide in TBE buffer electrophoresis and visualized under UV light on a transilluminator. Bands of the expected size were excised from agarose gel, purified with a commercial kit (NucleoSpin® Gel and PCR Clean-up, Macherey-Nagel, Germany) following the manufacturer's instructions, and finally sent for sequencing in both directions to a commercial service (Eurofins Genomics, Germany). Obtained sequences were manually assembled and compared to available *B. besnoiti* sequences using BLASTn software (https://www.ncbi.nlm.nih.gov/blast/).

Results

Herd study

Out of 217 sera analyzed for *B. besnoiti* antibodies, 60 resulted positive to ELISA and 51 of these (23.5%) were confirmed by Western Blot. *B. besnoiti* seroprevalence was higher in cows (47/108, *P* = 43.5%) than in calves (0/9, *P* = 0%) and heifers (1/97, *P* = 1.03%) and three newborn calves had antibodies anti-*B. besnoiti* (3/3, *P* = 100%). A strong agreement between ELISA and Western Blot tests was obtained (k = 0.89) (Table 1).

Tab. 1. Serological prevalence (P) of *Besnoitia besnoiti* infection in an infected dairy cattle herd in Italy according to both Western Blot (WB) results and the considered categories of animals.

Animal category	n	ELISA +	WB +	P %	CI 95%
Cows	108	56	47	43.5	34.1-53.4
Heifers (≥ 6 months)	97	1	1	1.03	0.05-5.6
Calves (> 3 weeks and < 6 months)	9	0	0	0	0-37.1
Newborn calves (≤ 3 weeks)	3	3	3	100	31-100
Total	217	60	51	23.5	18.1-29.8

CI 95=Confidence Interval 95%

Out of 33 seropositive cows clinically examined, 25 showed tissue cysts localized in the skin, sclera, and/or vulva: particularly, seven cows developed tissue cysts in the skin, 24 in scleral conjunctiva and/or in vulva, and eight did not evidence any tissue cysts. Furthermore, 15 and two cows presented nasal and ocular discharge, respectively. Any alteration in body temperature was not detected in any of the examined animals (mean = 38.4, SD = 0.34, min-max = 37.7–39.1) (Table 2).

Skin biopsies were collected from three of the seven cows presenting skin lesions suggestive of bovine besnoitiosis. The compression between glasses of skin biopsies from the region of the neck, rump, and hind legs from one of these cows revealed the presence of numerous cysts consistent with *B. besnoiti*. In the other two cows, no *B. besnoiti* tissue cyst was detected, but the presence of mites, morphologically identified as *Demodex bovis*, was evidenced.

Finally, data concerning reproductive performances and productive parameters were considered (Table 3).

Tab. 2. Clinical findings in seropositive cows from a *Besnoitia besnoiti* infected dairy cattle herd in Italy.

ID	Presence of tissue cysts and localization			Body temperature °C	Nasal discharge	Ocular discharge
	Sclera	*Vestibulum vaginae*	Skin			
1	yes	yes	no	38.3	no	no
2	no	yes	no	38.4	no	no
3	no	yes	no	38.8	no	no
4	yes	no	no	38.6	serous	no
5	yes	yes	yes	38.1	no	no
6	no	no	no	37.7	no	no
7	no	yes	no	38.3	no	no
8	no	yes	no	38.7	serous	no
9	yes	yes	yes	38.6	mucous	no
10	no	no	yes	38.7	no	no
11	yes	no	yes	38.2	serous	lacrimation
12	no	no	no	38.3	serous	no
13	no	no	no	38.8	serous	no
14	yes	no	no	38.1	mucous	no
15	yes	no	no	38.3	no	no
16	yes	no	no	38.3	no	no
17	no	yes	no	38.6	serous	no
18	no	no	no	38.2	no	no
19	no	no	no	38.3	no	no
20	no	yes	no	38.0	no	no
21	yes	no	no	38.2	no	no
22	no	yes	no	39.1	serous	no
23	yes	yes	no	38.2	no	no
24	yes	yes	yes	39.0	no	no
25	no	yes	no	37.7	serous	no
26	no	no	no	38.8	no	no
27	no	yes	no	38.3	serous	no
28	no	yes	no	38.3	serous	no
29	no	yes	yes	38.3	mucous	lacrimation
30	yes	no	no	37.8	serous	no
31	no	no	no	38.6	no	no
32	no	no	no	38.1	serous	no
33	yes	yes	yes	38.5	no	no
Number of animals with clinical findings	13	17	7		15	2

Tab. 3. Descriptive statistics (Mean, Standard Deviation, Minimum and Maximum) of age, reproductive and productive parameters sorted by the serological and clinical status of cows in a dairy cattle herd endemically infected by bovine besnoitiosis. Serological status (seronegative or seropositive) was determined according to Western Blot results while as clinically affected cows are meant those animals with the presence of tissue cysts in skin suggestive of a chronic form of the disease.

Variable	n	Cow group	Mean (SD)	Min-Max
Age (in months)	61	Seronegative	68.67 (181.73)	25.4-1435.4
	47	Seropositive	49.94 (20.1)	26.3-115.6
	7	Clinically affected	41.19 (17.10)	26.3-76.2
	108	Overall	60.7 (98.9)	25.4-1435.4
Number of parturitions	61	Seronegative	1.97 (1.23)	1-5
	46	Seropositive	2.29 (1.41)	1-6
	6	Clinically affected	1.86 (1.46)	1-5
	107	Overall	2.11 (1.31)	1-6
Number of inseminations	42	Seronegative	2.71 (1.67)	1-7
	33	Seropositive	2.00 (1.49)	1-5
	6	Clinically affected	2.00 (0.71)	1-3
	75 §	Overall	2.4 (1.45)	1-7
Number of days between calving	51	Seronegative	438.08 (101.95)	319-730
	42	Seropositive	410.45 (87.59)	337-677
	6	Clinically affected	404.8 (52.20)	340-482
	93 †	Overall	428.26 (88.82)	319-730
Mature Equivalent Milk Yield	54	Seronegative	11423.80 (2228.48)	5528-15996
	36	Seropositive	11804.11 (1956.52)	7353-16159
	6	Clinically affected	10865.00 (1921.19)	7353-13190
	90 ‡	Overall	11581 (2116)	5528-16159
Daily milk production (in kg)	54	Seronegative	31.43 (8.96)	16.3-53.2
	36	Seropositive	33.49 (9.16)	16-54.8
	6	Clinically affected	31.41 (5.57)	26.8-43
	90 ‡	Overall	32.34 (9.06)	16-54.8
Fat content in milk (%)	54	Seronegative	3.83 (0.83)	2.14-6.32
	36	Seropositive	3.70 (0.85)	1.56-6.53
	6	Clinically affected	3.69 (0.45)	3.09-4.15
	90 ‡	Overall	3.8 (0.84)	1.56-6.53
Protein content in milk (%)	54	Seronegative	3.33 (0.39)	2.62-4.37
	36	Seropositive	3.40 (0.38)	2.78-4.24
	6	Clinically affected	3.30 (0.28)	3.02-3.9
	90 ‡	Overall	3.4 (0.39)	2.62-4.37
Milk somatic cell count (cells/ml)	54	Seronegative	535.00 (1314.33)	10-5393
	36	Seropositive	269.45 (627.26)	14-3770
	6	Clinically affected	103.71 (164.13)	29-475
	90 ‡	Overall	416 (1068)	10-5953

§ Insemination data of only 75 cows are reported since the other cows calved but have not been inseminated yet.
† Data concerning days between calving are missing for 14 cows since these animals have calved but have not been inseminated or the diagnosis of pregnancy has not been done yet.
‡ Productive parameters of the 90 lactating cows at time of sampling are reported.
Reproductive and productive parameters are missing for the slaughtered cow with chronic besnoitiosis.

The seven animals showing skin cysts evidenced a decrease of some productive parameters (daily kg of milk, % of fat and protein, and mature equivalent milk yield) if compared both to seropositive and seronegative animals.

However, according to GLM analysis, any significative association between serology and age and reproductive and productive parameters was not detected ($P > 0.05$), even considering the subgroup of the cows with *B. besnoiti* tissue cysts in skin.

Case report

Concerning the slaughtered cow chronically infected by besnoitiosis, histology carried out on tissue samples confirmed the highest concentration of *Besnoitia* cysts in the skin of the neck, rump, hind legs, eyelid and vulva, in muzzle and in mucosal membranes of the upper respiratory tract. Fewer and smaller dimension cysts were also seen in the lung. No *Besnoitia* cysts were detected in the liver, heart, aorta wall, tonsils, mediastinal lymph nodes, spleen, ovaries, uterus, and vulvar mucosa. In the skin, hyperkeratosis and dermal inflammation with infiltration of macrophages, plasma cells, eosinophils, and lymphocytes were also present.

The presence of parasite DNA was confirmed in tissues where *B. besnoiti* cysts were evidenced by histological examination and also in other organs, i.e., masseter muscles, tonsils, mediastinal lymph nodes, liver, cardiac muscle, aorta wall, ovaries, uterus, and vulva (both in skin and mucosa) (Table 4).

Sequencing of 231-bp PCR fragments from all examined tissues confirmed that they belonged to *B. besnoiti* with a homology of 100%. One of the obtained sequences was submitted to GenBank under accession number MN104147.

Tab. 4. Histological and molecular findings of tissue samples analysis of a cow chronically infected by *B. besnoiti*.

Tissues	Tissue cysts by histopathology	*B. besnoiti* DNA by PCR
Skin of neck, rump and hind legs	3	+
Skin of eyelid	3	+
Muzzle	3	+
Masseters muscle	N.D.	+
Mucous membranes of the upper respiratory tract	3	+
Tonsils	0	+
Mediastinal lymph nodes	0	+
Lungs	1	+
Liver	0	+
Cardiac muscle	0	+
Aorta wall	0	+
Spleen	0	-
Ovaries	0	+
Uterus	0	+
Vulva (skin)	2	+
Vulva (mucosa)	0	+

Tissue cysts score: 0=no cysts; 1=1-9 cysts; 2=10-49 cysts; 3=more than 50 cysts
N.D.=not determined
+=positive to PCR; -=negative to PCR

Discussion

The study confirms the circulation of *B. besnoiti* infection among cattle in Italy, reporting a case of bovine besnoitiosis in a dairy farm in Northern Italy. High seroprevalence of antibodies against *B. besnoiti* with a part of the seropositive animals showing clinical signs, but only a few animals affected by a severe form of the disease, suggests that the infection might have been undetected in the herd since years. Indeed, in this herd, an overall seroprevalence of 23.5% was recorded. The percentage results higher when compared to a previous study conducted in a dairy farm in Central Italy reporting an overall seroprevalence of 9.7% and of 17% if only lactating cows were considered (Gentile et al., 2012). Previously, seropositivity to *B. besnoiti* in dairy cows was also detected in the Lombardy region, where Gazzonis et al. (2014) recorded an intra-herd prevalence of 5 and 5.2% in two dairy farms. Studies concerning *B. besnoiti* infection in dairy cattle in Europe are limited; moreover, higher prevalence values in dairy cattle farms were reported in Ireland (68%) and in France (Liénard et al., 2011; Ryan et al., 2016). Concerning Northern Italy, similar values of *B. besnoiti* infection were reported in a serological survey conducted in a beef

herd (36.5%) (Gazzonis et al., 2017), suggesting a diffusion of the protozoan infection in the study area higher than expected.

It is unclear how the infection entered in the herd being all seropositive animals born in Italy. However, it should be noticed that in a few tens of meters away from the infected herd, there was a beef farm regularly importing animals from France. Then, it is possible that some of these animals were infected and the parasite was mechanically transmitted from this farm by the bite of hematophagous insects that act as mechanical vectors of *B. besnoiti* (Olias et al., 2011). Indeed, the study farm did not apply a plan for the control of insects at that time. Based on both clinical and serological findings, the herd appears to have been endemically infected for some time. Furthermore, the farmer reported that the cow with chronic besnoitiosis had skin lesions compatible with *B. besnoiti* infection for at least 1 year, but the disease was misdiagnosed as a cutaneous infection and then the cow stayed in the farm from a long time before being slaughtered. Considering animal categories, a prevalence of 43.5% was recorded in cows; even if a statistical association between age and seropositivity to *B. besnoiti* was not evidenced, it is noteworthy to consider that seroreactive animals were almost all in this productive category. As previously demonstrated, age represents a risk factor for the parasite infection with older cattle having a higher probability of testing positive (Gazzonis et al., 2017). Indeed, only a heifer (1.03%) resulted seropositive to the parasite, whereas any calves did not react serologically for *B. besnoiti* antibodies. Furthermore, three newborn calves showed anti-*B. besnoiti* antibodies: these animals were about 15 days of age and showed no clinical signs of bovine besnoitiosis; besides, all of them were born from *B. besnoiti* positive cows. For that reason, seropositivity of these animals may be due only to maternal immunity transfer through colostrum. In fact, Hornok et al. (2015) observed that vertical transmission of *B. besnoiti* did not occur, but newborn calves could acquire passive immunity from seropositive mother cows. However, the infection in calves should be further monitored for the development of clinical signs and lesions, since a clinical case of besnoitiosis was recently reported in a calf younger than 6 months of age (Diezma-Díaz et al., 2017).

Out of 47 seropositive cows, 33 were clinically examined. Twenty-five (75.8%) of these animals showed lesions ascribable to the chronic phase of bovine besnoitiosis in at least one of the typical localizations (skin, vulva, or sclera). In particular, 17 and 13 cows presented tissue cysts in *vestibulum vaginae* and sclera, respectively, while only in seven animals skin lesions were observed. Fifteen cows evidenced nasal and two of these also ocular discharges. However, 12 of these animals with discharge also presented tissue cysts in at least one of the typical localizations. Otherwise, three cows with no evidence of tissue cysts showed nasal discharge. All examined cows

were normothermic. Clinical examination did not reveal animals with clinical signs ascribable to the acute phase of bovine besnoitiosis. It should also be considered that infected animals without detectable clinical signs and macroscopic lesions characteristic of the chronic phase, i.e., subclinically infected animals, are more frequently found than clinically affected animals in endemically infected herds. Indeed, where the infection is widespread, the proportion of infected cattle developing the clinical disease is lower. As previously reported (Liénard et al., 2011; Álvarez-García et al., 2014), also in this case study, the animals of the farm can be stratified in different categories according to both serology and clinical examination. The slaughtered cow, a clinical case of severe systemic chronic infection, represented the "tip of the iceberg" of bovine besnoitiosis; such cases are relatively sporadic in both endemic and epidemic situations. Only a small proportion of seropositive animals developed tissue cysts in the scleral conjunctiva, in the vulvar region or in the skin, as detected by clinical examination, without any systemic alteration. A larger subset includes seropositive sub-clinically infected animals without any clinical sign; this category poses a huge risk for parasite transmission, being a source of infection for the other animals in the farm. Finally, there is a last group represented by seronegative animals, exposed to the risk of acquiring the infection.

As regards the impact of *B. besnoiti* infection on herd efficiency, statistical analysis did not evidence any effect of seropositivity or evidence of the disease in chronic phase (i.e., presence of tissue cysts in the skin) on reproductive and productive parameters in cows. However, it is noteworthy to consider that cows showing tissue cysts in the skin, and then in a chronic form of bovine besnoitiosis, evidenced a decrease of certain productive parameters, i.e., daily kg of milk, % of fat and protein, and also mature equivalent milk yield (Table 3). It has been suggested that *B. besnoiti* infection may cause a decrease in milk production (Alvarez-Garcia et al., 2013; Cortes et al., 2014), but to the best of our knowledge, there are no studies reporting data supporting this hypothesis. Even if statistical evidence was lacking, it is reasonable to consider that a decrease in productivity could be correlated to the debilitation caused by the chronic phase of the disease. Besides, the detection of *Demodex bovis* infection in two cows seropositive for *B. besnoiti* contributes to supporting this hypothesis. It is known that these mites develop heavy infection mainly in dairy cows with increased stress; the occurrence of bovine demodicosis seems to be associated with debilitating factors or with receptive physiological states of the animal (pregnancy or lactation) (Ciurnelli and Ciarlantini, 1975; Manfredini et al., 1994). In fact, these infested cows have calved recently and were producing milk. Nevertheless, the small number of seropositive animals developing a chronic clinical form of the disease could have influenced the

statistical results; further studies in other dairy farms are thus needed to clearly understand the impact of the disease on the herd productivity.

Regarding the clinical case of the slaughtered cow affected by the chronic phase of bovine besnoitiosis, histopathology and molecular analyses evidenced a systemic form of the disease with severe clinical signs with a wide intra-organic distribution. Histology confirmed a high load of *B. besnoiti* tissue cysts in the skin of the region of the neck, rump, hind legs, eyelid and vulva, in the muzzle and in mucous membranes of the upper respiratory tract, as also pointed out by previous studies (Álvarez-García et al., 2014). The localization of parasite cysts in these body regions of infected animals emphasizes the possibility of parasite transmission through hematophagous insects, since these areas represent preferential feeding sites, but also for direct contact among animals (Olias et al., 2011). Furthermore, parasitic cysts were also detected in the lungs, even if in fewer amount and of smaller dimensions than those detected in other organs. The presence of tissue cyst in the lungs was previously only reported by Langenmayer et al. (2015) and also in a roe deer with systemic besnoitiosis (Arnal et al., 2017). Additionally, the presence of *Besnoitia* DNA was detected in lungs from infected cows by real-time PCR (Basso et al., 2013; Frey et al., 2013). Although respiratory disorders are common in the acute phase of the bovine besnoitiosis (Álvarez-García et al., 2014), it is still to be clarified if the evidence of tissue cysts in the lung, and also in the upper respiratory tract, could be associated to respiratory symptoms also in the chronic phase of the disease.

Furthermore, molecular analysis of the tissue samples showed a wider diffusion of the protozoan in other host organs, i.e., heart, liver, aorta wall, tonsil, ovary, uterus, and vulva. The presence of the parasite in reproductive organs of cows was already reported by both histopathology (Nobel et al., 1977; Nobel et al., 1981; Frey et al., 2013; Langenmayer et al., 2015) and molecular techniques (Basso et al., 2013; Frey et al., 2013; Diezma-Díaz et al., 2017). Although *B. besnoiti* is supposed to be a cause of abortion in pregnant dams due to the high fever of short duration in the acute phase of the disease (Álvarez-García et al., 2014), its effect on female reproductive system needs to be further investigated to elucidate the role of the parasite on cows' fertility and pregnancy. Finally, parasite DNA was also found in masseter muscles. *B. besnoiti* is generally scarcely investigated in muscle; however, the presence of *Besnoitia* spp. in muscles was previously reported in a few studies: in particular, it was detected by histopathology in muscle of a cattle (Langenmayer et al., 2015), by histopathology in fascia and muscle from nine *B. tarandi* infected reindeers (Dubey et al., 2004), and by both histopathology and molecular biology in gluteal muscle of a roe deer with systemic besnoitiosis (Arnal et al., 2017). All these records seem to demonstrate that the protozoan presence in muscle could not be occasional. *Besnoitia* spp. could be able to colonize

several kinds of muscles, and this may pose a question for food safety, even if the parasite is not considered zoonotic so far. At this regard, the Regulation (EC) No. 854/2004 of the European Parliament and of the Council of 29 April 2004 laying down specific rules for the organization of official controls on products of animal origin intended for human consumption, generically reported that "[…] meat is to be declared unfit for human consumption if it […] exhibits parasitic infestation, unless otherwise provided for in Section IV; […]". Actually, considering the poor knowledge regarding this infection and the frequent absence of evident clinical signs, it is possible that meat from cattle with besnoitiosis goes with no restrictions to free trade. In Europe, only in Switzerland bovine besnoitiosis is a notifiable disease: if an outbreak is diagnosed, affected farms are confiscated and suspected and infected animals must be euthanized (916.401 Ordinance on epizootic diseases (OFE) of the 27 June 1995, Art. 189a-d). The presence of *B. besnoiti* in muscle should be further investigated to clarify if the parasite is commonly found in the cattle muscles and if it should be considered as a novel food-borne parasite.

Conclusions

The study reports a case of bovine besnoitiosis in a dairy farm in Northern Italy. High intra-herd seroprevalence, clinical signs of the disease in a part of the seropositive animals, and a case of systemic besnoitiosis in a chronically affected cow were reported. The results demonstrated that bovine besnoitiosis continues to spread in the Italian cattle population. Breeders and veterinarians should be aware of this parasitic disease with consequences on the health and well-being of infected animals, as well as on the economy of affected farms. As already pointed out (Alvarez-Garcia et al., 2014; Gutiérrez-Expósito et al., 2017b), the surveillance of bovine besnoitiosis should be based on a standardized diagnostic procedure including both clinical and laboratory tests, i.e., combining a careful clinical inspection of sclera conjunctiva and *vestibulum vaginae* with the serological diagnosis. This is the basic prerequisite to designing specific control programs, to be adapted to the epidemiological situation of each herd or region. Finally, the study has also demonstrated that besnoitiosis can be considered a neglected parasitic disease of cattle and effective knowledge through dissemination plans among breeders and veterinarians is needed to implement specific control programs.

Declarations

Compliance with ethical standards

Conflict of interest

The authors declare that they have no conflict of interest.

Ethical approval

All procedures were approved by the Institutional Animal Care and Use Committee of Università degli Studi di Milano ("Organismo Preposto al Benessere degli Animali," Prot. no. OPBA_34_2017). This article does not contain any studies with human participants performed by any of the authors.

Informed consent

Informed consent was obtained from the owner of the animals and from all individual participants (farmers) included in the study. Informed consent was also obtained from the owner of the animal (cow) for the case study.

Exploring alterations of hematological and biochemical parameters, enzyme activities and serum cortisol in *Besnoitia besnoiti* naturally infected dairy cattle

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Parasite and Vectors, under revision.

Abstract

Besnoitia besnoiti is an Apicomplexan protozoa causative of bovine besnoitiosis, a chronic and debilitating disease of cattle, with a variety of pathological findings, that could alter some laboratory parameters. A study was conducted in a bovine besnoitiosis endemically infected dairy herd located in Italy characterized by high intra-herd seroprevalence and cattle with clinical signs of the disease. In the study, alterations of laboratory parameters, i.e., hematological and biochemical parameters, enzyme activities and serum cortisol levels in *Besnoitia besnoiti* naturally infected cows were deeply investigated.

Laboratory parameters of 107 cows, 61 seronegative and 46 seropositive to *B. besnoiti*, including 27 with clinical signs of bovine besnoitiosis, were compared. Generalized Linear Models were used to evaluate the effect of *Besnoitia* infection on the considered laboratory parameters.

Hematological analyses revealed that *B. besnoiti* infection determined a significant alteration of the leukocyte differential with a higher percentage of granulocytes and a lower percentage of lymphocytes in seropositive and clinically affected animals (Mann-Whitney *U* Test, $P = 0.022$); Erythrocyte and Platelet counts did not show any difference between the considered groups of cows. Biochemistry evidenced that the parasite infection influenced serum protein values in seropositive cows and GLDH values in clinically affected animals. No or only slight differences were revealed for all the other biochemical and enzyme activity parameters in *B. besnoiti* infected animals. Besides, despite the lack of statistical significance, seropositive and clinically affected cows evidenced higher concentrations of serum cortisol values if compared to seronegative animals: this may indicate that bovine besnoitiosis could be related to stress in infected animals.

Even if physiological, pathological, and farm-related factors could have influenced the results in investigated animals, further studies involving more animals from different farms would be advisable to infer the role of *B. besnoiti* on these alterations, since laboratory parameters could help veterinarians in the diagnosis of bovine besnoitiosis in cattle.

Keywords: Bovine besnoitiosis; Dairy cattle; Hematology; Biochemistry; Enzyme activities; Cortisol.

Introduction

Bovine besnoitiosis is a parasitic disease of cattle caused by the cystogenic coccidian parasite *Besnoitia besnoiti*. In Europe, it is considered an emerging or re-emerging disease, with an increasing geographical distribution and the number of cases of infection (EFSA, 2010). In Italy, in the last decade, outbreaks of bovine besnoitiosis were reported, particularly in Northern regions (Gentile et al., 2012; Gazzonis et al., 2014, Gazzonis et al., 2017; Villa et al., 2019).

Bovine besnoitiosis is a chronic and debilitating disease, characterized by both cutaneous and systemic manifestations, compromising animal welfare and responsible for economic losses on affected farms (EFSA, 2010; Alvarez-Garcia et al., 2013; Cortes et al., 2014). The disease progresses in two sequential clinical phases. Initially, the intense multiplication of tachyzoites within endothelial cells causes vasculitis, hyperplasia, thrombosis, necrosis of venules and arterioles, and increased vascular permeability, subsequently resulting in congestion, hemorrhages, and infarcts. The acute phase is thus characterized by hyperthermia, edemas, and other non-specific clinical signs, including depression, swelling of the superficial lymph nodes, the arrest of rumination, weight loss, anorexia, photophobia, epiphora, ocular and nasal discharge, tachycardia and tachypnea. The subsequent chronic phase is due to the slow replication of bradyzoites inside tissue cysts with tropism for connective tissues, surrounded by granulomatous inflammation. The skin becomes progressively thickened and wrinkled with alopecia, seborrhea, and hyperkeratosis, with the presence of pathognomonic subcutaneous thick-walled tissue cysts. Chronically infected cattle show loss of weight and progressive deterioration of the body condition (Jacquiet et al., 2010; Alvarez-Garcia et al., 2014). The pathological findings occurring during both the acute and chronic phases of bovine besnoitiosis could lead to alterations of laboratory parameters.

A few studies focused on the hematological and biochemical changes during *B. besnoiti* infection. A study investigated both the impact of naturally acquired acute, subacute, and chronic besnoitiosis on these parameters in three Simmental heifers and two Limousin cows and compared their changes in seronegative and seropositive Limousins from a German cow-calf operation, analyzing a high number of samples from a limited number of animals (224 samples from nine Simmental and 75 samples from 54 Limousin cattle) (Langenmayer et al., 2015). Alterations of blood parameters in 11 beef cattle from an outbreak of bovine besnoitiosis in southwestern Spain (Nieto-Rodriguez et al., 2016) and in a naturally infected bull from South Africa were reported (Dubey et al., 2013). Most of these studies concerned mostly beef cattle, except that of Alshehabat et al. (2016) evaluating selected metabolic biochemical parameters including enzyme activities

associated with *B. besnoiti* infection in dairy cattle from Jordan. Moreover, hematology and biochemistry changes were observed in goats affected by naturally acquired caprine besnoitiosis in Iran (Nazifi et al., 2002; Oryan et al. 2008).

Besides, to the best of our knowledge, no studies investigated the cortisol concentration in bovine besnoitiosis affected cows. Cortisol is a cholesterol-derived steroid synthesized in the adrenal cortex under the control of the HPA (Hypothalamic–Pituitary–Adrenal) axis, reacting to a wide range of stimuli from both internal and external origin. This glucocorticoid is the primary hormone involved in stress response: its main action is to activate biological functions to respond to stress and to restore homeostasis after exposure to stressors. It is commonly used as an indicator of stress and welfare in animals (Mormède et al., 2007). Prolonged elevation of cortisol influences negatively the immune response, causing suppression of the immune system and increased susceptibility to disease; it has also adverse effects on the reproductive success of animals (Brown and Vosloo, 2017). It was also reported that during immunosuppression *B. besnoiti* proliferation in cattle might be facilitated (Alvarez-Garcia et al., 2014).

Considering the spread of bovine besnoitiosis that could be underestimated (EFSA, 2010; Gutierrez-Exposito et al., 2017) and since there are no studies focused on alterations in laboratory parameters in Holstein Friesian dairy cows under intensive farming conditions, the main goal of this study was to investigate alterations of hematological and biochemical parameters, including muscle and liver enzyme activity, and variation of serum cortisol levels in *Besnoitia besnoiti* naturally infected cows from an intensive dairy herd endemically affected by bovine besnoitiosis. Further aims were i) to evaluate if the infection could be predicted by variations of laboratory parameters investigated and ii) to verify the effects of the parasite infection on the health status and welfare of infected animals.

Materials and methods

Background

The study was conducted in a bovine besnoitiosis endemically infected dairy herd hosting 217 Holstein Friesian cattle located in the province of Brescia (Lombardy, Northern Italy). A serological screening for *B. besnoiti* was performed on the whole herd; Western Blot positive results were considered as B. besnoiti seropositive animals. Besides, a part of the seropositive animals was clinically examined to reveal symptoms and lesions of besnoitiosis. The herd resulted affected by high intra-herd *B. besnoiti* seroprevalence (23.5% increasing up to 43.5% considering only cows) and a part (n = 27) of the seropositive animals suffered from clinical signs of the

chronic phase of the disease; indeed, clinically affected cattle evidenced the presence of tissue cysts in at least one of the typical localizations (sclera, vulva, or skin). Moreover, a case of severe systemic besnoitiosis in a chronically affected cow was also recorded (Villa et al., 2019). Besides, a form of generalized demodectic mange in two cows co-infected with *B. besnoiti* was also reported (Villa et al., 2020).

Sample and data collection

Overall, 107 cows from the infected herd, 46 seropositive to *B. besnoiti* and 61 seronegative, were included in the study. During the sampling for the serological screening, blood samples were collected in tubes with EDTA for hematological analyses. Besides, an aliquot of the sera collected for serological analyses was stored at -20° C for biochemistry, including liver and muscle enzyme activity analyses, and cortisol determination. Epidemiological data, including individual data (breed, sex, productive category, age, and origin of the animals) and information regarding reproductive (number of parturitions and phase of lactation) were noted.

Hematology

On blood samples preserved in tubes with EDTA, hematological analyses were performed within 24 hours from the collection time, using the analyzer Cell-Dyn 3500 (Abbott Laboratories, Abbott Park, IL, USA). The following hematological parameters were included: Red Blood Cells (RBC), Hemoglobin (Hb), Hematocrit (Ht), Mean Corpuscular Volume (MCV), Mean Corpuscular Hemoglobin (MCH), Mean Corpuscular Hemoglobin Concentration (MCHC), Red Cell Distribution Width (RDW), White Blood Cells (WBC), Lymphocytes, Granulocytes, Platelet Count (PLT), Mean Platelet Volume (MPV), Plateletcrit (Pct), Platelet Distribution Width (PDW).

Biochemistry and enzyme activity

Biochemistry and enzyme activity analyses were carried out on serum with the automated analyzer BT3500 (Biotecnica Instruments S.p.a., Rome, Italy) using reagents, controls and calibrators provided by Futurlab Srl (Limena, Italy) and Randox Laboratories Ltd. (Crumlin, Co. Antrium, UK) (exclusively for BOHB and NEFA). The following analytes were measured (acronyms and methods between brackets): albumin (albumin, bromochresol green), aspartate aminotransferase (AST, kinetic IFCC), β-hydroxybutyrate (BOHB, D-3-Hydroxybutyrate dehydrogenase), calcium (orthocresoftaleine), magnesium (xylidyl blue), creatine kinase (CK, kinetic IFCC), glutamate dehydrogenase (GLDH, kinetic IFCC), lactate (LO-POD), lactate dehydrogenase (LDH, kinetic IFCC), non-esterified fatty acid (NEFA, ACS-ACOD), phosphorous (phosphomolibdate), total

bilirubin (modified Jendrassik-Grof assay), total proteins (modified biuret). Globulin (globulin) concentration was calculated by subtracting albumin from total proteins whereas the albumin:globulin ratio (A/G) by dividing albumin by globulin.

Cortisol determination

Cortisol concentration was assessed in serum samples by commercially available enzyme-linked immunosorbent assay kit (Cortisol Enzyme-linked immunosorbent assay kit; art. CEA462Ge, Cloud Clone Corp, Katy, TX, USA). Each sample was prepared in duplicate, and absorbance measured using a wavelength of 405 nm, using a Labisystem Multiskan Ex (Nepean, ON, Canada) microplate reader, according to the relevant standard curves. The mean recovery was 102.8% ± 10.8, The average intra- and inter-assay coefficients of variation were 5.7% and 7.6% The assay sensitivity was 5.15 ng/ml. The laboratory technician was blinded to the hypotheses and conditions.

Statistical analysis

Values of hematology, biochemistry, enzyme activities and serum cortisol of seronegative and *B. besnoiti* seropositive dairy cattle were compared statistically. Besides, the same laboratory parameters were compared considering the groups of seronegative or asymptomatic seropositive cows and animals with clinical signs of chronic besnoitiosis. To assess normality, Shapiro-Wilk Test was used. To compare the two groups, the non-parametric Mann-Whitney *U* Test was performed; the level of significance was set at P values below 0.05.

Separate generalized linear models (GLMs) with linear distribution were performed for each analyte to verify the influence of *B. besnoiti* infection on hematological and biochemical parameters, enzyme activities and serum cortisol levels (dependent variables). As predictor, the outcome of *B. besnoiti* infection was considered both as i) seropositivity to *B. besnoiti* (dichotomous variable: presence vs. absence of antibodies based on Western Blot results) and ii) clinical signs of bovine besnoitiosis (dichotomous variable: seronegative or asymptomatic seropositive animals vs. clinically affected cows); these outcomes were evaluated singularly, entering them as independent variables in separate models. For the models containing the variable "seropositivity to *B. besnoiti*", all examined animals were included. For the models containing the variable "clinical signs of bovine besnoitiosis", 14 seropositive animals were excluded from the analysis, since it was not possible to perform a clinical examination and thus ascertain their clinical status. In addition, to considering the influence of the physiological status of the cows on the considered laboratory parameters, number of parturitions (dichotomous variable: primiparous

vs. multiparous cows) and phase of lactation (ordinal variable: early, mid, late, and dry) were also entered in each model as independent variables. Besides, the two-way interactions between *B. besnoiti* infection (seropositivity to *B. besnoiti* or clinical signs of bovine besnoitiosis), number of parturitions and phase of lactation were considered, to verify the overall influence of both the parasite infection and the physiological status of the cows on the considered laboratory parameters. All variables and their two-way interactions were entered in multivariate models developed through a backward selection procedure (significance level to remove variables from the model = 0.05), based on Akaike's information criterion (AIC) values. When retained in the final models, the estimated means of the variable "phase of lactation" and the interactions were compared through pairwise comparisons. Statistical analysis was performed using SPSS software (Statistical Package for Social Science, IBM SPSS Statistics for Windows, version 25.0., Chicago, IL, USA).

Results

In the study, laboratory parameters of 107 cows, 61 seronegative and 46 seropositive to *B. besnoiti*, including 27 with clinical signs of chronic bovine besnoitiosis, were investigated.

All sampled animals were female Holstein Friesian cows and all of them were born in the farm. The mean age of sampled cows was 46.8 months (SD=17.7, Min-Max=25.4-115.6). Forty-six of them were primiparous, whereas the remaining have had between two and six parturitions. Ninety-five animals were lactating at the time of sampling: 31 of them in the early, 37 in the mid and 27 in the late phase of lactation, respectively; the remaining 12 animals were in the dry phase.

Results of hematological and biochemical parameters, enzyme activities and serum cortisol levels are resumed in Tab. 1. Descriptive statistics were sorted considering the groups of seronegative and seropositive cows according to the Western Blot results for *B. besnoiti*; besides, among seropositive animals, a subgroup of cows presenting clinical signs of bovine besnoitiosis was also considered. Rating the quantity of the data, laboratory parameters results sorted also considering the number of parturitions (primiparous and multiparous cows) and the phase of lactation (early, mid, late, and dry) are reported in Supplementary Tables 1 and 2, respectively. For all the considered laboratory parameters specific reference ranges for dairy cows were used.

Tab. 1. Descriptive statistics (mean, standard deviation, minimum and maximum) of hematological, biochemical and enzyme activities analyses, and cortisol determination sorted by the serological and clinical status of cows in a dairy cattle herd endemically infected by bovine besnoitiosis. Serological status (seronegative or seropositive) was determined according to Western Blot results while as clinically affected cows are meant those animals with the presence of clinical signs of the disease. Laboratory parameters significantly ($P<0.05$) different versus seronegative cows (Mann-Whitney U Test) are highlighted in bold.

	Parameter	Unit	Seronegative		Seropositive				Range
					Overall		Clinically affected		
			Mean (SD)	Min-Max	Mean (SD)	Min- Max	Mean (SD)	Min-Max	
Hematology	**RBC**	$x10^6/\mu L$	6.11 (0.73)	4.67-8.53	6.15 (0.67)	4.80-7.14	6.07 (0.66)	4.80-7.02	6-10
	Hb	g/dl	12.46 (1.55)	8.86-15.88	12.18 (1.75)	3.81-14.89	12.04 (2.01)	3.81-14.45	8-12
	Ht	%	26.77 (2.60)	20.70-31.80	27.35 (2.73)	22.20-32.40	26.69 (2.30)	22.20-30.80	20-35
	MCV	μ^3	44.08 (3.97)	32.00-56.00	44.70 (3.22)	38.00-50.00	44.30 (3.45)	39.00-50.00	40-50
	MCH	pg	20.57 (2.91)	14.74-27.48	19.92 (3.12)	7.62-24.96	19.92 (3.46)	7.62-24.96	15-20
	MCHC	g/dl	46.74 (5.62)	35.95-62.35	44.64 (6.34)	17.17-56.71	45.08 (7.17)	17.17-56.71	30-40
	RDW	%	15.23 (0.99)	13.40-17.50	15.22 (0.79)	13.80-16.90	15.20 (0.84)	13.90-16.90	15-17
	WBC	$x10^3/\mu L$	9.35 (6.31)	2.70-48.10	9.69 (6.58)	4.70-37.20	9.70 (6.33)	4.70-35.80	6-12
	Lymphocytes	%	53.52 (13.69)	11.00-97.00	**45.78 (18.53)**	12.00-88.00	46.48 (18.46)	12.00-86.00	55-85
	Granulocytes	%	46.48 (13.69)	3.00-89.00	**54.22 (18.53)**	12.00-88.00	53.52 (18.46)	14.00-88.00	15-45
	PLT	$x10^3/\mu L$	307.77 (115.04)	17.10-490.00	286.41 (106.87)	90.00-557.00	286.74 (111.46)	90.00-557.00	200-800
	MPV	μ^3	13.43 (35.35)	8.20-285.00	8.82 (0.32)	8.10-9.50	8.80 (0.30)	8.30-9.40	5-9
	Pct	%	0.28 (0.09)	0.07-0.43	0.25 (0.09)	0.08-0.47	0.25 (0.10)	0.08-0.47	0.1-0.4
	PDW	%	13.39 (13.00)	9.70-113.00	11.66 (1.05)	9.70-14.20	11.63 (1.17)	9.70-14.20	10-14

Biochemistry	**Total proteins**	g/dl	8.73 (1.11)	6.30-11.50	8.34 (1.04)	5.60-10.50	8.28 (1.06)	5.60-10.50	5,9-7,7
	Albumin	g/dl	2.96 (0.52)	2.00-4.20	3.12 (0.60)	2.00-4.70	2.93 (0.52)	2.00-3.90	2,7-4,3
	Globulin	g/dl	5.78 (1.34)	2.90-8.90	5.22 (1.10)	3.20-7.70	5.36 (0.92)	3.60-6.80	1.6-5
	A/G ratio		0.56 (0.22)	0.26-1.28	0.63 (0.21)	0.31-1.19	0.56 (0.13)	0.37-0.82	0,7-1,5
	Total bilirubin	mg/dl	0.56 (0.36)	0.09-1.63	0.62 (0.84)	0.13-5.26	0.72 (1.05)	0.13-5.26	0,1-0,3
	Ca	mg/dl	9.56 (0.74)	7.00-11.10	9.54 (0.72)	7.10-10.80	9.40 (0.81)	7.10-10.80	7,9-10
	P	mg/dl	6.43 (1.21)	3.90-9.70	6.18 (1.21)	4.00-10.40	6.13 (1.35)	4.00-10.40	4,6-9
	Mg	mg/dl	2.14 (0.35)	1.38-3.10	2.15 (0.35)	1.22-2.93	2.17 (0.36)	1.22-2.65	1,4-2,3
	NEFA	mmol/l	0.32 (0.24)	0.06-1.05	0.34 (0.20)	0.09-0.76	0.33 (0.21)	0.12-0.76	0,4-0,8
	BOHB	mmol/l	0.64 (0.24)	0.29-1.54	0.60 (0.28)	0.05-1.48	0.65 (0.31)	0.05-1.48	0,03-0,3
	Lactate	mmol/l	2.82 (0.92)	1.47-5.23	2.81 (0.94)	1.10-5.39	2.78 (1.02)	1.10-5.39	<2
Enzymes	**AST**	U/l	74.40 (33.17)	43.00-252.00	74.07 (21.24)	47.00-159.00	75.26 (23.16)	48.00-159.00	48-100
	CK	U/l	303.58 (1278.29)	28.00-9971.00	173.76 (332.28)	37.00-2182.00	145.59 (161.06)	39.00-782.00	44-228
	LDH	U/l	870.42 (261.21)	434.00-2173.00	866.44 (253.36)	464.00-2113.00	904.44 (289.32)	535.00-2113.00	500-1500
	GLDH	U/l	33.42 (51.46)	6.40-367.00	31.49 (31.09)	0.00-137.70	36.69 (36.10)	0.00-137.70	<20
	Cortisol	ng/ml	6.81 (6.41)	0.77-35.48	9.11 (9.51)	0.56-43.86	8.54 (11.36)	0.56-43.86	<10

Hematology

Mean of RBC, Hb, Ht, and erythrocyte indices (MCV, MCH, MCHC, and RDW) showed similar values between seronegative, seropositive, and clinically affected cows (Table 1). However, considering overall both seronegative and *B. besnoiti* seropositive animals, alterations of some of these parameters were detected. Particularly, mean values of RBC (6.13±0.70), Ht (27.02±2.66) and RDW (15.23±0.90) resulted low and those of MCV (44.35±3.66) and MCH (20.29±3.01) high, but remained upper and below the limits of the reference interval, respectively (Table 1).

WBC counts resulted similar in the considered groups of animals (seronegative, seropositive and clinically affected cows) and mean values included within the reference interval (Table 1). Nonetheless, a modification in the leukocyte differential with a higher percentage of granulocytes and a lower percentage value of lymphocytes was evidenced in seropositive (54.22±18.53 and 45.78±18.53, respectively) and clinically affected (53.52±18.46 and 46.48±18.46, respectively) cows, above all in primiparous seropositive ones (62.19±16.51 and 37.81±16.51, respectively) and primiparous animals with clinical signs of besnoitiosis (56.77±23.61 and 43.23±23.61, respectively), if compared to seronegative and multiparous ones (Additional file 1: Table S1). Indeed, while seronegative animals were equally distributed considering the reference interval for the leukocyte differential, 69.6% of seropositive cows, increasing up to 87.5% rating only primiparous cows, showed a mean concentration of lymphocytes and granulocytes below and above the reference range, respectively.

The mean values of PLT, MPV, Pct, and PDW were slightly lower in seropositive (286.41±106.87, 8.82±0.32, 0.25±0.09, and 11.66±1.05, respectively) and clinically affected (286.74±111.46, 8.80±0.30, 0.25±0.10, and 11.63±1.17, respectively) cows when compared to seronegative animals (307.77±115.04, 13.43±35.35, 0.28±0.09, and 13.39±13.00, respectively), but with the mean values for all groups of cows within the reference intervals (Table 1).

Biochemistry and enzyme activity

Regarding serum total protein concentration, the mean of total proteins (8.57±1.09) resulted elevated in all the cows; albumin (3.03±0.55) and globulin (5.54±1.27) concentrations were at the lower and upper limit of the range, respectively, with a slightly lower value of the A/G ratio (0.59±0.22). Besides, seropositive (8.34±1.04) and clinically affected (8.28±1.06) cows showed lower values of total protein if compared to seronegative animals (8.73±1.11); globulin concentrations were lower in seropositive (5.22±1.10) and clinically affected (5.36±0.92) animals than seronegative cows (5.78±1.34). Albumin (3.12±0.60) and A/G ratio (0.63±0.21) values were higher in seropositive cows, but slightly lower in clinically affected animals (2.93±0.52 and

0.56±0.13), if compared to seronegative ones (2.96±0.52 and 0.56±0.22, respectively). Values of serum protein differed considering the lactation phase: in particular, total protein values (9.13±1.02) were highest in the late phase, albumin (3.68±0.34) and A/G ratio (0.87±0.26) were highest in dry cows, whereas globulin values were highest in the late phase of lactation (6.14±1.27) but lowest in dry animals (4.59±1.35).

Total bilirubin resulted elevated over the range limit in all cows (0.58±0.61), with higher values in seropositive (0.62±0.84) and clinically affected animals (0.72±1.05) than in seronegative ones (0.56±0.36).

Concerning minerals, the mean concentration of Ca, P, and Mg were within the limits, with similar values in seropositive and seronegative cows (Table 1).

NEFA, BOHB and lactate showed similar mean values between the groups of cows according to *B. besnoiti* seropositivity and clinical signs (Table 1). However, considering mean concentrations in all the animals, values of BOHB (0.62±0.25) and lactate (2.82±0.93) were above the reference range, while NEFA values (0.33±0.22) were within the reference interval.

Concerning enzyme activities, AST and LDH values were within the normal range and showed similar values in all the animals (Table 1), with only a slightly higher value in clinically affected animals (75.26±23.16 and 904.44±289.32, respectively) if compared with seronegative cows (74.40±33.17 and 870.42±261.21). CK resulted elevated over the upper limit in seronegative animals (303.58±1278.29), while the mean value was within the reference interval in seropositive cows (173.76±332.28). GLDH evidenced values above the reference limit in all the cows (32.58±43.65) with a slight major value in clinically affected animals (36.69±36.10).

Cortisol determination

Seropositive (9.11±9.51) and clinically affected (8.54±11.36) cows evidenced higher values of cortisol if compared to seronegative animals (6.81±6.41). Cortisol levels were generally higher in the early lactation phase (9.13±12.37) and dry cows (10.96±6.27), compared to the mid (5.62±4.87) and late (8.27±5.76) phase of lactation; instead, mean values resulted similar in primiparous (7.15±7.33) and multiparous (8.04±8.52) cows. However, overall mean cortisol levels (7.92±8.09) were generally elevated considering the recommended reference value (5-10 ng/mL) in all the animals.

Statistical analysis

Values of hematology, biochemistry, enzyme activities and serum cortisol were compared statistically using the non-parametric Mann-Whitney *U* Test considering the groups of

seronegative vs. *B. besnoiti* seropositive dairy cattle and seronegative or asymptomatic seropositive vs. clinically affected animals. The Mann-Whitney *U* Test revealed that the distribution of lymphocytes and granulocytes was significantly different in seronegative and seropositive cows ($P = 0.022$). On the contrary, none of the other parameters showed a significant difference between the considered groups of animals.

The effect of *Besnoitia* infection on hematological and biochemical parameters, enzyme activities and serum cortisol levels was subsequently considered using GLMs. Besides, the interactions between *Besnoitia* infection, "number of parturitions" and/or "phase of lactation" were considered to take into account both the influence of parasite infection and the physiological status of the cows on the considered laboratory parameters. Final models in which selected parameters resulted associated with *B. besnoiti* infection are reported in Tab. 2; final models in which only the variables "number of parturitions" or "phase of lactation" resulted statistically associated to the response variable are reported in Supplementary Table 3.

Considering hematological parameters, only the variables lymphocytes and granulocytes resulted influenced by both *B. besnoiti* seropositivity and clinical disease. In fact, the percentage of lymphocytes was evidenced to be lower in seropositive (Mean±SD=45.78±18.53, OR=0.93) compared to seronegative cows (53.52±13.69). Besides, in seropositive cattle, the granulocyte percentage (54.22±18.53) was 1.08 times higher than in seronegative cattle (46.48±13.69). In addition, the interaction between *B. besnoiti* seropositivity and the number of parturitions resulted statistically associated to the alterations in the leukocyte differential, i.e., the percentage values of lymphocytes (37.81±16.51, OR=0.85) were lower and percentage values of granulocytes (62.19±16.51, OR=1.18) were higher in seropositive primiparous cows than seropositive multiparous and their respective seronegative categories. Similarly, clinically affected cows evidenced lower percentages of lymphocytes (47.88±18.15, OR=0.86) and higher percentages of granulocytes (52.12±18.15, OR=1.04) if compared to seronegative or asymptomatic seropositive ones (51.70±16.12 and 48.30±16.12, respectively). Also, in this case, the interaction between clinical signs of besnoitiosis and the number of parturitions resulted associated with lower and higher percentage values of lymphocytes (38.75±17.98, OR=0.86) and granulocytes (61.25±17.98, OR=1.16), respectively, in clinically affected primiparous cows, when compared to multiparous animals with clinical signs of chronic besnoitiosis and seronegative or asymptomatic seropositive ones (Table 2).

Regarding biochemical analytes, alterations in serum protein values were associated with *B. besnoiti* serological status and the lactation phase. In fact, parasite infection resulted a predictor of lower values of total protein (8.34±1.04, OR=0.66) and globulin (5.22±1.10, OR=0.52) in

seropositive cows, if compared to seronegative animals (8.73±1.11 and 5.78±1.34, respectively). On the contrary, higher values of albumin (3.12±0.60, OR=1.26) and A/G ratio (0.63±0.21, OR=1.11) were evidenced in seropositive cows than seronegative ones (2.96±0.52 and 0.56±0.22, respectively). Besides, concerning the lactation phase, higher total protein values (9.13±1.02, OR=2.06) were evidenced in cows in the late phase of lactation, whereas dry cows showed more probability to have higher values for both albumin (3.68±0.34, OR=2.00) and A/G ratio (0.87±0.26, OR=1.33). Furthermore, cows in the late phase of lactation and dry animals had more probability to show higher and lower globulin values (6.15±1.27, OR=2.08 and 4.59±1.35, OR=0.44, respectively) than cows of other lactation categories (Table 2).

Among enzyme activity parameters, the presence of clinical signs of besnoitiosis and the number of parturitions resulted associated to alterations in GLDH values. Indeed, being clinically affected animals enhanced the probability to have higher mean values of GLDH (39.86±37.10, OR=1.15) if compared to seronegative or asymptomatic seropositive cows (31.22±48.68); similarly, being primiparous (37.72±36.78, OR=1.15) enhanced the probability to have higher GLDH values than being multiparous animals (23.20±18.64).

Tab. 2. Results of the significative variables to the GLM analysis regarding the variation on laboratory parameters according to *B. besnoiti* infection (seropositivity and clinical signs), number of parturitions and phase of lactation. Descriptive statistics (mean, standard deviation, minimum and maximum) of the considered parameters were also included. Mean values of each parameter per each phase of lactation with different superscript letters are statistically different from each other (p−value <0.05, GLM, pairwise comparison), while those with the same superscript letters are not statistically different from each other (p−value >0.05, GLM, pairwise comparison). The number of parturitions was classified as follows: Primiparous = one parturition, Multiparous = two or more parturitions. The phase of lactation was classified as follows: Early = 0-120 days, Mid = 121-250 days, Late= 251-305 days, and Dry.

Response Variable	Predictor	Category	Mean (SD)	Min-Max	β[a]	SE[b]	Wald Chi-Square	OR (95% CI)	*P*-value	AIC
Lymphocytes	Seropositivity to *B. besnoiti*	Seronegative	53.52 (13.69)	11.00-97.00	0			1		80.636
		Seropositive	45.78 (18.53)	12.00-88.00	-0.08	0.03	6.30	0.93 (0.87-0.98)	0.012	
	Seropositivity to *B. besnoiti* X Number of parturitions	Seronegative Primiparous	54.47 (14.36)	25.00-97.00	0			1		
		Seronegative Multiparous	52.29 (13.83)	11.00-81.00	-0.02	0.04	0.28	0.98 (0.90-1.06)	0.594	
		Seropositive Primiparous	37.81 (16.51)	12.00-65.00	-0.17	0.05	11.92	0.85 (0.77-0.93)	0.001	

		Seropositive Multiparous	50.14 (18.70)	15.00-88.00	-0.04	0.04	1.14	0.96 (0.88-1.04)	0.286	
	Clinical signs of besnoitiosis	Healthy	51.70 (16.12)	11-97	0			1		
		Clinically affected	47.88 (18.15)	12-86	-0.15	0.05	7.26	0.86 (0.78-0.96)	0.007	
	Clinical signs of besnoitiosis X Number of parturitions	Healthy Primiparous	53.39 (15.34)	21-97	0			1		-64.738
		Healthy Multiparous	49.86 (17.43)	11-88	-0.03	0.04	0.80	0.96 (0.89-1.04)	0.370	
		Clinically affected Primiparous	38.75 (17.98)	12-65	-0.15	0.05	7.26	0.86 (0.78-0.96)	0.007	
		Clinically affected Multiparous	57.00 (13.55)	29-86	0.4	0.05	0.44	1.04 (0.93-1.15)	0.506	
Granulocytes	Seropositivity to *B. besnoiti*	Seronegative	46.48 (13.69)	3.00-89.00	0			1		
		Seropositive	54.22 (18.53)	12.00-88.00	0.08	0.03	6.30	1.08 (1.02-1.15)	0.012	
	Seropositivity to *B. besnoiti* X Number of parturitions	Seronegative Primiparous	45.53 (14.36)	3.00-65.00	0			1		80.636
		Seronegative Multiparous	47.71 (13.83)	19.00-89.00	0.02	0.04	0.28	1.02 (0.94-1.12)	0.594	
		Seropositive Primiparous	62.19 (16.51)	35.00-88.00	0.17	0.05	11.92	1.18 (1.07-1.29)	0.001	
		Seropositive Multiparous	49.86 (18.70)	12.00-85.00	0.04	0.04	1.14	1.04 (0.96-1.13)	0.286	
	Clinical signs of besnoitiosis	Healthy	48.30 (16.12)	3-89	0			1		
		Clinically affected	52.12 (18.15)	14-88	0.15	0.05	7.26	1.16 (1.04-1.29)	0.007	
	Clinical signs of besnoitiosis X Number of parturitions	Healthy Primiparous	46.61 (15.34)	3-79	0			1		-64.738
		Healthy Multiparous	50.14 (17.43)	12-89	-0.03	0.04	0.80	1.04 (0.96-1.12)	0.370	
		Clinically affected Primiparous	61.25 (17.98)	35-88	-0.15	0.05	7.26	1.16 (1.04-1.29)	0.007	

		Clinically affected Multiparous	43.00 (13.55)	14-71	0.4	0.05	0.44	0.96 (0.87-1.07)	0.506	
Total proteins	Seropositivity to *B. besnoiti*	Seronegative	8.73 (1.11)	6.30-11.50	0			1		304.313
		Seropositive	8.34 (1.04)	5.60-10.50	-0.42	0.20	4.30	0.66 (0.44-0.98)	0.038	
	Phase of lactation	Early	8.40 (0.98)[a]	6.30-10.20	0			1		
		Mid	8.38 (1.06)[a]	5.60-11.00	-0.02	0.26	0.00	0.98 (0.59-1.61)	0.930	
		Late	9.13 (1.02)	7.40-11.50	0.72	0.28	6.84	2.06 (1.19-3.54)	0.009	
		Dry	8.27 (1.26)[a]	6.30-10.50	-0.13	0.35	0.13	0.88 (0.44-1.76)	0.717	
Albumin	Seropositivity to *B. besnoiti*	Seronegative	2.96 (0.52)	2.00-4.20	0			1		153.895
		Seropositive	3.12 (0.60)	2.00-4.70	0.23	0.09	5.75	1.26 (1.04-1.52)	0.017	
	Phase of lactation	Early	2.99 (0.50)[a]	2.1-3.9	0			1		
		Mid	2.88 (0.42)[a]	2.00-4.00	-0.11	0.12	0.86	0.89 (0.70-1.14)	0.354	
		Late	2.98 (0.65)[a]	2.00-4.70	-0.00	0.13	0.00	0.99 (0.76-1.29)	0.951	
		Dry	3.68 (0.34)	2.90-4.20	0.69	0.17	16.60	2.00 (1.43-2.79)	0.000	
Globulin	Seropositivity to *B. besnoiti*	Seronegative	5.78 (1.34)	2.90-8.90	0			1		326.879
		Seropositive	5.22 (1.10)	3.20-7.70	-0.65	0.22	8.21	0.52 (0.34-0.81)	0.004	
	Phase of lactation	Early	5.41 (1.14)[a]	3.80-8.00	0			1		
		Mid	5.50 (1.15)[a]	3.60-8.70	0.09	0.29	0.09	1.09 (0.62-1.95)	0.754	
		Late	6.14 (1.27)	4.40-8.90	0.73	0.31	5.36	2.08 (1.12-3.86)	0.021	

		Dry	4.59 (1.35)	2.90-7.60	-0.82	0.40	4.11	0.44 (0.19-0.97)	0.043	
A/G ratio	Seropositivity to *B. besnoiti*	Seronegative	0.56 (0.22)	0.26-1.28	0			1		42.458
		Seropositive	0.63 (0.21)	0.31-1.19	0.10	0.04	7.76	1.11 (1.03-1.19)	0.005	
	Phase of lactation	Early	0.58 (0.19)[a]	0.27-1.00	0			1		
		Mid	0.55 (0.16)[a]	0.26-0.95	-0.35	0.05	0.53	0.97 (0.88-1.06)	0.466	
		Late	0.52 (0.21)[a]	0.29-1.04	-0.65	0.05	1.59	0.94 (0.85-1.04)	0.207	
		Dry	0.87 (0.26)	0.38-1.27	0.28	0.06	18.74	1.33 (1.17-1.51)	0.000	
GLDH	Clinical signs of besnoitiosis	Healthy	31.22 (48.68)	6.4-367	0			1		33.850
		Clinically affected	39.86 (37.10)	0.00-137.7	0.14	0.07	4.51	1.15 (1.01-1.31)	0.034	
	Number of parturitions	Primiparous	37.72 (36.78)	0.00-163.9	0.14	0.06	6.04	1.15 (1.03-1.29	0.014	
		Multiparous	23.20 (18.64)	6.4-82	0			1		

[a] coefficients, [b] standard error (SE) of the coefficients

Discussion

The study provides data on laboratory parameters, including hematology, biochemistry, enzyme activity, and cortisol determination, in *B. besnoiti* naturally infected cows from an endemically affected dairy cattle herd in Italy. Results were analyzed considering both the serological status for *B. besnoiti* (seronegative and seropositive cows) and the presence of clinical chronic bovine besnoitiosis (healthy and clinically affected cows). Besides, the influence of the number of parturitions (primiparous and multiparous cows) and the phase of lactation (early, mid, late, and dry) on laboratory parameters were also considered. Finally, the two-way interactions between *Besnoitia* seropositivity or clinical disease, number of parturitions and phase of lactation were evaluated.

Hematology

The mean RBC counts resulted at the lower limit of the reference range in all the animals. However, it should be considered that out of 107 cows analyzed for blood parameters, 95 were lactating at the time of sampling, and lactating cows have normally consistently lower RBC than non-lactating cows (Wood and Quiroz-Rocha, 2010). A few authors demonstrated the occurrence of anemia both in beef cattle (Langenmayer et al., 2015) and in goats (Nazifi et al., 2002) with besnoitiosis, probably caused by the chronic inflammatory state.

An important alteration was evidenced in WBC counts. Even though the mean number of WBC fell within the normal range in all considered groups of animals, *B. besnoiti* infection caused an alteration in the leukocyte differential with a higher percentage of the granulocytes and lower percentage of the lymphocytes in seropositive and clinically affected cows. The same alteration was influenced by the number of parturitions and then enhanced for seropositive and clinically affected animals considering the group of primiparous cows. Besides, it was demonstrated that many seropositive animals (69.6%), and particularly primiparous cows (87.5%), presented this modification in the leukocyte differential. Indeed, a significative interaction was evidenced between parasite infection and physiological status of the cows: this alteration in the leukocyte differential with higher values of granulocytes and lower values of lymphocytes was influenced not only by *B. besnoiti* seropositivity or clinical disease, but also by the physiological status of the cows. This finding suggests that in primiparous cows the effects of the parasite infection may be enhanced if compared to multiparous cows, probably due to a higher level of stress and immunosuppression in younger animals.

Langenmayer et al. (2015) reported leukopenia in the acute phase of the disease followed by higher WBC concentration values after seroconversion in beef cattle. Other two studies evidenced leukocytosis in cattle infected with *B. besnoiti*: Dubey et al. (2013) reported also a left shift neutrophilia in the clinically affected bull, whereas in another study lymphocytosis was detected in some infected beef animals (Nieto-Rodriguez et al., 2016). Nazifi et al. (2002) evidenced that compared to normal goats, WBC, and neutrophils were significantly higher and lymphocytes significantly lower in goats with clinical besnoitiosis.

It is recognized that in adult cattle lymphocytes remain the dominant cell type, with a neutrophil-to-lymphocyte ratio of approximately 1:2. A shift in this ratio (stress leukogram) may be due to several causes; inflammatory granulocytosis is reported in viral, bacterial, protozoal, parasitic, and fungal infections, whereas stress, infections, immune suppression, also related to corticosteroids, are among the causes for lymphopenia (Roland et al., 2014). Besides, aggregation of lymphocytes around *Besnoitia* cysts in affected organs could also induce lymphopenia (Nazifi et al., 2002).

At this regard, it should be emphasized that the farm is officially free of tuberculosis, brucellosis, and leucosis. All the animals of the herd are vaccinated against Bovine Viral Diarrhea Virus; besides, the farm adheres to the plan for the control of infectious bovine rhinotracheitis and paratuberculosis. Further, after the diagnosis of some cases of Q Fever also causing abortions in the herd one year before, all the animals were vaccinated against *Coxiella burnetii*. Moreover, it is noteworthy to underline that a form of generalized demodectic mange in two dairy cows co-infected with *B. besnoiti* was diagnosed and then *Demodex bovis* mites could also affect other animals causing subclinical forms of the disease, possibly contributing to the detected alterations in WBC parameters (Villa et al., 2020). Therefore, the observed alteration in the leukocyte differential could be a predictor of the parasite infection, suggesting the need to include bovine besnoitiosis in the differential diagnoses along with other diseases.

Any relevant alteration was not detected in platelet indexes: only a slight reduction of mean values of PLT counts, MPV, Pct, and PDW in seropositive and clinically affected animals was evidenced, thus suggesting that slight thrombocytopenia may indicate the presence of an inflammatory response. Besides, it could be that increase of platelets consumption for their destruction during the chronic inflammatory process due to the parasite infection may be factors determining these findings (Russell, 2010). However, also considering that none of the previous studies investigated these parameters, it is not possible to draw significant conclusions.

Biochemistry and enzyme activity

Serum total proteins were elevated in all cows with a slightly higher value in seronegative animals; the parasite infection caused lower total proteins mean values in seropositive animals. Besides, a higher level of albumin and a lower level of globulin resulted associated with *B. besnoiti* infection, as detected in seropositive cows. Langenmayer et al. (2015) reported higher values of total proteins, albumin and globulin in seropositive cattle, and some other animals with clinical besnoitiosis high levels of total proteins and albumin were evidenced (Nieto-Rodriguez et al., 2016); similar findings were also shown in clinically infected goats (Oryan et al., 2008). Moderate hypoalbuminemia and mild hyperglobulinemia were reported during the acute phase in a clinically affected bull (Dubey et al., 2013). However, it should be considered that these results refer to studies performed on beef cattle. Instead, Alshehabat et al. (2016) reported a lower value in total proteins with a higher value of albumin in *B. besnoiti* infected dairy cows.

Regarding total proteins, it could not be excluded that seropositive cows may manifest edematous phenomena associated with hypoproteinemia conditions, not detectable at the clinical examination, since the infection in these animals could be subclinical. Higher mean values of albumin in

seropositive animals could indicate slight dehydration; instead, clinically affected animals showed slightly lower albumin concentration if compared to the other groups, probably due to inflammation (as albumin acts as a negative acute phase protein) and negative energy balance. In any case, mean values of albumin were at the lower limit of the range for all the animals. The lower values of globulin in seropositive animals, even if with mean values elevated in all the animals, could be due to immunosuppression also considering the higher levels of serum cortisol and the lower values of lymphocytes (Stockham and Scott, 2008). Moreover, it was evidenced that also the phase of lactation influenced serum protein parameters, reflecting the physiological changes occurring during the lactation period.

The higher values of total bilirubin, particularly in seropositive and clinically affected cows, evidenced also in beef cattle by Langenmayer et al. (2015) could be related to both prehepatic icterus due to hemorrhages, and anorexia, sickness, and debilitation.

No alterations were detected in Ca, P, and Mg mean concentrations that resulted within reference ranges and without any difference between seronegative and seropositive cows. However, it should be considered that blood concentrations of minerals are influenced by physiological factors, such as food intake and hydration, and can also change daily (Langenmayer et al., 2015).

Concerning metabolites and ketones, any difference was underlined between seronegative and seropositive and clinically affected cows. BOHB and lactate were both mildly higher, indicating a reduced energy balance in milk-producing dairy cows, whereas NEFA values resulted within the reference range in all the animals.

Regarding enzyme activities, any relevant alteration was not detected for AST, CK and LDH in *B. besnoiti* seropositive and clinically affected animals. Inconsistent results were also reported in previous studies. AST values were similar in all the considered groups of animals; CK recorded a higher activity in seronegative animals, but it should be considered that some animals had very high values distorting the mean results. According to Langenmayer et al. (2015) the effect of tissue cysts on muscle fibers could be too low leading to the only mild and not detectable release of muscle enzymes. LDH mean value was slightly higher in clinically affected animals if compared to seronegative ones; it could be associated with tissue damage. GLDH recorded a mild high level above the reference range in all the animals with a higher mean value in clinically affected animals and particularly in primiparous cows; these findings could be due to the metabolic condition of the cows.

Cortisol determination

Higher cortisol mean values were detected in seropositive and clinically affected cows, if compared to seronegative animals, even if with no statistical significance.

Mormède et al. (2007) reported that mean baseline cortisol values in cattle are typically lower than 10 ng/mL, and in recent studies often under 5 ng/mL. In the present study, the mean values of cortisol resulted quite high for all the herd animals: this finding could be related to poor farm management practices that were evidenced during the visits to the farm. Even if Mann-Whitney *U* Test did not evidence any significant difference between seronegative and seropositive cows and GLMs did not show any relation between *B. besnoiti* infection and cortisol levels, it is hypothesized that, among other physiological, pathological and farm-related factors, higher values of cortisol in *B. besnoiti* seropositive and clinically affected animals may be due also to the stress related to the disease; indeed, bovine besnoitiosis is a chronic and debilitating disease, compromising animal welfare. In the same farm, a decrease of certain productive parameters (i.e., daily kg of milk, % of fat and protein, and also mature equivalent milk yield) was previously evidenced in cows showing tissue cysts in the skin, that could be correlated to the debilitation caused by the chronic phase of the disease (Villa et al., 2019). The detection of *D. bovis* infestation in two cows, seropositive for *B. besnoiti,* contributes to supporting this hypothesis: it is known that these mites develop heavy infection mainly in dairy cows with stress and the occurrence of bovine demodicosis seems to be associated with debilitating factors or with receptive physiological states of the animal, i.e. pregnancy or lactation. Since bovine besnoitiosis is a chronic and debilitating disease, it should not be neglected its role in determining an immune imbalance, possibly leading to other infections, including *D. bovis* (Villa et al., 2020). Stress could be a consequence of *B. besnoiti* infection, but it was also experimentally demonstrated that immunodepression related to corticosteroids could facilitate the parasite proliferation (Alvarez-Garcia et al., 2014). In this regard, it is noteworthy to consider that in seropositive cows a significant shift with the decrease of lymphocytes was evidenced. To the best of the Authors' knowledge, this is the first study investigating the cortisol determination in *B. besnoiti* seropositive and clinically affected cattle. Even if only preliminary, the obtained results may suggest that *B. besnoiti* may cause a higher value of cortisol and then stress in infected animals: the consequences on animal welfare should not be neglected. Besides, its effect on both the disease onset and progression should be considered. However, also considering the lack of statistical significance, the association between *B. besnoiti* infection and cortisol levels could not be demonstrated, and this hypothesis should be further investigated.

Conclusions

The study provides data on alterations of laboratory parameters in *B. besnoiti* seropositive and clinically affected cows from an endemically infected dairy herd in Italy. Only mild changes were revealed in hematology, biochemistry, and enzyme activities. *B. besnoiti* infection seemed to be associated with a modification in the leukocyte differential characterized by higher percentage of the granulocytes and a lower value of the lymphocytes' population, particularly in primiparous cows. Besides, considering biochemistry and enzyme activity, the parasite infection resulted causative of alterations in serum proteins parameters in seropositive animals and GLDH values in clinically affected ones. Even if with no statistical significance, higher cortisol levels in seropositive and clinically affected animals may suggest that bovine besnoitiosis could be related to stress in infected cows: this may lead to consequences not only on animal welfare but also on the disease onset and progression. However, laboratory values are influenced by both physiological and pathological factors. Besides, it should be considered that farm-related factors could influence results on investigated animals. Further studies involving a higher number of animals from different farms, including seropositive cattle and clinically affected cows in both acute and chronic phase of the disease, could help to figure out the role of *B. besnoiti* in alterations of laboratory parameters, and to aid veterinarians in the diagnosis of bovine besnoitiosis to optimize a control plan for the disease in the infected herds.

Declarations

Ethics approval and consent to participate

Biological samples collection was performed by qualified veterinarians applying adequate procedures of handling and disinfection to minimize pain or distress in sampled animals. All procedures for the collection of biological specimens from live animals were accomplished following good clinical practices in the respect of animal welfare according to all applicable international, national, and institutional guidelines for the care and use of animals. The study was conducted with the approval of the Institutional Animal Care and Use Committee of Università degli Studi di Milano (Prot. n° OPBA_34_2017).

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Supplementary Tab. 1. Descriptive statistics (mean, standard deviation, minimum and maximum) of hematological, biochemical and enzyme activities analyses, and cortisol determination sorted by the number of parturitions and the serological and clinical status of cows in a dairy cattle herd endemically infected by bovine besnoitiosis. The number of parturitions was classified as follows: Primiparous = one parturition, Multiparous = two or more parturitions. Serological status (seronegative or seropositive) was determined according to Western Blot results while as clinically affected cows are meant those animals with the presence of clinical signs of the disease.

	Parameters	Number of Parturitions	Seronegative				Seropositive							
							Overall				Clinically affected			
			Mean	SD	Min	Max	Mean	SD	Min	Max	Mean	SD	Min	Max
Hematology	**RBC**	Primiparous	6.31	0.56	5.28	7.55	6.47	0.55	5.18	7.02	6.35	0.60	5.18	7.02
		Multiparous	5.76	0.63	4.67	7.23	5.97	0.68	4.80	7.14	5.81	0.59	4.80	7.02
		Total	6.04	0.65	4.67	7.55	6.14	0.68	4.80	7.14	6.06	0.64	4.80	7.02
	Hb	Primiparous	12.79	1.71	8.86	15.88	12.42	1.06	10.92	14.89	12.25	0.83	10.92	13.86
		Multiparous	12.13	1.38	9.63	14.71	12.00	2.05	3.81	14.63	11.81	2.62	3.81	14.45
		Total	12.47	1.58	8.86	15.88	12.15	1.76	3.81	14.89	12.01	1.98	3.81	14.45
	Ht	Primiparous	27.35	2.40	21.50	31.80	27.92	1.67	24.00	30.20	27.37	1.65	24.00	30.00
		Multiparous	26.01	2.57	20.70	31.00	26.87	3.03	22.20	32.40	26.02	2.58	22.20	30.80
		Total	26.70	2.56	20.70	31.80	27.24	2.66	22.20	32.40	26.65	2.27	22.20	30.80
	MCV	Primiparous	43.40	3.04	37.00	49.00	43.44	3.16	39.00	49.00	43.38	3.53	39.00	49.00
		Multiparous	45.39	3.51	40.00	56.00	45.21	3.03	38.00	50.00	45.00	3.21	39.00	50.00
		Total	44.36	3.40	37.00	56.00	44.58	3.16	38.00	50.00	44.25	3.40	39.00	50.00
	MCH	Primiparous	20.38	2.99	15.55	25.84	19.37	2.55	15.69	24.36	19.47	2.31	15.69	24.36
		Multiparous	21.20	2.62	16.12	27.48	20.19	3.45	7.62	24.96	20.28	4.17	7.62	24.96
		Total	20.78	2.83	15.55	27.48	19.89	3.15	7.62	24.96	19.90	3.40	7.62	24.96
	MCHC	Primiparous	46.88	5.91	36.77	62.35	44.64	4.75	37.38	54.35	44.87	3.39	39.45	52.51
		Multiparous	46.82	5.13	38.38	56.51	44.73	7.23	17.17	56.71	45.22	9.26	17.17	56.71
		Total	46.85	5.50	36.77	62.35	44.70	6.40	17.17	56.71	45.05	7.04	17.17	56.71
	RDW	Primiparous	15.38	0.94	14.00	17.30	15.46	0.69	13.90	16.50	15.42	0.79	13.90	16.50
		Multiparous	14.98	0.96	13.40	17.00	15.10	0.83	13.80	16.90	14.95	0.83	13.90	16.90
		Total	15.19	0.96	13.40	17.30	15.22	0.79	13.80	16.90	15.17	0.83	13.90	16.90
	WBC	Primiparous	10.38	8.02	2.70	48.10	10.19	3.71	6.80	17.70	10.57	4.00	6.80	17.70
		Multiparous	8.36	4.13	4.20	26.20	9.57	7.83	4.70	37.20	9.06	7.73	4.70	35.80
		Total	9.40	6.46	2.70	48.10	9.79	6.62	4.70	37.20	9.76	6.22	4.70	35.80
	Lymphocytes	Primiparous	54.47	14.36	25.00	97.00	37.81	16.51	12.00	65.00	43.23	23.61	12.00	97.00
		Multiparous	52.29	13.83	11.00	81.00	50.14	18.70	15.00	88.00	52.67	16.93	20.00	86.00
		Total	53.41	14.02	11.00	97.00	45.76	18.73	12.00	88.00	48.29	20.48	12.00	97.00
	Granulocytes	Primiparous	45.53	14.36	3.00	75.00	62.19	16.51	35.00	88.00	56.77	23.61	3.00	88.00

		Multiparous	47.71	13.83	19.00	89.00	49.86	18.70	12.00	85.00	47.33	16.93	14.00	80.00
		Total	46.59	14.02	3.00	89.00	54.24	18.73	12.00	88.00	51.71	20.48	3.00	88.00
	PLT	Primiparous	291.10	127.36	17.10	488.00	319.63	135.95	90.00	557.00	319.23	140.15	90.00	557.00
		Multiparous	326.86	97.84	102.00	490.00	274.10	81.11	115.00	449.00	272.00	88.98	115.00	449.00
		Total	308.36	114.50	17.10	490.00	290.29	104.76	90.00	557.00	293.93	115.80	90.00	557.00
	MPV	Primiparous	18.19	50.39	8.60	285.00	8.83	0.32	8.30	9.20	8.78	0.34	8.30	9.20
		Multiparous	8.75	0.33	8.20	9.50	8.80	0.30	8.10	9.50	8.79	0.26	8.50	9.40
		Total	13.63	36.26	8.20	285.00	8.81	0.31	8.10	9.50	8.79	0.30	8.30	9.40
	Pct	Primiparous	0.27	0.10	0.07	0.42	0.28	0.12	0.08	0.47	0.28	0.12	0.08	0.47
		Multiparous	0.29	0.08	0.08	0.43	0.24	0.07	0.10	0.38	0.23	0.08	0.10	0.38
		Total	0.28	0.09	0.07	0.43	0.25	0.09	0.08	0.47	0.25	0.10	0.08	0.47
	PDW	Primiparous	11.93	0.90	10.40	14.30	11.73	1.25	9.70	14.20	11.61	1.37	9.70	14.20
		Multiparous	15.00	19.23	9.70	113.00	11.59	0.95	9.80	13.10	11.59	1.00	10.30	13.10
		Total	13.41	13.34	9.70	113.00	11.64	1.05	9.70	14.20	11.60	1.17	9.70	14.20
Biochemistry	**Total protein**	Primiparous	8.69	1.04	6.30	11.50	8.26	0.88	5.60	9.30	8.15	0.92	5.60	9.10
		Multiparous	8.86	1.21	6.60	11.00	8.40	1.15	6.30	10.50	8.39	1.16	6.30	10.50
		Total	8.77	1.12	6.30	11.50	8.35	1.05	5.60	10.50	8.28	1.04	5.60	10.50
	Albumin	Primiparous	2.95	0.45	2.40	3.90	3.02	0.51	2.00	3.70	2.95	0.53	2.00	3.70
		Multiparous	2.88	0.52	2.00	3.90	3.15	0.63	2.10	4.70	2.87	0.52	2.10	3.90
		Total	2.91	0.48	2.00	3.90	3.10	0.59	2.00	4.70	2.91	0.52	2.00	3.90
	Globulin	Primiparous	5.75	1.19	3.10	8.90	5.24	0.72	3.60	6.30	5.20	0.73	3.60	6.30
		Multiparous	5.98	1.45	2.90	8.70	5.25	1.29	3.20	7.70	5.51	1.04	3.80	6.80
		Total	5.86	1.32	2.90	8.90	5.24	1.11	3.20	7.70	5.37	0.91	3.60	6.80
	A/G ratio	Primiparous	0.54	0.18	0.29	1.03	0.59	0.13	0.41	0.80	0.58	0.12	0.42	0.80
		Multiparous	0.53	0.23	0.26	1.28	0.65	0.24	0.31	1.19	0.54	0.15	0.37	0.82
		Total	0.54	0.21	0.26	1.28	0.63	0.20	0.31	1.19	0.56	0.14	0.37	0.82
	Total bilirubin	Primiparous	0.60	0.43	0.18	1.63	0.61	0.67	0.13	3.01	0.64	0.75	0.13	3.01
		Multiparous	0.51	0.29	0.09	1.34	0.63	0.95	0.13	5.26	0.76	1.26	0.23	5.26
		Total	0.56	0.37	0.09	1.63	0.62	0.85	0.13	5.26	0.70	1.04	0.13	5.26
	Ca	Primiparous	9.71	0.62	8.00	10.50	9.78	0.83	7.10	10.80	9.65	0.89	7.10	10.80
		Multiparous	9.30	0.78	7.00	10.40	9.38	0.62	7.40	10.40	9.19	0.66	7.40	10.00

		Total	9.51	0.73	7.00	10.50	9.53	0.72	7.10	10.80	9.40	0.79	7.10	10.80
	P	Primiparous	6.35	1.13	4.30	8.30	6.29	1.15	4.40	8.30	6.14	1.21	4.40	8.30
		Multiparous	6.28	1.09	3.90	7.90	6.08	1.26	4.00	10.40	6.08	1.46	4.00	10.40
		Total	6.32	1.10	3.90	8.30	6.16	1.21	4.00	10.40	6.11	1.33	4.00	10.40
	Mg	Primiparous	2.17	0.32	1.38	2.73	2.17	0.34	1.22	2.64	2.18	0.36	1.22	2.64
		Multiparous	2.12	0.38	1.42	3.10	2.13	0.36	1.46	2.93	2.14	0.36	1.46	2.65
		Total	2.15	0.35	1.38	3.10	2.14	0.35	1.22	2.93	2.16	0.35	1.22	2.65
	NEFA	Primiparous	0.34	0.24	0.09	1.05	0.33	0.19	0.12	0.71	0.32	0.20	0.12	0.71
		Multiparous	0.31	0.25	0.06	1.02	0.34	0.21	0.09	0.76	0.32	0.21	0.14	0.76
		Total	0.33	0.24	0.06	1.05	0.34	0.20	0.09	0.76	0.32	0.20	0.12	0.76
	BOHB	Primiparous	0.62	0.25	0.29	1.54	0.62	0.20	0.32	1.06	0.58	0.17	0.32	0.98
		Multiparous	0.66	0.23	0.32	1.20	0.60	0.31	0.05	1.48	0.70	0.39	0.05	1.48
		Total	0.64	0.24	0.29	1.54	0.61	0.28	0.05	1.48	0.65	0.31	0.05	1.48
	Lactate	Primiparous	2.77	1.01	1.47	5.23	2.87	1.05	1.10	5.39	2.96	1.09	1.10	5.39
		Multiparous	2.80	0.78	1.51	4.50	2.77	0.90	1.21	4.84	2.66	0.95	1.21	4.84
		Total	2.78	0.89	1.47	5.23	2.80	0.95	1.10	5.39	2.80	1.01	1.10	5.39
Enzymes	**AST**	Primiparous	81.07	40.76	43.00	252.00	73.63	25.77	49.00	159.00	74.46	28.89	49.00	159.00
		Multiparous	68.00	24.13	44.00	171.00	73.46	18.56	47.00	131.00	74.53	17.65	48.00	110.00
		Total	74.65	33.98	43.00	252.00	73.52	21.16	47.00	159.00	74.50	23.08	48.00	159.00
	CK	Primiparous	468.21	1828.68	28.00	9971.00	104.94	31.82	62.00	183.00	106.69	32.16	62.00	183.00
		Multiparous	153.07	214.93	57.00	1137.00	141.36	161.56	37.00	782.00	178.40	211.53	39.00	782.00
		Total	313.40	1311.32	28.00	9971.00	128.11	130.60	37.00	782.00	145.11	158.07	39.00	782.00
	LDH	Primiparous	912.90	223.61	434.00	1362.00	962.44	340.17	601.00	2113.00	975.46	367.74	601.00	2113.00
		Multiparous	826.89	305.39	453.00	2173.00	804.18	171.01	464.00	1094.00	849.07	179.40	535.00	1094.00
		Total	870.65	268.05	434.00	2173.00	861.73	254.28	464.00	2113.00	907.75	284.45	535.00	2113.00
	GLDH	Primiparous	34.75	34.02	10.00	163.90	42.91	41.83	0.00	137.70	47.35	45.10	0.00	137.70
		Multiparous	20.63	14.90	6.40	75.40	25.78	21.72	6.90	82.00	26.91	21.76	6.90	81.30
		Total	27.69	26.98	6.40	163.90	32.01	31.24	0.00	137.70	36.40	35.46	0.00	137.70
	Cortisol	Primiparous	6.12	4.63	0.77	19.10	8.65	10.09	1.25	40.41	8.73	11.14	1.25	40.41
		Multiparous	6.96	7.56	1.02	35.48	8.96	9.30	0.56	43.86	8.00	11.62	0.56	43.86
		Total	6.55	6.24	0.77	35.48	8.85	9.47	0.56	43.86	8.34	11.18	0.56	43.86

Supplementary Tab. 2. Descriptive statistics (mean, standard deviation, minimum and maximum) of hematological, biochemical and enzyme activities analyses, and cortisol determination sorted by the lactation phase and the serological and clinical status of cows in a dairy cattle herd endemically infected by bovine besnoitiosis. The lactation phase was classified as follows: Early = 0-120 d, Mid = 121-250 d, Late= 251-305 d, and Dry. Serological status (seronegative or seropositive) was determined according to Western Blot results while as clinically affected cows are meant those animals with the presence of clinical signs of the disease. Blank spaces (-) are due to the lack of clinically affected cows in the dry phase.

	Parameters	Lactation Phase	Seronegative				Seropositive							
							Overall				Clinically affected			
			Mean	SD	Min-	Max	Mean	SD	Min-	Max	Mean	SD	Min-	Max
Hematology	**RBC**	Early	5.68	0.72	4.67	7.30	5.62	0.68	4.80	7.01	5.56	0.68	4.80	6.96
		Mid	6.27	0.66	5.10	7.55	6.48	0.45	5.69	7.02	6.46	0.50	5.69	7.02
		Late	6.19	0.48	5.32	6.84	6.31	0.48	5.62	7.14	6.04	0.34	5.62	6.65
		Dry	6.45	0.98	5.51	8.53	6.14	1.09	4.93	7.04	-	-	-	-
		Total	6.11	0.73	4.67	8.53	6.15	0.67	4.80	7.14	6.07	0.66	4.80	7.02
	Hb	Early	11.94	1.63	8.86	14.34	11.27	2.50	3.81	13.83	10.75	2.89	3.81	13.83
		Mid	12.94	1.48	9.71	15.88	12.36	1.30	10.30	14.89	12.57	1.08	10.30	14.45
		Late	12.60	1.63	9.67	15.59	12.73	0.71	11.29	13.68	12.91	0.71	11.69	13.68
		Dry	12.14	1.25	10.41	14.89	13.19	1.67	11.36	14.63	-	-	-	-
		Total	12.46	1.55	8.86	15.88	12.18	1.75	3.81	14.89	12.04	2.01	3.81	14.45
	Ht	Early	25.55	2.76	20.70	31.00	25.84	2.73	22.20	31.20	25.78	2.70	22.20	29.10
		Mid	27.34	2.56	21.50	31.10	27.36	1.98	23.60	30.80	27.16	2.13	23.60	30.80
		Late	26.63	1.97	23.60	30.40	28.54	2.48	24.70	32.20	27.12	1.90	24.70	29.70
		Dry	28.01	2.72	23.90	31.80	29.67	4.65	24.30	32.40	-	-	-	-
		Total	26.77	2.60	20.70	31.80	27.35	2.73	22.20	32.40	26.69	2.30	22.20	30.80
	MCV	Early	45.12	2.85	40.00	49.00	46.07	2.81	39.00	50.00	46.44	3.28	39.00	50.00
		Mid	43.80	3.83	38.00	56.00	42.35	2.37	38.00	46.00	42.25	2.38	39.00	46.00
		Late	43.27	3.24	37.00	49.00	45.50	3.00	41.00	49.00	45.17	3.60	41.00	49.00
		Dry	44.11	6.70	32.00	50.00	48.33	2.08	46.00	50.00	-	-	-	-
		Total	44.08	3.97	32.00	56.00	44.70	3.22	38.00	50.00	44.30	3.45	39.00	50.00
	MCH	Early	21.21	3.13	15.55	27.48	20.11	4.36	7.62	24.96	19.35	5.12	7.62	24.96
		Mid	20.78	2.67	16.05	25.96	19.20	2.74	15.32	24.36	19.61	2.61	16.34	24.36
		Late	20.47	3.06	16.12	25.50	20.29	2.11	16.31	22.87	21.41	1.41	18.85	22.87
		Dry	19.08	2.69	14.74	22.98	21.61	1.25	20.79	23.05	-	-	-	-
		Total	20.57	2.91	14.74	27.48	19.92	3.12	7.62	24.96	19.92	3.46	7.62	24.96
	MCHC	Early	46.93	6.39	36.92	62.35	43.47	8.54	17.17	53.21	41.35	9.79	17.17	49.73
		Mid	47.44	4.63	38.64	56.33	45.39	5.83	37.50	56.71	46.53	5.33	39.40	56.71
		Late	47.42	5.87	38.38	59.96	44.93	5.01	37.38	53.29	47.76	3.64	44.91	53.29

	Dry	43.66	5.59	35.95	53.69	44.64	2.42	42.01	46.76	-	-	-	-
	Total	46.74	5.62	35.95	62.35	44.64	6.34	17.17	56.71	45.08	7.17	17.17	56.71
RDW	Early	14.94	0.97	13.40	17.30	14.54	0.67	13.80	15.90	14.39	0.56	13.90	15.70
	Mid	15.47	0.94	13.70	17.20	15.70	0.57	14.70	16.90	15.86	0.56	15.00	16.90
	Late	15.49	0.84	14.00	16.70	15.29	0.68	14.60	16.60	15.08	0.44	14.60	15.60
	Dry	14.82	1.23	13.60	17.50	15.37	0.72	14.90	16.20	-	-	-	-
	Total	15.23	0.99	13.40	17.50	15.22	0.79	13.80	16.90	15.20	0.84	13.90	16.90
WBC	Early	11.61	10.61	4.20	48.10	7.79	3.00	5.00	17.70	8.16	3.68	5.00	17.70
	Mid	9.18	3.98	4.50	23.50	12.69	9.63	5.70	37.20	11.52	8.52	5.70	35.80
	Late	7.49	2.59	2.70	12.90	8.36	2.83	4.70	14.60	8.38	3.57	4.70	14.60
	Dry	8.58	2.42	5.00	12.20	6.83	2.19	5.10	9.30	-	-	-	-
	Total	9.35	6.31	2.70	48.10	9.69	6.58	4.70	37.20	9.70	6.33	4.70	35.80
Lymphocytes	Early	57.00	18.66	22.00	97.00	46.36	16.72	27.00	88.00	45.56	13.76	27.00	64.00
	Mid	52.80	10.49	25.00	68.00	50.12	20.93	12.00	86.00	50.00	21.20	12.00	86.00
	Late	54.20	9.31	41.00	71.00	40.92	17.06	12.00	64.00	40.83	20.28	12.00	64.00
	Dry	47.44	15.05	11.00	59.00	38.00	20.07	15.00	52.00	-	-	-	-
	Total	53.52	13.69	11.00	97.00	45.78	18.53	12.00	88.00	46.48	18.46	12.00	86.00
Granulocytes	Early	43.00	18.66	3.00	78.00	53.64	16.72	12.00	73.00	54.44	13.76	36.00	73.00
	Mid	47.20	10.49	32.00	75.00	49.88	20.93	14.00	88.00	50.00	21.20	14.00	88.00
	Late	45.80	9.31	29.00	59.00	59.08	17.06	36.00	88.00	59.17	20.28	36.00	88.00
	Dry	52.56	15.05	41.00	89.00	62.00	20.07	48.00	85.00	-	-	-	-
	Total	46.48	13.69	3.00	89.00	54.22	18.53	12.00	88.00	53.52	18.46	14.00	88.00
PLT	Early	331.71	104.47	112.00	490.00	293.29	82.92	90.00	449.00	289.78	101.58	90.00	449.00
	Mid	304.41	139.51	17.10	464.00	293.47	133.92	137.00	557.00	288.17	135.50	137.00	557.00
	Late	302.80	110.95	75.00	463.00	278.25	95.10	115.00	453.00	279.33	87.26	115.00	355.00
	Dry	278.33	85.95	129.00	426.00	247.00	124.74	112.00	358.00	-	-	-	-
	Total	307.77	115.04	17.10	490.00	286.41	106.87	90.00	557.00	286.74	111.46	90.00	557.00
MPV	Early	8.81	0.38	8.30	9.50	8.66	0.31	8.10	9.20	8.69	0.33	8.30	9.20
	Mid	22.70	61.74	8.20	285.00	8.88	0.28	8.40	9.40	8.90	0.30	8.40	9.40
	Late	8.90	0.27	8.40	9.40	8.88	0.32	8.50	9.50	8.75	0.22	8.50	9.10
	Dry	9.10	0.38	8.60	9.80	9.03	0.35	8.70	9.40	-	-	-	-

		Total	13.43	35.35	8.20	285.00	8.82	0.32	8.10	9.50	8.80	0.30	8.30	9.40
	Pct	Early	0.29	0.09	0.11	0.43	0.25	0.07	0.08	0.38	0.25	0.08	0.08	0.38
		Mid	0.28	0.11	0.08	0.40	0.25	0.12	0.12	0.47	0.25	0.12	0.12	0.47
		Late	0.27	0.10	0.07	0.40	0.25	0.08	0.10	0.40	0.24	0.07	0.10	0.31
		Dry	0.25	0.09	0.12	0.42	0.22	0.11	0.11	0.32	-	-	-	-
		Total	0.28	0.09	0.07	0.43	0.25	0.09	0.08	0.47	0.25	0.10	0.08	0.47
	PDW	Early	11.39	0.94	9.90	12.90	11.25	1.20	9.80	14.20	11.30	1.41	9.80	14.20
		Mid	16.89	22.64	10.40	113.00	12.07	1.00	9.70	13.10	12.04	1.00	9.70	13.10
		Late	11.80	1.14	9.70	14.30	11.55	0.85	10.30	13.00	11.32	1.01	10.30	13.00
		Dry	12.07	0.86	10.70	13.50	11.73	0.93	11.10	12.80	-	-	-	-
		Total	13.39	13.00	9.70	113.00	11.66	1.05	9.70	14.20	11.63	1.17	9.70	14.20
	Total Protein	Early	8.64	0.95	6.70	10.20	8.11	0.98	6.30	9.90	8.06	0.98	6.30	9.30
		Mid	8.64	1.09	6.40	11.00	8.08	0.97	5.60	9.30	8.06	1.03	5.60	9.10
		Late	9.29	1.13	7.40	11.50	8.92	0.87	7.60	10.50	9.07	1.01	7.60	10.50
		Dry	8.17	1.16	6.30	9.60	8.60	1.77	7.00	10.50	-	-	-	-
		Total	8.73	1.11	6.30	11.50	8.34	1.04	5.60	10.50	8.28	1.06	5.60	10.50
	Albumin	Early	2.89	0.47	2.20	3.90	3.11	0.54	2.10	3.70	3.00	0.57	2.10	3.70
		Mid	2.81	0.33	2.30	3.70	2.95	0.51	2.00	4.00	2.83	0.45	2.00	3.60
		Late	2.75	0.48	2.00	3.80	3.27	0.74	2.40	4.70	3.02	0.62	2.50	3.90
Biochemistry		Dry	3.73	0.26	3.20	4.20	3.53	0.55	2.90	3.90	-	-	-	-
		Total	2.96	0.52	2.00	4.20	3.12	0.60	2.00	4.70	2.93	0.52	2.00	3.90
	Globulin	Early	5.75	1.15	3.80	8.00	5.00	1.02	4.00	7.20	5.06	0.81	4.20	6.80
		Mid	5.83	1.26	3.60	8.70	5.12	0.90	3.60	6.30	5.23	0.87	3.60	6.30
		Late	6.54	1.27	4.60	8.90	5.65	1.15	4.40	7.70	6.05	0.95	4.60	6.80
		Dry	4.43	1.05	2.90	5.70	5.07	2.27	3.20	7.60	-	-	-	-
		Total	5.78	1.34	2.90	8.90	5.22	1.10	3.20	7.70	5.36	0.92	3.60	6.80
	A/G ratio	Early	0.53	0.18	0.28	1.00	0.65	0.18	0.37	0.88	0.60	0.14	0.37	0.82
		Mid	0.51	0.16	0.26	0.95	0.60	0.16	0.41	0.95	0.55	0.12	0.41	0.78
		Late	0.44	0.16	0.29	0.80	0.62	0.23	0.31	1.04	0.51	0.16	0.39	0.80
		Dry	0.89	0.23	0.68	1.28	0.82	0.41	0.38	1.19	-	-	-	-
		Total	0.56	0.22	0.26	1.28	0.63	0.21	0.31	1.19	0.56	0.13	0.37	0.82

Total Bilirubin	Early	0.55	0.39	0.09	1.63	0.64	0.75	0.22	3.01	0.72	0.89	0.23	3.01
	Mid	0.62	0.40	0.22	1.57	0.43	0.21	0.13	0.98	0.47	0.23	0.13	0.98
	Late	0.59	0.35	0.23	1.49	0.93	1.41	0.25	5.26	1.23	1.98	0.35	5.26
	Dry	0.41	0.24	0.19	0.96	0.39	0.19	0.21	0.59	-	-	-	-
	Total	0.56	0.36	0.09	1.63	0.62	0.84	0.13	5.26	0.72	1.05	0.13	5.26
Ca	Early	9.47	0.62	8.00	10.40	9.37	0.78	7.40	10.40	9.21	0.86	7.40	10.00
	Mid	9.51	0.84	7.00	10.50	9.45	0.83	7.10	10.80	9.42	0.94	7.10	10.80
	Late	9.45	0.72	8.00	10.20	9.78	0.47	9.00	10.40	9.63	0.38	9.10	9.90
	Dry	9.99	0.70	8.50	11.10	9.77	0.40	9.30	10.00	-	-	-	-
	Total	9.56	0.74	7.00	11.10	9.54	0.72	7.10	10.80	9.40	0.81	7.10	10.80
P	Early	6.10	0.91	5.10	8.30	5.74	1.10	4.00	8.30	5.68	1.24	4.00	8.30
	Mid	6.12	1.31	3.90	8.10	5.99	0.97	4.40	7.50	5.98	1.07	4.40	7.50
	Late	6.77	0.79	5.20	7.90	7.00	1.36	5.50	10.40	7.10	1.71	5.80	10.40
	Dry	7.16	1.69	4.40	9.70	5.90	1.23	5.00	7.30	-	-	-	-
	Total	6.43	1.21	3.90	9.70	6.18	1.21	4.00	10.40	6.13	1.35	4.00	10.40
Mg	Early	2.14	0.31	1.42	2.65	2.19	0.35	1.54	2.64	2.20	0.37	1.54	2.64
	Mid	2.15	0.38	1.41	2.83	2.04	0.35	1.22	2.64	2.09	0.38	1.22	2.64
	Late	2.11	0.33	1.38	2.72	2.22	0.36	1.64	2.93	2.29	0.29	1.91	2.65
	Dry	2.14	0.44	1.78	3.10	2.31	0.25	2.02	2.48	-	-	-	-
	Total	2.14	0.35	1.38	3.10	2.15	0.35	1.22	2.93	2.17	0.36	1.22	2.65
NEFA	Early	0.51	0.33	0.06	1.05	0.48	0.21	0.13	0.76	0.45	0.22	0.13	0.76
	Mid	0.28	0.14	0.09	0.60	0.25	0.16	0.12	0.71	0.25	0.17	0.12	0.71
	Late	0.23	0.19	0.10	0.84	0.32	0.19	0.16	0.71	0.30	0.21	0.16	0.71
	Dry	0.24	0.07	0.12	0.35	0.26	0.14	0.09	0.34	-	-	-	-
	Total	0.32	0.24	0.06	1.05	0.34	0.20	0.09	0.76	0.33	0.21	0.12	0.76
BOHB	Early	0.71	0.31	0.33	1.54	0.67	0.36	0.32	1.48	0.78	0.38	0.32	1.48
	Mid	0.67	0.23	0.29	1.20	0.62	0.23	0.34	1.06	0.61	0.22	0.34	1.03
	Late	0.58	0.17	0.35	0.97	0.54	0.27	0.05	1.12	0.54	0.35	0.05	1.12
	Dry	0.49	0.09	0.35	0.63	0.50	0.12	0.36	0.58	-	-	-	-
	Total	0.64	0.24	0.29	1.54	0.60	0.28	0.05	1.48	0.65	0.31	0.05	1.48
Lactate	Early	2.60	0.68	1.69	3.87	2.63	0.90	1.10	3.62	2.58	0.97	1.10	3.62

	Mid	2.84	1.08	1.47	5.23	2.77	0.82	1.34	4.84	2.74	0.92	1.34	4.84
	Late	2.62	0.66	1.64	3.93	3.02	1.21	1.23	5.39	3.17	1.35	1.60	5.39
	Dry	3.50	1.09	2.24	5.22	3.07	0.75	2.25	3.73	-	-	-	-
	Total	2.82	0.92	1.47	5.23	2.81	0.94	1.10	5.39	2.78	1.02	1.10	5.39
AST	Early	72.75	29.04	46.00	171.00	78.46	24.09	48.00	131.00	75.33	21.26	48.00	110.00
	Mid	76.15	26.49	43.00	144.00	73.71	24.16	49.00	159.00	76.00	28.60	49.00	159.00
	Late	66.53	19.37	45.00	104.00	70.83	12.72	53.00	95.00	73.67	16.17	53.00	95.00
	Dry	86.56	62.58	56.00	252.00	70.00	25.87	47.00	98.00	-	-	-	-
	Total	74.40	33.17	43.00	252.00	74.07	21.24	47.00	159.00	75.26	23.16	48.00	159.00
CK	Early	123.56	67.70	57.00	285.00	184.77	228.12	37.00	782.00	229.33	264.54	39.00	782.00
	Mid	165.35	235.34	54.00	1137.00	102.29	32.50	57.00	183.00	108.83	35.11	65.00	183.00
	Late	114.53	72.62	28.00	318.00	113.33	51.27	61.00	204.00	93.50	32.95	61.00	139.00
	Dry	1245.89	3274.62	62.00	9971.00	772.67	1220.57	57.00	2182.00	-	-	-	-
	Total	303.58	1278.29	28.00	9971.00	173.76	332.28	37.00	2182.00	145.59	161.06	39.00	782.00
LDH	Early	931.06	372.51	453.00	2173.00	889.85	154.22	603.00	1094.00	886.67	178.60	603.00	1094.00
	Mid	881.20	217.56	504.00	1362.00	914.00	356.91	464.00	2113.00	949.92	393.06	601.00	2113.00
	Late	800.40	210.15	434.00	1185.00	791.58	141.84	535.00	1090.00	840.17	183.99	535.00	1090.00
	Dry	855.33	190.29	657.00	1305.00	795.00	275.09	524.00	1074.00	-	-	-	-
	Total	870.42	261.21	434.00	2173.00	866.44	253.36	464.00	2113.00	904.44	289.32	535.00	2113.00
GLDH	Early	22.47	18.50	6.40	75.40	35.59	38.07	0.00	121.00	31.59	41.32	0.00	121.00
	Mid	31.86	35.21	7.20	163.90	40.10	34.42	9.40	137.70	47.16	38.39	13.10	137.70
	Late	32.55	26.76	12.80	105.60	20.10	12.78	8.00	55.30	23.38	16.74	10.10	55.30
	Dry	57.72	116.37	9.00	367.00	10.43	3.02	8.40	13.90	-	-	-	-
	Total	33.42	51.46	6.40	367.00	31.49	31.09	0.00	137.70	36.69	36.10	0.00	137.70
Cortisol	Early	7.17	9.39	1.02	35.48	10.94	14.76	0.56	43.86	11.50	17.56	0.56	43.86
	Mid	4.26	2.72	0.77	10.08	6.81	6.02	1.25	19.36	6.34	5.66	1.25	19.36
	Late	7.10	5.84	1.89	20.14	9.66	5.61	2.90	19.12	8.07	6.61	2.90	19.12
	Dry	10.75	5.37	5.55	18.39	11.45	9.47	1.28	20.01	-	-	-	-
	Total	6.81	6.41	0.77	35.48	9.11	9.51	0.56	43.86	8.54	11.36	0.56	43.86

Enzymes

Supplementary Table 3. Results of the significative variables to the GLM analysis regarding the variation on laboratory parameters according to number of parturitions and phase of lactation. Descriptive statistics (mean, standard deviation, minimum and maximum) of the considered parameters were also included.

Response Variable	Predictor	Category	Mean (SD)	Min-Max	β[a]	SE[b]	Wald Chi-Square	OR (95% CI)	P-value	AIC
RBC	Number of parturitions						11.038		0.001	183.072
		Primiparous	6.36 (0.56)	5.18-7.55	0.378	0.1137		1.459 (1.168-1.823)	0.001	
		Multiparous	5.86 (0.66)	4.67-7.23	0			1		
	Phase of lactation						21.433		0.0001	
		Early	5.65 (0.67) [a]	4.67-7.30	-0.292	0.2204		0.746 (0.485-1.150)	0.185	
		Mid	6.36 (0.57) [b]	5.10-7.55	0.307	0.2179		1.359 (0.887-2.083)	0.159	
		Late	6.24 (0.47) [b]	5.32-7.14	0.245	0.2236		1.277 (0.824-1.980)	0.274	
		Dry	6.37 (0.97) [ab]	4.93-8.53	0			1		
Hb	Phase of lactation						8.794		0.032	397.236
		Early	11.64 (2.05) [a]	3.81-14.34	-0.685	0.6287		0.504 (0.147-1.729)	0.276	
		Mid	12.67 (1.41) [b]	9.71-15.88	0.350	0.6182		1.419 (0.423-4.767)	0.571	
		Late	12.66 (1.28) [b]	9.67-15.59	0.336	0.6382		1.399 (0.401-4.888)	0.599	
		Dry	12.40 (1.36) [ab]	10.40-14.89	0			1		
Ht							12.343		0.006	486.657

	Phase of lactation	Early	25.68 (2.70)	20.70-31.20	-2.419	0.9704		0.089 (0.013-0.596)	0.013	
		Mid	27.35 (2.28) [a]	21.50-31.10	-0.751	0.9542		0.472 (0.073-3.061)	0.431	
		Late	27.48 (2.37) [a]	23.60-32.20	-0.622	0.9851		0.537 (0.078-3.701)	0.528	
		Dry	28.42 (3.14) [a]	23.90-32.40	0			1		
MCV	Number of parturitions	Primiparous	43.41 (3.04)	37-49	-1.427	0.6035	5.587	0.240 (0.074-0.784)	0.018	526.958
		Multiparous	45.30 (3.25)	38-56	0			1		
	Phase of lactation	Early	45.55 (2.83) [ac]	39-50	-1.572	1.1699	12.943	0.208 (0.021-2.055)	0.005 0.179	
		Mid	43.14 (3.28) [b]	38-56	-3.552	1.1567		0.29 (0.003-0.277)	0.002	
		Late	44.26 (3.28) [ab]	37-49	-2.642	1.1871		0.071 (0.007-0.730)	0.026	
		Dry	45.17 (6.09) [c]	32-50	0			1		
RDW	Phase of lactation	Early	14.76 (0.86) [b]	13.40-17.30	-0.200	0.2826	18.852	0.819 (0.470-1.424)	0.000 0.479	274.083
		Mid	15.57 (0.79) [a]	13.70-17.20	0.617	0.2761		1.854 (1.079-3.185)	0.025	
		Late	15.40 (0.76) [ac]	14.00-16.70	0.445	0.2884		1.561 (0.887-2.747)	0.122	
		Dry	14.96 (1.12) [bc]	13.60-17.50	0			1		
Ca							7.995		0.005	217.610

	Number of parturitions	Primiparous	9.73 (0.69)	7.1-10.8	0.390	0.1381		1.478 (1.127-1.937)	0.005	
		Multiparous	9.34 (0.70)	7.0-10.4	0			1		
P	Phase of lactation						12.834		0.005	311.434
		Early	5.94 (0.99) [a]	4.0-8.3	-0.162	0.4297		0.850 (0.366-1.974)	0.706	
		Mid	6.06 (1.15) [a]	3.9-8.1	-0.041	0.4196		0.960 (0.422-2.185)	0.923	
		Late	6.87 (1.06) [b]	5.2-10.4	0.774	0.4332		2.169 (0.928-5.069)	0.074	
		Dry	6.84 (1.64) [ab]	4.4-9.7	0			1		
NEFA	Phase of lactation						27.916		0.000	30.288
		Early	0.49 (0.28)	0.06-1.05	0.268	0.0790		1.307 (1.120-1.526)	0.001	
		Mid	0.27 (0.15) [a]	0.09-0.71	0.041	0.0771		1.042 (0.896-1.212)	0.596	
		Late	0.27 (0.19) [a]	0.10-0.84	0.048	0.0800		1.049 (0.897-1.227)	0.548	
		Dry	0.24 (0.09) [a]	0.09-0.35	0			1		
AST	Number of parturitions						8.204		0.004	38.728
		Primiparous	78.42 (36.01)	43-252	0.679	0.1958		1.971 (1.343-2.893)	0.001	
		Multiparous	70.73 (21.50)	44-171	0			1		
	Phase of lactation						5.083		0.166	
		Early	75.31 (26.62) [ab]	46-171	0.169	0.1334		1.184 (0.911-1.538)	0.206	
		Mid	75.03 (25.12) [ab]	43-159	0.110	0.1385		1.116 (0.851-1.464)	0.427	

		Late	68.44 (16.59) [a]	45-104	0.062	0.1385		1.064 (0.811-1.396)	0.654	
		Dry	82.42 (55.01) [b]	47-252	0			1		
							10.750		0.013	
		Primiparous Early	71.75 (15.15)	51-94	-0.728	0.2253		0.483 (0.311-0.751)	0.001	
		Primiparous Mid	77.91 (30.21)	43-159	-0.608	0.2154		0.545 (0.357-0.831)	0.005	
		Primiparous Late	71.50 (20.68)	45-104	-0.624	0.2216		0.536 (0.347-0.828)	0.005	
	Number of parturitions X Phase of lactation	Primiparous Dry	127.67 (108.09)	56-252	0			1		
		Multiparous Early	76.67 (30.08)	46-171	0			1		
		Multiparous Mid	70.80 (14.89)	44-108	0			1		
		Multiparous Late	66.00 (12.68)	48-95	0			1		
		Multiparous Dry	59.80 (8.81)	47-70	0			1		
							16.221		0.000	
	Number of parturitions	Primiparous	339.04 (1469.46)	28-9971	32.329	5.8866		1.098E+14 (1070923246-1.125E+19)	0.000	
CK		Multiparous	147.21 (188.48)	37-1137	0			1		740.220
							28.171		0.000	
	Phase of lactation	Early	151.00 (160.37) [a]	37-782	-0.102	3.9935		0.903 (0.000-2265.046)	0.980	

		Mid	136.38 (175.26) [a]	54-1137	0.118	4.1625		1.125 (0.000-3929.539)	0.977	
		Late	114.00 (62.87) [a]	28-318	-0.644	4.1625		0.525 (0.000-1834.041)	0.877	
		Dry	1127.58 (2848.74)	57-9971	0			1		
							28.298		0.000	
		Primiparous Early	128.75 (64.88)	62-256	-32.707	6.6951		6.244E-15 (1.249E-20-3.121E-9)	0.000	
		Primiparous Mid	110.59 (38.69)	54-230	-32.965	6.4759		4.823E-15 (1.483E-20-1.569E-9)	0.000	
	Number of parturitions X Phase of lactation	Primiparous Late	134.00 (73.14)	28-318	-31.969	6.6632		1.306E-14 (2.781E-20-6.132E-9)	0.000	
		Primiparous Dry	3395.33 (5694.78)	77-9971	0			1		
		Multiparous Early	159.48 (185.09)	37-782	0			1		
		Multiparous Mid	174.20 (272.32)	57-1137	0			1		
		Multiparous Late	98.00 (50.20)	57-204	0			1		
		Multiparous Dry	162.40 (189.53)	57-499	0			1		
							5.144		0.023	
LDH	Number of parturitions	Primiparous	930.51 (268.04)	434-2113	1.150	0.5069		3.157 (1.169-8.528)	0.023	480.299
		Multiparous	815.54 (245.50)	453-2173	0			1		

First report of *Demodex bovis* infestation in bovine besnoitiosis co-infected dairy cattle in Italy

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Abstract

A form of generalized demodectic mange in two dairy cows infected with *Besnoitia besnoiti* is described. The herd was endemically infected with bovine besnoitiosis; an overall seroprevalence of *B. besnoiti* antibodies of 23.5%, that increased up to 43.5% considering only cows, was reported. Two out of the cows seropositive to *B. besnoiti*, at clinical examination presented skin nodules, widespread all over the body, and in particular in anterior regions. Skin biopsies from the region of the neck were collected and the nodules were microscopically examined through compression method. *B. besnoiti* tissue cysts were not revealed but a semi-solid yellowish content was evidenced with the presence of several mites, morphologically identified as *Demodex bovis*. Histological examination of skin biopsies evidenced slight acanthosis and hyperkeratosis of the epidermis and superficial dermatitis with oedema and macrophagic and eosinophilic infiltration. Cystic formations located in the deep dermis were lined by metaplastic squamous epithelium and severe cellular infiltration. A treatment with eprinomectin was attempted and clinical improvement of both cows was observed, particularly at the fifteenth day after treatment, with nodules reduced in size and mites in there degenerated. This is the first report of the co-infection of *D. bovis* infestation and bovine besnoitiosis in cattle. Furthermore, it was demonstrated that *D. bovis* circulates in the Italian cattle population, but subclinical forms could be underdiagnosed.

Keywords: Demodex bovis; *Besnoitia besnoiti*; Co-infection; Dairy cows; Histology; Eprinomectin.

Introduction

Demodex bovis (Stiles, 1892) is a mite that lives in hair follicles and sebaceous glands. It causes bovine demodicosis or demodectic mange. The disease is characterized by follicular papules and nodules, especially in anterior body regions (Matthes et al., 1994). It can occur both in subclinical and in a generalized form. Transmission occurs through close contact between animals, and mainly from infested dams to offspring. Bovine demodicosis is a quite common disease in tropical areas, but it is rare in temperate regions (Mullen and Durden, 2019). Only scarce, not updated and scattered data are available concerning *D. bovis* in cattle in Italy. Mange is included among notifiable diseases according to Italian veterinary rules (D.P.R. 8 Febbraio 1954, n. 320, Titolo II, Capo XXVII – Rogna, Art. 146–149). Among others, nutritional deficiencies, physiological stress due to pregnancy and lactation, presence of other infections or infestations, are factors associated with episodes of bovine demodicosis. Concerning individual factors, the disease seems to be more prevalent in female young animals (Faccini et al., 2004).

Bovine besnoitiosis, caused by the cyst-forming apicomplexan protozoa *Besnoitia besnoiti*, is a chronic and debilitating parasitic disease of cattle, characterized by both cutaneous and systemic manifestations, compromising animal welfare and responsible for economic losses on affected farms. In Europe, including Italy, bovine besnoitiosis is an emerging or re-emerging disease, with an increasing geographical distribution and the number of cases of infection (EFSA, 2010). Bovine besnoitiosis was previously reported both in dairy cows and in beef cattle in Northern Italy (Gentile et al., 2012; Gazzonis et al., 2014; 2017). Furthermore, *Besnoitia* spp. specific antibodies were recently detected for the first time in Italy also in horses and donkeys reared in Northern regions (Villa et al., 2018). However, the identity of *Besnoitia* species circulating in Italian equids is unknown to date.

A case of generalized bovine demodicosis in two *B. besnoiti* seropositive cows from a dairy farm in Northern Italy is reported.

Materials and methods

The study was conducted in a dairy farm hosting 217 Holstein Friesian cattle located in the province of Brescia (Lombardy, Northern Italy), where clinical cases of bovine besnoitiosis were previously reported (Villa et al., 2019). At that time, a serological screening for *B. besnoiti* was performed on the whole herd using the recommended approach of ELISA (ID Screen® Besnoitia Indirect 2.0, IDVET, Montpellier, France) and confirmatory Western Blot. Western Blot was

performed and interpreted according to Fernandez-Garcia et al. (2009). Briefly, a total of 4×10^7 *B. besnoiti* tachyzoites under non reducing condition were employed for electrophoresis. Tachyzoite antigens were transferred to nitrocellulose membranes and incubated with sera from cattle at a 1:20 dilution, followed by a peroxidase-conjugated anti-Bovine IgG antibody diluted at 1:1200 (Sigma-Aldrich, Saint Louis, USA). The presence of at least three bands in at least two of the three principal antigenic areas (area I: 72.5, 58.9 and 51.4 kDa; area II: 38.7, 31.8 and 28.5 kDa; area III: 23.6, 19.1, 17.4, 14.5 kDa) was considered as a positive result. A seroprevalence of *B. besnoiti* of 23.5% was recorded, which increased up to 43.5% considering only adult cows (Villa et al., 2019). The farm is officially free of tuberculosis, brucellosis and leucosis. All the animals of the herd are vaccinated against BVDV; besides, the farm adheres to the plan for the control of IBR and paratuberculosis. Further, some cases of Q Fever were diagnosed in the herd one year before and after those episodes all the animals were vaccinated against *Coxiella burnetii*.

During the clinical examination of the cows, which resulted seropositive to *B. besnoiti*, skin biopsies from the region of the neck were collected from three out of seven cows presenting skin lesions suggestive of bovine besnoitiosis. Skin biopsies were collected using 4- to 6-mm disposable punch instruments. An aliquot of the nodules was microscopically examined through compression between glasses to detect the presence of *B. besnoiti* tissue cysts. The material included in the nodules was dissolved in 5% potassium hydroxide (KOH) solution at room temperature for 2 h to clear the mites. Subsequently, the material was filtered and washed from the colloidal remains and observed under a light microscope (MAFF, 1986). Mites were identified according to morphological characteristics as described by Bukva (1986). Another part was processed for histological examination: samples were fixed in 10% buffered formalin, embedded in paraffin wax, sectioned at 5 μm, stained with hematoxylin and eosin (HE) and microscopically examined.

Results

Two three years old lactating Holstein Friesian cows resulted seropositive for *B. besnoiti*: ELISA S/P% was 132 and 162, respectively, and Western Blot positive for both cows (Villa et al., 2019). These animals were clinically examined to reveal signs and lesions ascribable to bovine besnoitiosis. Any clinical alteration of the acute phase of the disease was not detected. Regarding the presence of *B. besnoiti* tissue cysts in typical localizations, i.e. skin, sclera and vulva, one of these cows presented tissue cysts in *scleral conjunctiva* and *vestibulum vaginae*. Besides, both cows evidenced the presence of skin nodules of varying sizes (0.5–2 cm), widespread all over the

body, in particular in the regions of head, neck, back and flanks (Fig. 1.A). However, these skin lesions did not show the typical aspect of those characteristic of chronic bovine besnoitiosis. Indeed, the compression between glasses of the nodules did not reveal the presence of *B. besnoiti* tissue cysts, whereas a semi-solid yellowish content was found. The microscopic observation of this material after washing with 5% KOH evidenced the presence of mites, morphologically identified as *D. bovis* (Bukva, 1986). A huge quantity of mites at various stages of development was observed (Fig. 1.B). Histology showed slight acanthosis and hyperkeratosis of the epidermis and superficial dermatitis with oedema and macrophagic and eosinophilic infiltration. Cystic formations located in the deep dermis were lined by a layer of metaplastic squamous epithelium and severe infiltration of macrophages, plasma cells, eosinophils and lymphocytes (Fig. 2.A). The material included consisted of degenerated and necrotic granulocytes with parts of hair, keratin flakes and sectioned mites (Fig. 2.B). Any *B. besnoiti* tissue cyst was not detected at histological examination.

In these animals a treatment with eprinomectin (EPRINEX Pour-On 0.5%) was attempted (Döpfer et al., 2013). This treatment was chosen since there is no milk withdrawal period for lactating dairy cattle and then milk from cows may be used for human consumption at any time following treatment. Two weeks later, these cows evidenced a clinical improvement: nodules were reduced in size and mites in there degenerated (Fig. 1.C). Another dose of treatment was administered one month after the first one, resulting in a further improvement of the clinical signs.

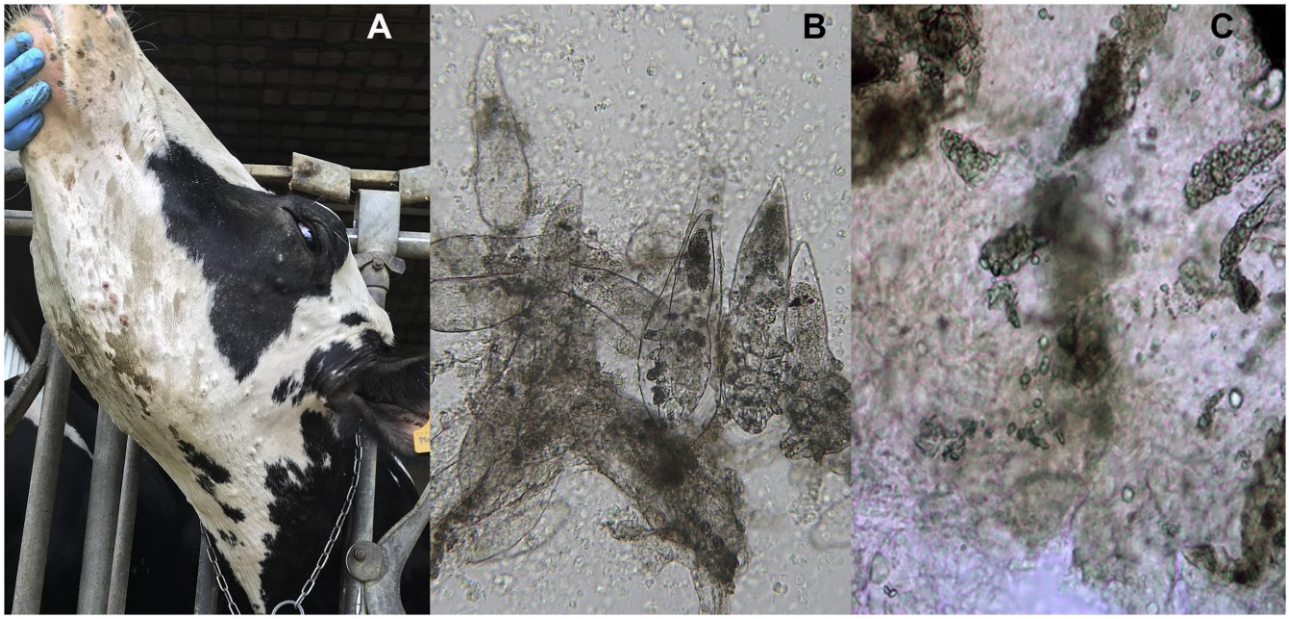

Fig. 1. A. Head and neck of the infested cow with evidence of several skin nodules. B. Specimens of *Demodex bovis* at various stages of development before treatment. C. Degenerate mites after treatment with eprinomectin.

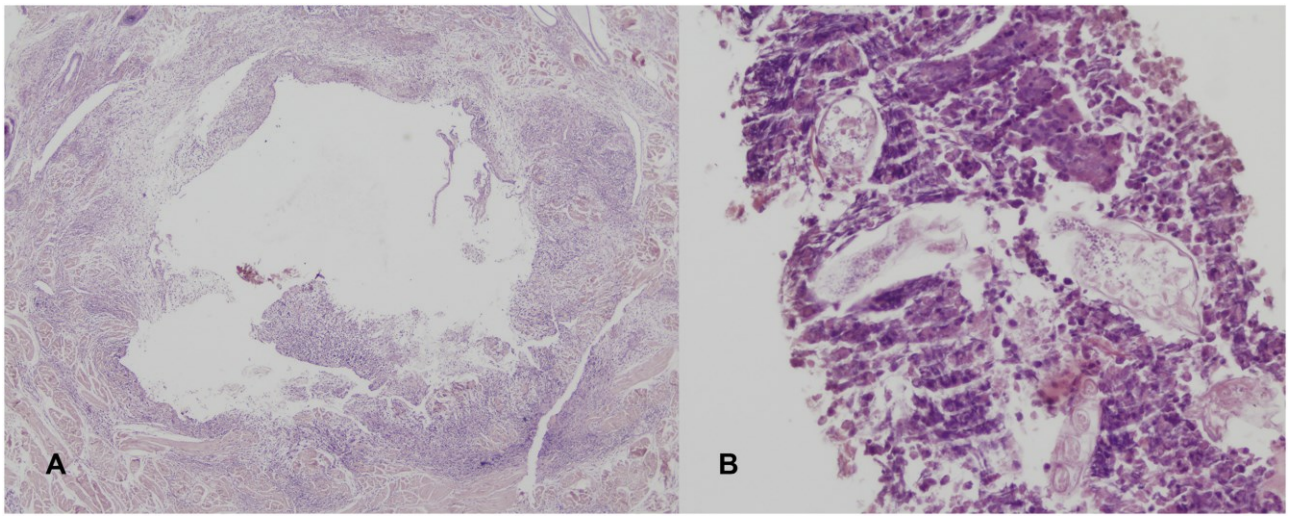

Fig. 2. Histopathology of skin nodules´ biopsies collected from *D. bovis* infested cows co-infected with *B. besnoiti*. A. Slight acanthosis and hyperkeratosis of the epidermis and superficial dermatitis. Cystic formations in the deep dermis lined by a layer of metaplastic squamous epithelium and severe cell infiltration (40×). B. Degenerated and necrotic granulocytes with parts of hair, keratin flakes and sectioned mites contained in cystic formations (400×).

Discussion

A generalized form of bovine demodectic mange was confirmed in two *B. besnoiti* co-infected cows from an endemically infected dairy cattle herd in Northern Italy. In the study farm, a high intra-herd seroprevalence of *B. besnoiti* antibodies and the presence of clinical signs of bovine besnoitiosis in a part of the seropositive animals were previously reported (Villa et al., 2019). Furthermore, this study highlighted the infestation with *D. bovis* in two cows positive to *B. besnoiti*. Unfortunately, it was not possible to understand how long the animals have been affected by *B. besnoiti* and no relevant clinical sign was reported in the past. The presence of lesions ascribable to bovine demodicosis was not observed in any other animal of the herd at the clinical examination; however, subclinical forms of the disease could not be excluded, since the presence of *D. bovis* was not systematically investigated in all cattle in the farm.

Only scarce, not updated and scattered data are available concerning *D. bovis* in cattle in Italy, where cases of bovine demodicosis were reported in cattle farms in Northern and Central Italy (Sani et al., 1925; Marastoni and Rossini, 1961; Ciurnelli and Ciarlantini, 1975; Manfredini et al., 1994; Giammarino et al., 1996).

In temperate climatic zones, bovine demodicosis generally occurs in a subclinical chronic form with only a moderate nodule number; on the contrary, a generalized form with hundreds to thousands of nodules was reported in 2–5% of cattle under intensive condition in Europe (Matthes et al., 1994).

The distribution of the nodules on the body surface of parasitized cows reflected what previously reported by Matthes (1994), i.e. a predilection for anterior body regions (shoulders, arms, neck, dewlap and hypochondriac region). However, in these cows, many nodules were also detected in the cephalic region, as previously reported in three cows by Giammarino (1996). On the other side, only a few nodules were evidenced in back regions in both animals.

Bovine demodicosis is reported mainly in dairy cows with increased stress: the occurrence of the disease seems to be correlated to debilitating factors or to receptive physiological states of the animal (pregnancy or lactation). In this regard, it is important to underline that both cows were lactating. In particular, one cow was at the end of lactation (290 days), but still not pregnant, and was producing 33.9 kg of milk per day; instead, the other one was at the beginning of lactation (65 days) with a milk daily production of 46.6 kg. Furthermore, even this is not the case, immunosuppression during the peripartum period may also facilitate *D. bovis* infestation and skin colonization. Besides, the eventual interference between *B. besnoiti* and bovine demodicosis and/or other infectious diseases should be clarified. In fact, since bovine besnoitiosis is a chronic and debilitating disease (Alvarez-Garcia et al., 2014), it should not be neglected its role in determining an immune imbalance possibly leading to other infections, including *D. bovis*. Concerning *D. bovis*, both natural and acquired immunity contribute to reducing mite numbers and associated clinical signs in infested cattle. However, generalized demodicosis could be due to diseases altering the immune system. Indeed, the co-infection with bovine besnoitiosis may interfere with the immune response leading to severe form of the disease as described in the case of these two cows. It should also be considered that these cows were not only seropositive to *B. besnoiti*, but one of them was also clinically affected by the chronic form of bovine besnoitiosis, as demonstrated by the detection of tissue cysts in sclera and vulva. Concerning skin lesions, in these two cows, in all collected skin biopsies the presence of *B. besnoiti* was not evidenced both through compression between glasses and histology. However, it is not possible to determinate, only based on clinical signs, if any of the skin lesions may harbor also parasite tissue cysts, since visually it is not easy to distinguish between *B. besnoiti* tissue cysts and nodules due to *D. bovis* infestation. Moreover, Abu-Samra et al. (2014) investigated the association between *Demodex* mites and bacteria involved in skin lesions of bovine demodicosis. Also in this case, the nature of the exudate suggested the involvement of bacteria, even if isolation of skin bacteria was not attempted.

This report demonstrated that *D. bovis* does circulate in the Italian cattle population. The disease should be further investigated. However, since bovine demodicosis is more frequently only subclinical, clinical forms could be underdiagnosed. Even if the disease does not compromise the

life of infested animals, it could reduce the productivity of the herd. The treatment with Eprinomectin 0.5% Pour on lead to an improvement of the clinical signs in both cows; however, a mite-free status of the animals cannot be guaranteed.

Conclusions

The co-infection of *B. besnoiti* and *D. bovis* was confirmed in two dairy cows. This is the first detection of demodectic mange in bovine besnoitiosis infected cattle. Both diseases in cattle should be further investigated, also in relation to the immune response of infected animals.

Declarations

Ethical statement

Biological samples were collected by qualified veterinarians applying adequate procedures of handling and disinfection to minimize pain or distress in sampled animals. All procedures for the collection of biological specimens from live animals were accomplished following good clinical practices in the respect of animal welfare according to current legislation. The study was conducted with the approval of the Institutional Animal Care and Use Committee of Università degli Studi di Milano (Permission OPBA_34_2017).

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Declaration of Competing Interest

The Authors declare they have no conflict of interest.

Research Line 1 bis:

Exposure of Italian equids to selected protozoa infections and investigation on clinical besnoitiosis in donkeys

First detection of anti-*Besnoitia* spp. specific antibodies in horses and donkeys in Italy

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Abstract

Among Apicomplexa protozoa infecting equids, *Besnoitia* spp., *Toxoplasma gondii* and *Neospora* spp. represent important issues from a sanitary and zootechnical viewpoint. However, only scarce epidemiological data are available on the spread of the infections in horses and donkeys in Europe. Therefore, a serosurvey was planned to estimate the prevalence of these Sarcocystidae species in Italian equids. Serum samples from 268 horses and 18donkeys raised in Italy were collected and serologically analyzed to detect anti-*Besnoitia* spp., anti-*T. gondii* and anti-*Neospora* spp. antibodies: an approach based on an initial screening by in-house ELISA followed by a confirmatory WB was used. Two horses (0.7%) and four donkeys (22.2%), showed antibodies anti-*Besnoitia* spp. Ten horses (3.7%) resulted positive to *T. gondii* and one of these (0.4%) was seropositive also to *Neospora* spp. This is the first detection of anti-*Besnoitia* spp. specific antibodies in Italian horses and donkeys. The study confirmed the circulation of *Besnoitia* spp. among equids in Europe. Low prevalence of *T. gondii* and *Neospora* spp. in horses raised in Italy was reported. Nevertheless, it is noteworthy to consider that consumption of horsemeat could represent a source for human toxoplasmosis.

Keywords: *Besnoitia* spp.; *Toxoplasma gondii*; *Neospora* spp.; Equids; Italy.

Introduction

In equids, infections by Apicomplexa protozoa are of concern from a veterinary and zootechnical viewpoint: particularly, *Besnoitia* spp., *Toxoplasma gondii* and *Neospora* spp. were reported to affect both horses and donkeys. Besnoitiosis in equids, caused by *Besnoitia bennetti*, is considered an emerging disease of donkeys in the United States (Ness et al., 2014). *Besnoitia* spp. specific antibodies were detected for the first time in Europe in equids from areas where bovine besnoitiosis is endemic in Spain (Gutierrez-Exposito et al., 2017). The infection was never explored in Italian equids, even if outbreaks of the disease in cattle were recently diagnosed in Northern regions (Gazzonis et al., 2014; 2017). Anti-*T. gondii* antibodies were demonstrated in both species in serological surveys worldwide, although there is not any confirmed report of clinical disease in equids (Dubey, 2010). On the contrary, *Neospora hughesi* is recognized as an etiological agent of the equine protozoal myeloencephalitis (EPM), an important neurological disease of horses (Dubey et al., 2017). Worldwide, a range of seroprevalence values between <1 and 65.6% and between 0 and 85.7% was reported for *T. gondii* and *Neospora* spp. infections in equids, respectively (Dubey, 2010; Dubey et al., 2017). In Italy, the presence of antibodies anti-*T. gondii* (3–8%) and anti-*Neospora* spp. (2.3–28%) has been reported in equids reared in Southern Italy (Ciaramella et al., 2004; Machacova et al., 2014; Machacova et al., 2015; Bartova et al., 2015). In Northern Italy data are limited to *T. gondii* infection in horses destined for human consumption (17.6%) (Papini et al., 2015), although the presence of the parasite in this area was recently reported in other domestic and wild species (Gazzonis et al., 2015; 2016; 2018a; 2018b). Therefore, the study aimed to contribute to the knowledge of *Besnoitia* spp., *T. gondii* and *Neospora* spp. by estimating their seroprevalence in equids from Italy.

Materials and methods

A minimum sample size of 246 horses was determined considering a population in Lombardy and Piedmont of 78,490 animals (National Zootechnical Database, https://www.vetinfo.sanita.it/), a 20% expected prevalence, a 95% confidence interval and a 5% desired absolute precision. From April 2016 to March 2017, blood samples from 268 horses (*Equus caballus*) apparently healthy from 33 stables located in Northern Italy (Lombardy and Piedmont regions) were collected by puncturing of the jugular vein using a Vacutainer® sterile collection system and preserved refrigerated in tubes without anticoagulants during the transportation to the laboratory. Once in laboratory, sera were separated by centrifugation (2120*g*, 15 min) and then stored at −20 °C until

serological analysis. Moreover, serum samples from 18 donkeys *(Equus asinus*) previously referred to the laboratory for routine parasitological examinations were also included in the study. Epidemiological data were collected interviewing the owners at sampling times; to avoid bias in the data collection, farms were visited by the same investigator. To detect anti-*Besnoitia* spp., anti-*T. gondii* and anti-*Neospora* spp. antibodies, all serum samples were initially screened by ELISA; subsequently, positive results were confirmed by Western Blot (WB), as recommended (Gutierrez-Exposito et al., 2017; Garcia-Lunar et al., 2015; 2017). For both serological tests, antigens of *B. besnoiti* and *N. caninum* originally isolated from cattle were used, because strong cross reactions with the respective species infecting equids (*B. bennetti* and *N. hughesi*) were demonstrated (Gutierrez-Exposito et al., 2017). An 'in-house' indirect ELISA was used to detect antibodies of *Besnoitia* spp., *T. gondii* and *Neospora* spp. in serum samples, as previously described (Gutierrez-Exposito et al., 2017; Garcia-Lunar et al., 2017; Gonzales-Warleta et al., 2014). A blocking solution of Phosphate Buffered Saline (PBS) containing 0.05% Tween 20 and 5% Bovine Serum Albumin was used; Protein G (recombinant peroxidase labeled, Sigma-Aldrich®, Saint Louis, USA) diluted at 1:1500 was used as conjugate. Absorbance was measured as Optical Density (OD) values at 405 nm using a microplate reader. Samples were analyzed in duplicate and the mean value of the OD was converted into a relative index per cent (RIPC) using the following formula: RIPC = (OD sample-OD negative control)/(OD positive control-OD negative control) × 100. For each pathogen, the cut-off value was calculated as the mean plus three standard deviations of RIPC values considering a panel of negative control sera ($n = 20$). For *T. gondii*, serum samples from horses previously referred to the laboratory for diagnostic purposes, were analyzed using a commercial indirect immunofluorescence antibody assay (IFAT), according to Gazzonis et al. (2015), using a FITC anti-horse IgG (MegaCor Diagnostik, Horbranz, Austria) as conjugate and considering 1:20 dilution as the cut-off (Papini et al., 2015). For *Neospora* spp. and *Besnoitia* spp., negative sera previously analyzed were included in the panels (Gutierrez-Exposito et al., 2017). Samples having a RIPC value higher than 12.3, 18.7 and 21.5 were considered ELISA-positive results respectively for *Besnoitia* spp., *T. gondii* and *Neospora* spp. and then submitted to confirmatory WB, performed and interpreted as previously described (Alvarez-Garcia et al., 2002; Chávez-Velásquez et al., 2005; Fernandez-Garcia et al., 2009). A total of 4×10^7 *B. besnoiti* tachyzoites under non-reducing condition and 2×10^7 *T. gondii*- and *N. caninum* tachyzoites under reducing condition were employed for electrophoresis. Tachyzoite antigens were transferred to nitrocellulose membranes and incubated with sera from horses and donkeys at a 1:20 dilution, followed by a peroxidase-conjugated anti-horse IgG (H + L) antibody diluted at 1:1500 (INGENASA®, Madrid, Spain). For

both serological tests, positive and negative control sera were included. In particular, for *T. gondii*, ovine positive and negative control sera were employed; for *Neospora* spp. and *Besnoitia* spp., both equine (horse and donkey, respectively) and bovine control sera were used (Gutierrez-Exposito et al., 2017).

Results

The presence of anti-*Besnoitia* spp. antibodies was demonstrated by ELISA in 21 horses and in four donkeys. Antibodies against *T. gondii* and *Neospora* spp. were detected in 19 and 22 horses, respectively. Seropositivity against *Besnoitia* spp. was confirmed by WB in six equids, specifically four donkeys and two horses. WB analysis confirmed seropositivity to *T. gondii* in ten horses and to *Neospora* spp. only in one horse, contemporary infected also by *T. gondii* (Table 1, Supplementary Fig. 1, Supplementary Fig. 2). Seroprevalence of the investigated parasites in different cohorts of the examined equids are reported in Table 2. Individual and managerial data regarding the seropositive animals are reported in Supplementary Table 1.

Table 1. Seropositivity to *Besnoitia* spp., *Toxoplasma gondii* and *Neospora* spp. in ELISA and Western Blot (WB) in 268 horses and 18 donkeys examined.

		Overall		**Horses**		**Donkeys**	
		ELISA	WB	ELISA	WB	ELISA	WB
***Besnoitia* spp.**	Pos/Ex [a]	25/286	6/286	21/268	2/268	4/18	4/18
	P% [b]	8.7	2.1	7.8	0.7	22.2	22.2
	(95% CI) [c]	(6-12.6)	(1-4.5)	(5.2-11.7)	(0.2-2.7)	(9-45.2)	(9-45.2)
Toxoplasma gondii	Pos/Ex [a]	19/286	10/286	19/268	10/268	0/18	0/18
	P% [b]	6.6	3.5	7.1	3.7	0	0
	(95% CI) [c]	(4.3-10.1)	(1.9-6.3)	(4.6-10.8)	(2-6.7)	(0-17.6)	(0-17.6)
***Neospora* spp.**	Pos/Ex [a]	22/286	1/286	22/268	1/268	0/18	0/18
	P% [b]	7.7	0.3	8.2	0.4	0	0
	(95% CI) [c]	(5.1-11.4)	(0.1-1.9)	(5.5-12.1)	(0.1-2.1)	(0-17.6)	(0-17.6)

[a] Pos/Ex, Positive/Executed, [b] P%, Prevalence %, [c] 95% CI, 95% Confidence interval.

Table 2. Seropositivity to *Besnoitia* spp., *Toxoplasma gondii* and *Neospora* spp. in examined equids related to considered individual and managerial data.

Variable	Category	*Besnoitia* spp.		*T. gondii*		*Neospora* spp.	
		Pos/Ex [a]	P% (95% CI) [b]	Pos/Ex [a]	P% (95% CI) [b]	Pos/Ex [a]	P% (95% CI) [b]
Species	Donkeys	4/18	22.2 (9-45.21)	0/18	0 (0-17.59)	0/18	0 (0-17.59)
	Horses	2/268	0.7 (0.21-2.69)	10/268	3.7 (2.04-6.73)	1/268	0.4 (0.06-2.08)
Gender	Male	2/146	1.4 (0.38-4.86)	8/146	5.5 (2.8-10.44)	1/146	0.7 (0.12-3.77)
	Female	4/140	2.9 (1.12-7.12)	2/140	1.4 (0.39-5.06)	0/140	0 (0-2.67)
Neutered	Yes	1/97	1 (0.18-5.61)	2/97	2.1 (0.57-7.21)	0/97	0 (0-3.81)
	No	5/184	2.7 (1.17-6.21)	8/184	4.3 (2.22-8.35)	1/184	0.5 (0.09-3.01)
Age (years)	0-4	0/92	0 (0-4.01)	5/92	5.4 (2.34-12.09)	0/92	0 (0-4.01)
	5-10	1/67	1.5 (0.26-7.98)	0/67	0 (0-5.42)	0/67	0 (0-5.42)
	11-15	3/46	6.5 (2.24-17.5)	1/46	2.2 (0.38-11.33)	0/46	0 (0-7.71)
	>15	2/81	2.5 (0.68-8.56)	4/81	4.9 (1.94-12.02)	1/81	1.2 (0.22-6.66)
Attitude	Reproduction	0/63	0 (0-5.75)	6/63	9.5 (4.44-19.26)	1/63	1.6 (0.28-8.46)
	Companion	2/32	6.2 (1.73-20.15)	0/32	0 (0-10.72)	0/32	0 (0-10.72)
	Hippo- onotherapy	2/13	15.4 (4.32-42.23)	0/13	0 (0-22.81)	0/13	0 (0-22.81)
	Walking	1/33	3 (0.54-15.32)	0/33	0 (0-10.43)	0/33	0 (0-10.43)
	Showjumper	1/19	5.3 (0.93-24.63)	0/19	0 (0-16.82)	0/19	0 (0-16.82)
	Saddle	0/112	0 (0-3.32)	2/112	1.8 (0.49-6.28)	0/112	0 (0-3.32)
	School	0/14	0 (0-21.53)	2/14	14.3 (4.01-39.95)	0/14	0 (0-21.53)
Box	Yes	3/202	1.5 (0.51-4.28)	4/202	2 (0.77-4.98)	0/202	0 (0-1.87)
	No	3/84	3.6 (1.22-9.98)	6/84	7.14 (3.31-14.72)	1/84	1.19 (0.21-6.44)
Housing	Only indoor	1/45	2.2 (0.39-11.56)	2/45	4.4 (1.23-14.82)	0/45	0 (0-7.87)
	Only outdoor	4/84	4.76 (1.87-11.61)	6/84	7.14 (3.31-14.72)	1/84	1.19 (0.21-6.44)
	Indoor in night	1/157	0.6 (0.11-3.52)	2/157	1.3 (0.35-4.52)	0/157	0 (0-2.39)
Paddock	Yes	5/237	2.1 (0.09-4.84)	8/237	3.4 (1.72-6.52)	1/237	0.4 (0.07-2.35)
	No	1/49	2 (0.36-10.69)	2/49	4.1 (1.13-13.71)	0/49	0 (0-7.27)
Bedding	Straw	1/69	1.4 (0.08-8.89)	0/69	0 (0-5.27)	0/69	0 (0-5.27)
	Wood shavings	1/103	9.7 (0.17-5.29)	4/103	3.9 (1.52-9.56)	0/103	0 (0-3.6)
	Ground	0/30	0 (0-11.35)	0/30	0 (0-11.35)	0/30	0 (0-11.35)
	No	4/84	4.8 (1.87-11-61)	6/84	7.1 (3.31-14.72)	1/84	1.2 (0.21-6.44)

[a] Pos/Ex, Positive/Executed, [b] 95% CI, 95% Confidence interval.

Discussion

The present study provided serological data on selected cystogenic coccidia in Italian equids, for which only scarce data are available in Europe. This is the first detection of anti-*Besnoitia* spp. specific antibodies in Italian horses and donkeys. Moreover, the circulation of *T. gondii* and *Neospora* spp. was confirmed in horses raised in Italy. European countries should be aware of these parasitic diseases. Surveillance should be implemented; harmonized diagnostic procedures and standardized techniques are needed in order to get comparable results and infer reliable conclusions. The diagnostic approach used in this study consisted of an initial screening by ELISA followed by a confirmatory WB: the use of a confirmatory technique is recommended due to the possibility of cross-reactions between closely related Apicomplexa, i.e. *T.*

gondii, *Neospora* spp., *Besnoitia* spp. and *Sarcocystis* spp. (Garcia-Lunar et al., 2015). In this study, six equids resulted positive to *Besnoitia* spp., with an overall seroprevalence of 2.1% (0.7% in horses and 22.2% in donkeys). Seropositive horses were raised in two farms in the south-western part of the study area, where outbreaks of bovine besnoitiosis were recently reported (Gazzonis et al., 2014; 2017); besides, it is noteworthy consider that in this area cattle are more frequently maintained on extensive pasture, in contrast to the Po Valley, where animals are kept mainly in intensive farms. Concerning *Besnoitia* spp. infected donkeys, these animals came from both northern and southern areas: interestingly, in all these farms the co-presence of other species, in particular domestic ruminants, was reported. Other countries where there are cases of bovine besnoitiosis should consider the possibility that also equids could be seropositive to *Besnoitia* spp. Indeed, *Besnoitia* spp. specific antibodies were recently detected in Spanish equids (Gutierrez-Exposito et al., 2017) with a seroprevalence of 7.1% in areas where bovine besnoitiosis is endemic. Similar to the present study, also in Spain the seroprevalence of *Besnoitia* spp. infection was higher in donkeys (15.3%) than in horses (2.9%); however, a higher susceptibility of donkeys for *Besnoitia* spp. infection was not demonstrated. Considering individual data, anti-*Besnoitia* spp. antibodies were found only in animals older than 5 years. Furthermore, equids housed only outdoor and without box resulted more infected with *Besnoitia* spp., probably because these animals could be at greater risk of exposure to the bite of vector insects. Outside of Europe, *Besnoitia* spp. infections in equids were attributed to *B. bennetti* in sub-Saharan countries and in the United States besnoitiosis is considered an emerging disease of donkeys (Ness et al., 2014). Molecular studies would be advisable to clarify which *Besnoitia* species is involved in the infection of Italian horses and donkeys. Concerning *T. gondii* infection, ten horses were positive to the parasite, resulting a prevalence of 3.7%; whereas no donkey showed antibodies against *T. gondii*. Epidemiological studies conducted in Southern Italy using IFAT reported a seroprevalence of 3% in horses (Bartova et al., 2015) and 8% in donkeys (Machacova et al., 2014). A similar seroprevalence of *T. gondii* infection in equids (2–4%) was reported in Greece and in Switzerland; in other European countries a range of prevalence between 7 and 23% was reported (Dubey, 2010). All seropositive horses examined in the present study were apparently healthy; in fact, there is no evidence that *T. gondii* causes clinical disease in equids and confirmed cases of clinical toxoplasmosis were never reported in horses and/or donkeys worldwide (Dubey, 2010). Considering individual data, regarding age, the parasite infection was more prevalent in the categories of young (0–4 years old) and older horses (>15 years old), suggesting both a vertical transmission, but also a higher risk of exposure to the parasite (horizontal transmission) with the increase of the age of the animals (Papini et al., 2015). Besides, horses kept only outdoor and not recovered indoor neither during

the night presented a higher *T. gondii* seroprevalence. Furthermore, it is noteworthy that horses bred for reproduction were more infected with *T. gondii* than sport horses. Farming is a significant risk factor for *T. gondii* infection in horses, because it is an activity that keeps the animals close to a potencial contaminated environment (Dubey, 2010). On the contrary, horses used for sport purposes are animals of considerable economic value, and for this reason they are usually more carefully managed. Since a part of the sampled horses was bred for meat production and of these some resulted positive to the parasite infection (6/63; 9.5%), it is noteworthy to consider the importance of *T. gondii* as zoonotic agent: indeed, the consumption of raw or undercooked horse meat represent a possible source of *T. gondii* infection for humans. Although prevalence is lower in horses when compared to other species such as small ruminants and domestic and wild pigs (Gazzonis et al., 2015; 2018a; 2018b) and the burden of tissue cysts is less consistent if compared to pigs, horse meat is often used for preparation of dishes containing raw meat, therefore also equids could represent a risk of parasite infection for humans. Regarding the relationship between detection of antibodies and presence of *T. gondii* tissue cysts in meat, only limited studies are available for horses and data suggest a lack of concordance (Opsteegh et al., 2016). For this reason, it is underlined the need of monitoring and surveillance of *T. gondii* in equine meat for human consumption to correctly assess the consequent risk of transmission of toxoplasmosis, with both serological and molecular techniques. Only one horse was confirmed positive to *Neospora* spp., with a prevalence of 0.4%; none of the donkeys resulted positive to the parasite. The rate of infection resulted lower when compared to studies recently conducted in Southern Italy, where values of prevalence of 2.3 and 28% in horses (Ciaramella et al., 2004; Bartova et al., 2015) and a prevalence of 11.8% in donkeys (Machacova et al., 2013) were reported. A similar seroprevalence of *Neospora* infection (0.4%) was reported in healthy horses from the Czech Republic; other studies conducted in different European countries revealed a range of prevalence between approximately 1 and 10% (Dubey et al., 2017). The positive horse, a 25 years old stud bred for reproduction and kept in pasture throughout the year, did not show clinical signs of neosporosis; in fact, the detection of *Neospora* spp. specific antibodies does not confirm the diagnosis of EPM, since the disease occurs only in a small proportion of infected horses. Besides, this horse was also co-infected by *T. gondii*. Further studies focused on the molecular characterization of *Neospora* spp. are necessary to determinate which species circulate in Italian equids.

Conclusions

The study reported the first detection of *Besnoitia* spp. specific antibodies in horses and donkeys from Italy and confirmed the circulation of *Besnoitia* spp. among equids in Europe only recently evidenced. Low prevalence of *T. gondii* and *Neospora* spp. in horses raised in Italy was also recorded. The results emphasized both the risk for humans to acquire toxoplasmosis through horse meat consumption and the need of further studies to investigate the epidemiology and also for the isolation and molecular characterization of the species of Sarcocystidae infecting Italian equids.

Declarations

Ethical statement

All procedures for collection of biological specimens from live animals were accomplished following good clinical practices in the respect of animal welfare according to current legislation. The study was conducted with the approval of Institutional Animal Care and Use Committee of Università degli Studi di Milano (Permission OPBA_34_2017).

Conflict of interest statement

The authors declare they have no conflict of interest.

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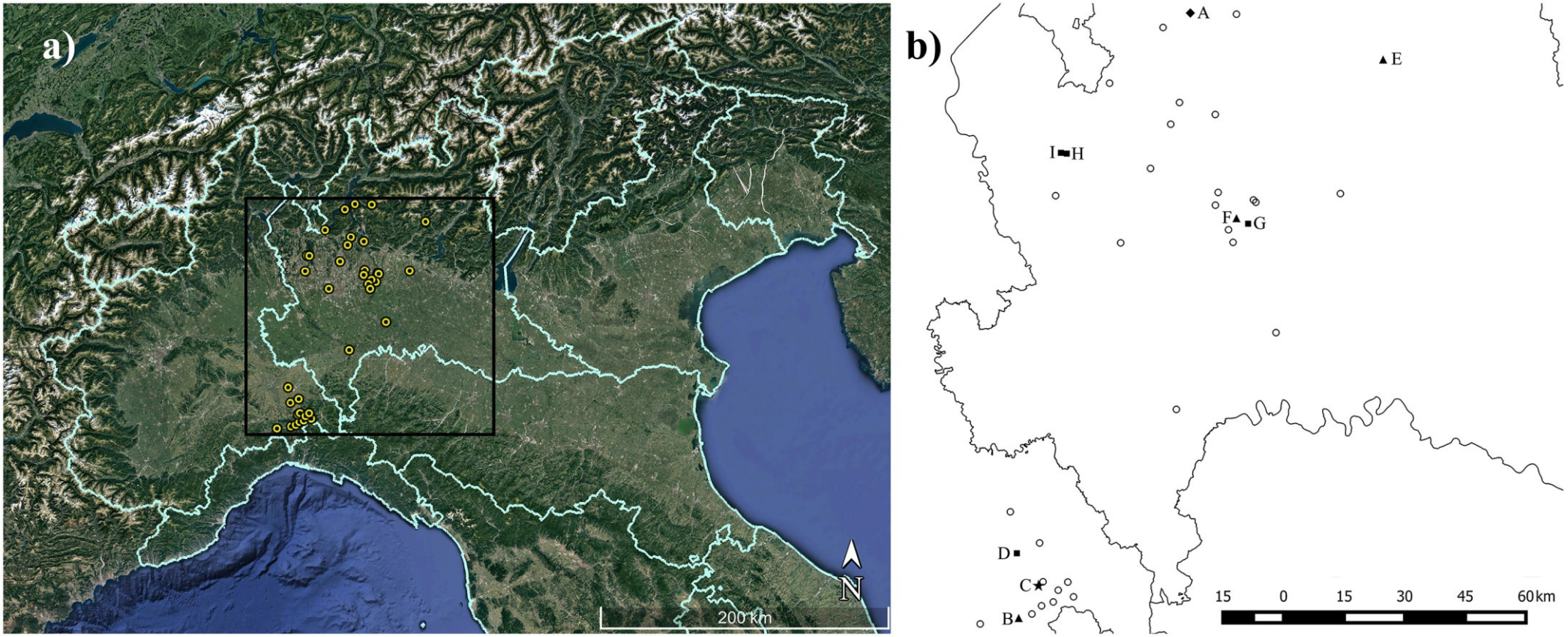
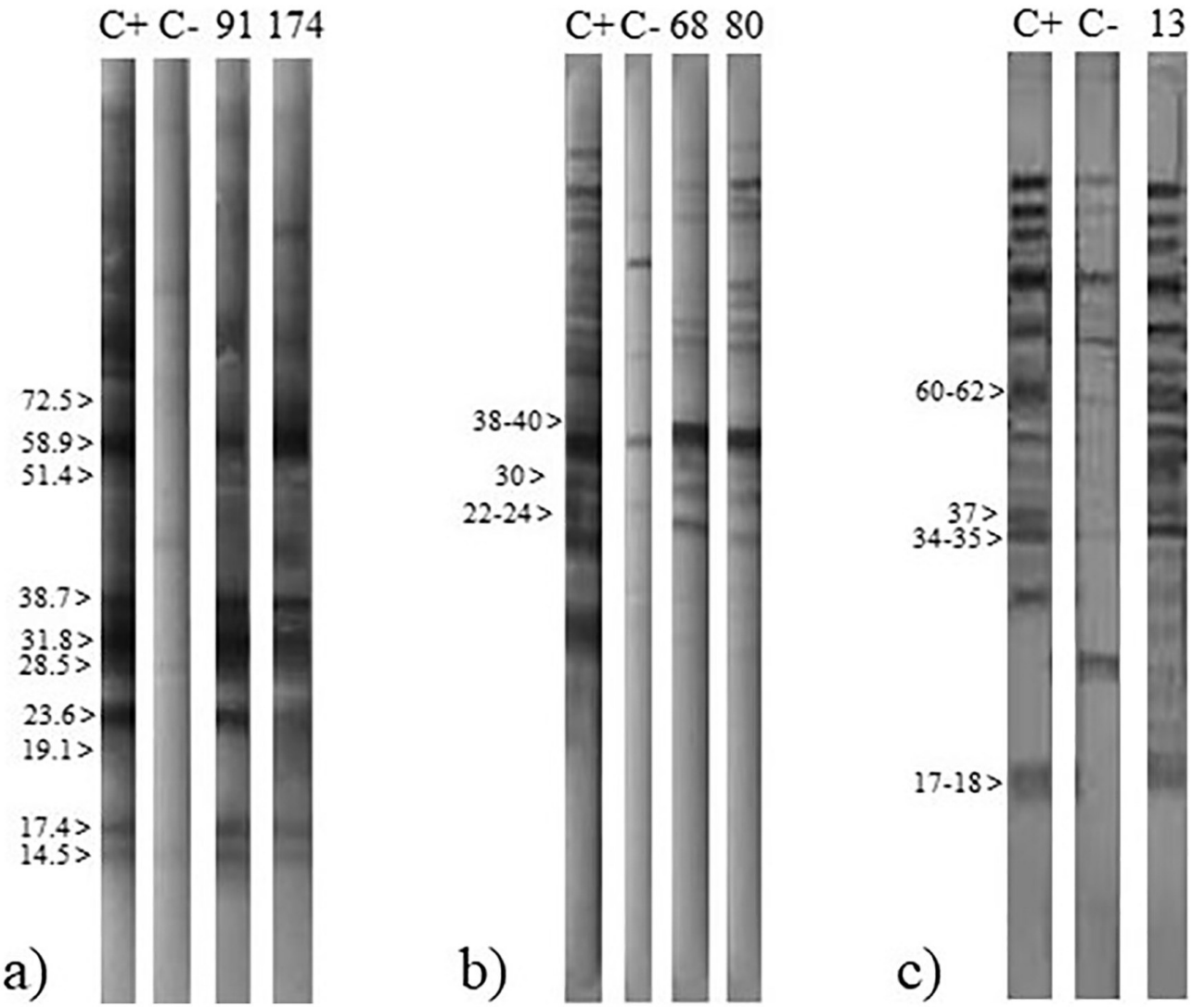


Supplementary Fig. 1. (a) Spatial distribution of stables of horses and donkeys located in Lombardy and Piedmont regions (Italy) included in the study. (b) Seropositivity to investigated Sarcocystidae is indicated with different symbols: square = *Besnoitia* spp., triangle = *T. gondii*, star = *T. gondii* and *Besnoitia* spp., diamond = *T. gondii* and *Neospora* spp., circle = negative.



Supplementary Fig. 2. Pattern of recognition of (a) *Besnoitia* spp., (b) *Toxoplasma gondii* and (c) *Neospora* spp. tachyzoite antigens in serum samples from naturally infected horses and donkeys by Western Blot.

Supplementary Table 1. Baseline characteristics related to individual data and farm management of seropositive equids to selected Sarcocystidae.

	ID	Farm	Breed	Attitude	Origin	Gender [a]	Age (years)	Housing	Box	Paddock	Bedding	Presence of other animals	*Besnoitia* spp. [b]	*Toxoplasma gondii* [b]	*Neospora* spp. [b]
Horse	13	A	Crossbred	Breeding Reproduction	Italy	M	25	Only outdoor	No	Yes	No	Wild animals Rodents	N	P	P
	31		Crossbred		Italy	M	1						N	P	N
	37		Crossbred		Italy	M	1						N	P	N
	63		Crossbred		Italy	M	3						N	P	N
	65		Crossbred		Italy	M	3						N	P	N
	68		Crossbred		Italy	M	4						N	P	N
	80	B	Shetland	School	Italy	F	8	Indoor in night	Yes	Yes	Wood shavings	No	N	P	N
	89	C	Bardigiano	School	Italy	NM	21	Only indoor	Yes	No	Wood shavings	No	N	P	N
	91		Crossbred	Showjumper	Italy	F	12						P	N	N
	120	D	Pure Spanish	Walk	Spain	F	14	Only outdoor	Yes	Yes	Ground	No	P	N	N
	282	E	Dutch Warmblood	Saddle	Italy	NM	14	Only indoor	Yes	No	Wood shavings	Cats, dogs, ruminants, pigs, poultry	N	P	N
	286	F	Dutch Warmblood	Saddle	Italy	F	18	Indoor in night	Yes	Yes	Wood shavings	No	N	P	N
Donkey	170	G	Sicilian Grey	Onotherapy	Italy	NM	19	Only outdoor	No	Yes	Ground	Dogs, rabbits, poultry, ruminants	P	N	N
	174		Amiata		Italy	F	11						P	N	N
	184	H	Crossbred	Companion	Italy	M	5	Only outdoor	No	Yes	No	No	P	N	N
	185	I	Crossbred	Companion	Italy	F	17	Indoor in night	Yes	Yes	Straw	Cats, rabbits, ruminants	P	N	N

[a] M, Male, F, Female, NM, Neutered Male; [b] P, Positive, N, Negative

Besnoitiosis in donkeys: an emerging parasitic disease of equids in Italy

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Abstract

Besnoitiosis is an emerging parasitic disease of equids. Italy is one of the few European countries where the circulation of *Besnoitia* spp. antibodies was demonstrated. In this study, a case of clinical besnoitiosis in two donkeys in northern Italy is reported. The two animals were clinically examined. Serum and blood samples were analyzed for the detection of *Besnoitia* spp. antibodies and for hematology, biochemistry, and enzyme activity, respectively. ITS-1 PCR and sequencing were carried out on DNA extracted from skin biopsies.

Clinical examination revealed numerous scleral pearls in eyes of both animals; alopecia and hyperkeratosis with skin nodules in the region of the neck, hind leg, and on the pinnae were detected. No cysts were evidenced by endoscopy in respiratory and genital tracts. Both animals resulted seropositive to *Besnoitia* spp. antibodies by Western Blot. Hematology evidenced light anemia, leukocytosis with eosinophilia, and lymphocytosis; biochemistry and enzyme activity revealed hypoalbuminemia with decreased albumin/globulin ratio and elevated alkaline phosphatase values. Parasitic DNA extracted from skin biopsies of both donkeys demonstrated a homology of 100% with *Besnoitia* spp.

This first clinical case of besnoitiosis in two donkeys in Italy both confirms the circulation of *Besnoitia* spp. in Italian equids and demonstrates that the distribution area of equine besnoitiosis in Europe could be wider than expected. Further studies are needed to infer its relevance, in relation to seroprevalence and clinical disease, and to identify the species infecting donkeys. Besnoitiosis may be a neglected disease of donkeys in Europe: an early and accurate diagnosis is fundamental to implement adequate control measures to prevent a “silent” spread of *Besnoitia* spp. infection in equids populations.

Keywords: Besnoitia spp., Donkey, Italy, Case report, Clinical features, Serology, PCR.

Introduction

Among 10 recognized species, the genus *Besnoitia* includes four closely related species (*B. besnoiti*, *B. caprae*, *B. bennetti* and *B. tarandi*) infecting domestic and wild ungulates (cattle, goats, equids and deers, respectively). *Besnoitia besnoiti*, the most reported species in Europe, is the causative agent of bovine besnoitiosis. The disease is chronic and debilitating, characterized by both cutaneous and systemic manifestations, compromising animal welfare, and responsible for economic losses on affected farms (Alvarez-Garcia et al. 2013; Cortes et al. 2014). Bovine besnoitiosis is a (re)emerging disease of cattle in Europe, with an increasing geographical distribution and the number of cases of infection (EFSA 2010).

Besnoitiosis in equids is caused by *Besnoitia bennetti*. The life cycle of the parasite is not completely clear: in fact, the definitive host, and the mode of transmission in equine infection remain still unknown (Dubey et al. 2005). Clinical signs and lesions are similar to those observed in bovine besnoitiosis. Indeed, the disease is characterized by multifocal white pinpoint miliary parasitic cysts in the skin of the face and body, in the nares, on the pinnae, and on the limbs and perineum. *Besnoitia* lesions are frequently found on mucous membranes, particularly in the upper respiratory tract. A typical feature of the disease is the development of parasitic cysts within the sclera and conjunctiva of the eye (scleral pearls). With the progression of the disease infected animals develop poor hair coat and skin lesions consisting of alopecia, hypotrichosis, hyperpigmentation, thickening, and crusting, involving the face, muzzle, eyes, ears, neck, flanks, legs, and perineum. Some infected animals remain otherwise healthy, others become cachexic and debilitated (Dubey et al. 2005; Ness et al. 2012).

The disease was historically limited to donkeys and horses in Africa, where outbreaks of the disease were reported in both species (Bennett 1927; Schultz and Thorburn, 1955; Bigalke 1970; Van Heerden et al. 1993). Outside of Africa, outbreaks of *Besnoitia* spp. infection were reported in donkeys in the USA where besnoitiosis may be considered an emerging disease of these equids (Terrell et al. 1973; Davis et al. 1997; Dubey et al. 2005; Elsheikha et al. 2005; Ness et al. 2012; Ness et al. 2014). Concerning Europe, the first case of besnoitiosis in a horse was reported in northern France (Henry and Masson 1922). Recently, the disease was suspected in seven donkeys from southern Spain since tissue cysts were detected by histopathology (Zafra et al. 2013). Clinical cases of besnoitiosis were also reported in two and 20 donkeys in Belgium and the UK, respectively (Lienard et al. 2018, Elsheikha et al. 2020): in both these reports, the diagnosis was molecularly confirmed. Furthermore, *Besnoitia* spp. specific antibodies were detected in equids in Spain (Gutierrez-Exposito et al. 2017), Portugal (Waap et al. 2020), and also Italy (Villa et al.

2018), in areas where outbreaks of bovine besnoitiosis were previously reported. Indeed, in the last decade, in northern Italy, outbreaks of bovine besnoitiosis were reported (Gentile et al. 2012; Gazzonis et al. 2017; Villa et al. 2019; Villa et al. 2020), and a serological survey on the spread of *B. besnoiti* in cattle was carried out (Gazzonis et al. 2014).

This study reports the diagnosis of a case of besnoitiosis in two donkeys, for the first time in Italy, using clinical, serological, and molecular tools.

Materials and methods

Ethical statement

Biological samples were collected by qualified veterinarians applying adequate procedures of handling and disinfection to minimize pain or distress in sampled animals. All procedures for the collection of biological specimens from live animals were accomplished following good clinical practices in the respect of animal welfare according to current legislation. The study was conducted with the approval of the Institutional Animal Care and Use Committee of Università degli Studi di Milano (Prot. n° OPBA_34_2017).

Background

In March 2019, a private veterinarian (C.P.) referred to the Parasitology Laboratory (Department of Veterinary Medicine, University of Milan, Lodi, Italy) two donkeys with poor body condition and suspected skin lesions. The animals, two one-year-old Amiatina donkeys, one male and one female, were reared by a private owner as companion animals in Brescia suburbs (northern Italy) (45°29′48″N 10°12′18″E) after being purchased from a farm located in the mountains nearby (Val Camonica, Brescia, Italy) (46°00′27″N 10°20′51″E) three months before. The two donkeys were kept in a fenced area during the day and recovered indoor during the night.

Clinical examination and sample collection

The two donkeys were hospitalized in the facilities of the Equine Isolation Unit of the Veterinary Teaching Hospital of the University of Milan (Lodi, Italy). Here, the animals were clinically examined, and body temperature (°C) was measured. The presence of tissue cysts ascribable to besnoitiosis was checked in the skin, sclera, and the vulva for the female donkey. The coat and skin of the animals were inspected for the presence of ectoparasites (lice and mites), eventually identified according to morphological characteristics (Taylor et al. 2016). Endoscopy of the upper respiratory tract and the vagina for the female animal was performed using an equine flexible

video-endoscope (Fujinon - DBE EN-450P5/20, Fujifilm, Australia). Bronchoalveolar lavage was also carried out. Each donkey was injected with Detomidine HCl (Medesan, Virbac s.r.l., Italy; 0.2 mg/kg b.w. intravenous) used as a sedative.

Blood samples were collected both in tubes with EDTA and without anticoagulants by puncturing of the jugular vein using a Vacutainer® sterile collection system. Once in the laboratory, sera were separated by centrifugation (2120 g, 15 min) and then stored at −20°C until serological analysis. Skin biopsies were collected by punch biopsy from the region of the neck and hindlimb using a 6-mm disposable punch instrument (GIMA, Italy). Tissue samples were mechanically homogenized and stored at -20°C for subsequent molecular analyses. Fecal samples were collected individually from both donkeys, stored refrigerated at +4° C, and analyzed within 24 hours

After hospital discharge, the two donkeys were regularly revisited for clinical follow-up.

Serology

Western Blot for *Besnoitia* spp. was performed and interpreted as previously described (Villa et al. 2018). Briefly, a total of 4×10^7 *B. besnoiti* tachyzoites under non-reducing conditions were employed for electrophoresis. Tachyzoite antigens were transferred to nitrocellulose membranes and incubated with sera from the two donkeys at a 1:20 dilution, followed by a peroxidase-conjugated anti-donkey IgG (H+L) antibody diluted at 1:1500 (Novus Biologicals Europe, Abingdon, United Kingdom). Positive and negative control sera, both from donkey (Villa et al. 2018) and bovine (Villa et al. 2019), were included. The presence of at least three bands in at least two of the three principal antigenic areas (area I: 72.5, 58.9 and 51.4 kDa; area II: 38.7, 31.8 and 28.5 kDa; area III: 23.6, 19.1, 17.4, 14.5 kDa) was considered as a positive result for *Besnoitia* spp. infection (Garcia-Lunar et al. 2012).

Immunofluorescence antibody tests for other protozoal diseases, including *Babesia caballi*, *Theileria equi*, *Toxoplasma gondii*, *Neospora* spp. and *Leishmania infantum* were also carried out (MegaFLUO®, MEGACOR Diagnostik GmbH, Austria), following the manufacturer's instructions. Cut-off values suggested by the producer were used for all pathogens, except for *L. infantum*, for which a cut-off of 1:40 was applied.

Molecular analysis

Tissue sample homogenates were processed to extract genomic DNA using a commercial kit (NucleoSpin® Tissue, Macherey-Nagel, Germany), following the manufacturer's instructions. DNA samples were analyzed using a conventional PCR targeting a region of 231 bp of the ITS-1 region as described by Cortes et al (2007). Positive and negative (non-template) controls were

inserted in each run: positive samples consisted of DNA extracted from tissues' samples from a bovine besnoitiosis chronically affected dairy cow (Villa et al. 2019). PCR products were run on 1.5% agarose gel containing 0.05% ethidium bromide in TBE buffer electrophoresis and visualized under UV light on a transilluminator. Bands of the expected size were excised from agarose gel, purified with a commercial kit (NucleoSpin® Gel and PCR Clean-up, Macherey-Nagel, Germany) following the manufacturer's instructions, and finally sent for sequencing in both directions to a commercial service (Eurofins Genomics, Germany). Obtained sequences were manually assembled and compared to available *Besnoitia* spp. sequences using BLASTn software (https://www.ncbi.nlm.nih.gov/blast/).

Hematology, biochemistry, and enzyme activity

On blood samples preserved in tubes with EDTA, hematological analyses were performed within 24 hours from the collection time, using the automated laser hematology analyzer ADVIA 120 with multispecies software for veterinary use (Siemens Healthcare Diagnostics, Milan, Italy). The following hematological parameters were included: Red Blood Cells (RBC), Hemoglobin (Hb), Hematocrit (Ht), Mean Corpuscular Volume (MCV), Mean Corpuscular Hemoglobin (MCH), Mean Corpuscular Hemoglobin Concentration (MCHC), White Blood Cells (WBC), Neutrophils, Monocytes, Eosinophils, Basophils, Lymphocytes, Platelet Count (PLT). The leukocyte differential provided by the instrument was checked microscopically on Romanowsky stained blood smears (Dif-stain kit, Titolchimica S.P.A., Rovigo, Italy).

Biochemistry and enzyme activity analyses were carried out on serum with the automated analyzer BT3500 (Biotecnica Instruments S.p.a., Rome, Italy) using reagents, controls and calibrators provided by Futurlab Srl (Limena, Italy). The following analytes were measured (acronyms and methods between brackets): albumin (albumin, bromochresol green), alkaline phosphatase (ALP, kinetic IFCC), aspartate aminotransferase (AST, kinetic IFCC), creatinine (Jaffè), creatine kinase (CK, kinetic IFCC), glucose (GOD-POD), total proteins (modified biuret), urea (urease). Globulin (globulin) concentration was calculated by subtracting albumin from total proteins whereas the albumin:globulin ratio (A/G) by dividing albumin by globulin.

Quantitative copromicroscopic examination

Quantitative coprological examination was performed using FLOTAC Dual Technique. Flotation solutions of Satured Sodium Chloride (Specific gravity 1200) and Zinc Sulphate (Specific gravity 1.350) were used, recommended for the detection of nematodes, cestodes, and trematodes eggs, nematodes larvae, and coccidian oocysts (Cringoli et al. 2010).

Results

The two donkeys referred for suspected besnoitiosis were in poor body condition and presented dull and rough haircoat (Fig. 1.A). Alopecia and hyperkeratosis with skin nodules in the region of the neck (Fig 1.B), hind leg (Fig. 1.C) and on the pinnae (Fig. 1.D) were detected. Skin nodules were solid, consistent, non-fistulizing, and with a size of about 0.5-1 cm. The surrounding skin was clear with no signs of reactive. A few specimens of bloodsucking lice, morphologically identified as *Haemotopinus asini*, were present on the coat of both donkeys. No lesions due to rubbing or scratching were found. Numerous typical scleral pearls in the eyes of both animals were detected (Fig. 1.E). No cysts were revealed by endoscopy in the nares and in mucosa of the larynx and nasopharynx. In the female donkey, no cysts were detected in the vulva and in the vagina neither by visual inspection nor by endoscopy. Body temperature was normal in both donkeys (37.3 and 37.5 °C in male and female animals, respectively).

Fig. 1. A. Two donkeys affected by besnoitiosis showed poor body condition and presented dull and rough haircoat. B. Alopecia and hyperkeratosis on the neck of the female donkey. C. Alopecia and hyperkeratosis on the hind leg of the male donkey. D. Alopecia on the pinnae of the female donkey. E. Numerous typical scleral pearls (indicated by the arrows) in the eye of the male donkey.

Both animals resulted seropositive to *Besnoitia* spp. according to Western Blot results (Fig. 2).

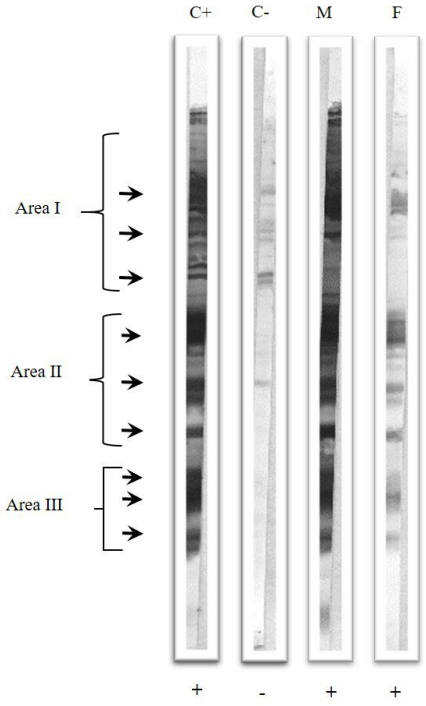

Fig. 2. Pattern of recognition of Besnoitia spp. tachyzoite antigens in serum samples from donkeys affected by besnoitiosis by Western Blot. M = male donkey, F = female donkey, C+ = positive control, C- = negative control.

Skin biopsies collected from both donkeys resulted positive for the presence of parasitic DNA. Sequencing of 231-bp PCR-fragments demonstrated no nucleotide variations between the two sequences (100% identity), and homology of 100% with *Besnoitia* spp. sequences deposited in GenBank. In particular, ITS-1 sequencing evidenced that the T insertion at position 148, reported for *B. bennetti* (Lienard et al. 2018), was not evidenced, suggesting that our isolates may be identified as another *Besnoitia* species, i.e. *B. besnoiti*, *B. caprae* or *B. tarandi*, even if in any case *B. bennetti* could not be discarded (Supplementary File 1). However, a conclusive species identification could not be achieved. The obtained sequence was submitted to GenBank. Therefore, based on both the clinical examination and the results obtained by serology and molecular analysis, the diagnosis of besnoitiosis was confirmed in both donkeys.

Regarding the remaining laboratory tests, the animals were seronegative to *B. caballi*, *T. equi*, *T. gondii*, *Neospora* spp. and *L. infantum*. Hematology revealed light anemia in the male donkey, leukocytosis with eosinophilia, and lymphocytosis in both animals. Biochemistry and enzyme activities evidenced hypoalbuminemia with decreased A/G ratio and elevated ALP values in both donkeys (Tab. 1).

Tab. 1. Results of hematological, biochemical, and enzyme activities analyses of the donkeys.

	Parameter	Male	Female	Unit	Range
Hematology	**RBC**	**5.11**	7.15	$x10^6/\mu L$	6.8-9.3
	WBC	**13.19**	**15.96**	$x10^3/\mu L$	5.5-9
	Hb	**9.7**	13.5	gr/dl	11.3-16.5
	Ht	32.8	42.4	%	32-45
	MCV	**64.2**	**59.3**	μ^3	37-59
	MCH	19.0	18.9	pg	15-19
	MCHC	**29.6**	31.8	g/dl	31-40
	PLT	140	146	$x10^3/\mu L$	90-200
	Neutrophils	35	30	%	30-65
	Lymphocytes	**51**	**50**	%	25-40
	Monocytes	2	2	%	1-3
	Eosinophils	**12**	**18**	%	0-2
	Basophils	0	0	%	0-1
	Neutrophils	4.6	4.8	$x10^3/\mu L$	2.2-8.1
	Lymphocytes	**6.7**	**8**	$x10^3/\mu L$	1.7-5.8
	Monocytes	0.3	0.3	$x10^3/\mu L$	0-1
	Eosinophils	**1.6**	**2.9**	$x10^3/\mu L$	0-0.8
	Basophils	0	0	$x10^3/\mu L$	0-0.3
Biochemistry and Enzyme activitity	**Urea**	41	37	mg/dL	15-45
	Creatinine	0.7	0.7	mg/dL	<1.6
	Glucose	38	54	mg/dL	80-110
	Total protein	5.5	6.9	g/dL	5.5-8
	Albumin	**1.5**	**2.1**	g/dL	2.9-3.6
	Globulin	4	4.8	g/dL	2.6-4.4
	A/G ratio	**0.4**	**0.4**		0.7-1.5
	AST	122	186	U/L	<300
	ALP	**422**	**397**	U/L	<180
	CK	123	134	U/L	<180

Finally, copromicroscopic analyses revealed that both donkeys were highly infected by strongyles (EPG=1716 and 916 in male and female donkey, respectively), *Parascaris equorum* (EPG= 244 and 1012 in male and female donkey, respectively), and *Dictyocaulus arnfieldi* (LPG=16 and 60 in male and female donkey, respectively). Larvae of lungworms were also found in bronchoalveolar lavage. Then, both donkeys were dosed with ivermectin at 200 µg/kg b.w (EQVALAN® Ivermectine Paste, Boehringer Ingelheim Animal Health Italia S.p.A) and their body condition quickly improved.

After hospital discharge, the donkeys were revisited four times, one month apart and regularly every six months. Their body condition was improved; however, seropositivity and all clinical signs of besnoitiosis were still present.

Discussion

In this study, the diagnosis of besnoitiosis in two donkeys was confirmed for the first time in Italy. The diagnosis was achieved by a multidisciplinary approach, based on clinical features and laboratory findings, including serological and molecular analyses. Moreover, blood parameters (hematology, biochemistry and enzyme activity) and coprological examination were also performed.

The two infected donkeys were one year old. According to previous studies, clinical cases were reported both in young donkeys with an age range between one and seven years old (Ness et al., 2012), but the disease was also reported in older animals (Dubey et al. 2005; Lienard et al. 2018; Elsheikha et al. 2020). The animals showed clinical signs typical of the disease, i.e., scleral pearls in the eyes and tissue cysts in the skin, particularly in the region of the neck and the leg and on the pinnae. As previously demonstrated, the sclera and the nares are the most common localization sites for *Besnoitia* spp. lesions in donkeys (Elsheikha et al. 2005, Ness et al. 2014). However, no lesions in the nares were detected. Endoscopy did not reveal lesions in the larynx and nasopharynx; indeed, these lesions were identified only in half of confirmed case animals (Ness et al. 2012). Further, parasitic cysts were not detected in the vulvar mucous membranes, while these signs were described in previous studies (Dubey et al. 2005; Ness et al. 2012). The presence of skin lesions is widely reported in donkeys affected by besnoitiosis, even if the disease severity ranges from mild signs with animals in good condition to more serious clinical forms also leading to compromised health status (Dubey et al. 2005; Elsheikha et al. 2005; Ness et al. 2012, 2014). Only mild clinical signs were evidenced in these animals, probably also due to their young age. The donkeys should be followed-up over time to discern the evolution of the disease.

Both animals resulted seropositive to *Besnoitia* spp. antibodies by Western Blot, whereas they were seronegative to other protozoal diseases (*B. caballi*, *T. equi*, *T. gondii*, *Neospora* spp. and *L. infantum*). Molecular biology confirmed the presence of parasitic DNA in skin biopsies collected from both donkeys and sequencing demonstrated the identity of *Besnoitia* spp.

The donkeys showed some hematological disorders as leukocytosis with eosinophilia and lymphocytosis; besides, the male donkey presented also light anemia. These alterations were previously reported in cases of besnoitiosis in donkeys (Dubey et al. 2005; Lienard et al. 2018).

Interestingly, similar alterations in hematological parameters were reported in *B. besnoiti* infected cattle (Langenmayer et al. 2015; Villa et al. submitted). Besides, the animals showed hypoalbuminemia, probably due to intestinal protein loss but also inflammation, since albumin acts as a negative acute-phase protein. The same alteration was reported in a donkey with besnoitiosis (Dubey et al. 2005), and in clinically affected cows (Villa et al. submitted). Both donkeys also showed elevated ALP values: this finding may be related to liver and gut suffering. Blood parameters may be useful to aid veterinarians in the diagnosis of besnoitiosis in donkeys. However, it should be considered that donkeys were infected by gastrointestinal strongyles, ascarids, and lungworms, and an infestation by bloodsucking lice was evidenced. Indeed, these parasites could influence both the blood parameters and body condition. Donkeys showed a moderate infection by *D. arnfieldi* but they did not exhibit any respiratory clinical signs; according to Matthews et al. (2013), these equids seem to be resistant to lungworm infection in contrast to horses.

Regarding the origin of the infection, the life cycle and transmission modes are not clear for *Besnoitia* species infecting equids. In analogy to besnoitiosis in cattle, it is suspected that insects could act as mechanical vectors for the parasite and direct contact between animals may act a role in the spread of the infection (Dubey et al. 2005; Ness et al. 2012). Particularly, the donkeys involved in the study were born in a herd living in the mountains (lower Val Camonica, Central Alps) and then moved to their current location at the age of nine months. In the origin farm, apart from horses and donkeys, also other animal species were bred, including cattle, sheep, and goats; dogs were also present. Besides, also wild species lived in the area. In this context, donkeys may have come into contact with other infected animals. Further, in both locations the donkeys have been living outside during the day and recovered indoor during the night, then the contact with vector insects could have been possible also because programs for the control of insects were not applied. Finally, it should be emphasized that the current location of the donkeys is distant about 20 kilometers from a dairy herd endemically infected with bovine besnoitiosis and it cannot be excluded that the animals acquired the infection in their current residence (Villa et al. 2019). Indeed, epidemiological data together with molecular results may suggest that the donkeys could have been infected by *B. besnoiti*, the species infecting cattle. Indeed, considering European scenario, *B. caprae* was only reported in goats in Iran (Oryan and Azizi 2008), whereas *B. tarandi* in reindeer in Finland (Dubey et al. 2004). Analogously, *B. besnoiti* etiology was demonstrated by genotyping in a roe deer affected by systemic besnoitiosis (Arnal et al. 2017), thus confirming the possibility for *B. besnoiti* to infect and cause clinical disease in species other than cattle. However, even if *B. bennetti* was not reported in Italy so far, the lack of T insertion at position 148 of ITS-1

sequencing is not sufficient to discard *B. bennetti*, because it could be just a variation of the isolate. For all these reasons, the etiological diagnosis of *B. besnoiti* in these donkeys is just a hypothesis since in this case it was not possible to achieve a conclusive species identification. Then, the diagnosis of besnoitiosis in these donkeys would confirm the circulation of the parasite in the study area. Nonetheless, the possibility for donkeys to get infected with *B. besnoiti* would be of concern for the transmission of besnoitiosis among cattle and equids populations.

Besnoitiosis in equids was previously reported in Europe (Henry and Masson 1922; Zafra et al. 2013). Recently, besnoitiosis was diagnosed in two donkeys in Belgium, and *B. bennetti* was recognized by partial rDNA sequencing (Lienard et al. 2018). In the UK, the infection of *B. bennetti* was confirmed by microsatellite genotyping of DNA isolated from a dermal mass in one out of 20 infected donkeys (Elsheikha et al. 2020). Furthermore, serosurveys for *Besnoitia* spp. infection were performed in equids from southern Europe and donkeys seem to be more affected than horses. In Spain, a seroprevalence of 7.1% was recorded in equids, with a value of 15.3% in donkeys (Gutierrez-Exposito et al. 2017); in Portugal, one horse was seropositive with a prevalence of 0.3% (Waap et al., 2020). In Italy, the circulation of *Besnoitia* spp. infection both in horses and donkeys was recently confirmed: a value of seroprevalence of 2.1% increasing up to 22.2% if considering only donkeys was detected (Villa et al. 2018). As previously underlined (Dubey et al. 2005), parasite antibodies were found in equids without any clinical signs of the disease, suggesting that *Besnoitia* spp. infection could be more spread than realized in USA. Similarly, also in Europe, the presence of anti-*Besnoitia* spp. specific antibodies was reported in apparently healthy horses and donkeys (Gutierrez-Exposito et al. 2017; Villa et al. 2018; Waap et al. 2020).

For this reason, in analogy to bovine besnoitiosis, it should also be considered that infected animals without detectable clinical signs and macroscopic lesions, i.e. subclinically infected animals, could be more frequently found than clinically affected animals. In this way, only a small proportion of seropositive animals develop clinical signs. Instead, a larger subset includes seropositive sub-clinically infected animals without any clinical sign: this category poses a huge risk for parasite transmission, being a source of infection for the other animals (Villa et al. 2019).

Besnoitiosis in equids could be almost as spread as bovine besnoitiosis in Europe. However, due to difficulties in the diagnosis, besnoitiosis could be underdiagnosed and underreported, thus favoring a silent spread of the disease in European equids. Besnoitiosis should be included among differential diagnoses when detecting skin lesions in equids: indeed, ectoparasites, such as lice in this case, can be frequently found, and this initial diagnosis might preclude further investigations for *Besnoitia* spp. infection diagnosis. Besides, other frequently found parasitic infections could

conceal *Besnoitia* spp. infection (e.g., parasites causing poor body condition, parasites in the respiratory tract). A better understanding of the epidemiology of *Besnoitia* spp. infection in the donkey population in Italy and also in Europe would be advisable. It is to be discerned if this case report of clinical besnoitiosis in Italian donkeys is an unusual cluster of infection or may reflect a wider distribution of subclinical infections, largely undetected to date. The wide-spread distribution of *Besnoitia* spp. infection in equids could be of concern not only in Italy but in all Europe. To date, the species implicated in cases of besnoitiosis in southern Europe remains unknown: considering the wide distribution of *B. besnoiti*, for the control of *Besnoitia* spp. infection in equids, it would be advisable to elucidate if the species involved is *B. besnoiti* or *B. bennetti*. Therefore, microsatellite genotyping and whole parasite sequencing should be further performed. Further studies are needed to infer the relevance of besnoitiosis in equids in Europe, both in relation to the seroprevalence, but also to the clinical infection, considering the need to investigate parasite phylogenetic and genomic properties, biology, life cycle, and transmission modes. *Besnoitia* spp. infection may be more common in equids in Italy and Europe than realized since it is scarcely known and then diagnosed by veterinarians, who should be aware of this parasitic disease of equids due to the consequences for the health and well-being of animals.

Conclusions

In the study, the diagnosis of besnoitiosis was achieved in two donkeys in Italy. Both animals showed typical clinical signs, including scleral pearls in the eyes, alopecia and hyperkeratosis with skin nodules on the neck, hind leg, and the pinnae. Anti-*Besnoitia* spp. antibodies were evidenced in both animals by Western Blot and some alterations in hematological and biochemical parameters were detected. Molecular analysis confirmed the presence of parasitic DNA belonging to *Besnoitia* spp. from skin biopsies of both donkeys.

This first clinical case of besnoitiosis in two donkeys in Italy confirms the circulation of *Besnoitia* spp. in Italian equids. Besides, together with other recent clinical and serological studies, it is confirmed the circulation of the parasite in equids in Europe.

Knowledge of clinical features of besnoitiosis in horses and donkeys could assist clinicians in the diagnosis and prevention of the disease, since an early and accurate diagnosis, also considering the absence of vaccines and treatments, is fundamental to implement adequate control measures to prevent a “silent” spread of *Besnoitia* spp. infection in equids populations.

Declarations

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Conflict of Interest Statement

The Authors declare they have no conflict of interest.

Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Alignement of ITS1

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                          10         20         30         40         50         60         70         80         90        100

JF314861 B. besnoiti  TGACATTTAA TAACAATCAA CCCTTGAATC CCTATTACAA CAATAAGCTT GCATCTCTCG TTTCGAGGGG TGCATTCGAG AAGTGTGCTG CCCTCTTGTT
HM008988 B. caprae    .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
AY665400 B. tarandi   .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
MG652473 B. bennetti  .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
JQ013812 B. bennetti  .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
AY665399 B. bennetti  .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
Donkey Italy Female   .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
Donkey Italy Male     .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........

                         110        120        130        140        150        160        170        180        190        200

JF314861 B. besnoiti  GTCATTTTTG ACAACAAGAG CATCGCCTTC TTTTTTTTT- CCAACACCGT TTAACTAAAC CAACGATCTG TTGTTTAGCG GGCGGGGATC CACCTCCTCA
HM008988 B. caprae    .......... .......... .......... .........- .......... .......... .......... .......... .......... ..........
AY665400 B. tarandi   .......... .......... .......... .........- .......... .......... .......... .......... .......... ..........
MG652473 B. bennetti  .......... .......... .......... .........T .......... .......... .......... .......... .......... ..........
JQ013812 B. bennetti  .......... .......... .......... .........T .......... .......... .......... .......... .......... ..........
AY665399 B. bennetti  .......... .......... .......... .........T .......... .......... .......... .......... .......... ..........
Donkey Italy Female   .......... .......... .......... .........- .......... .......... .......... .......... .......... ..........
Donkey Italy Male     .......... .......... .......... .........- .......... .......... .......... .......... .......... ..........

                         210        220        230

JF314861 B. besnoiti  CTCTGCTATC ACGGATTGGT TAATACAAAC C
HM008988 B. caprae    .......... .......... .......... .
AY665400 B. tarandi   .......... .......... .......... .
MG652473 B. bennetti  .......... .......... .......... .
JQ013812 B. bennetti  .......... .......... .......... .
AY665399 B. bennetti  .......... .......... .......... .
Donkey Italy Female   .......... .......... .......... .
Donkey Italy Male     .......... .......... .......... .
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Research Line 2:

Genetic characterization of *Neospora caninum* isolates in cattle and impact of neosporosis on herd performances

Neospora caninum infection in dairy cattle in Italy: serological investigation in two study herds

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Abstract

Neospora caninum is recognized as a major cause of abortion in cattle, determining economic losses to both dairy and beef industries. With the aim of evaluating the spread of neosporosis and its effects on herd efficiency, an epidemiological study was designed in two dairy farms recruited as case-study. Both selected farms, located in Lombardy region, performed genetic improvement of Holstein Friesian, and reported cases of abortions ascribable to *N. caninum*.

Blood samples were collected from 540 animals, including cows and heifers above 24 months, and analyzed by indirect immunofluorescent antibody test. Epidemiological data (individual, reproductive and productive data) were noted.

Overall, 94 animals (17.4%) resulted positive to *N. caninum* (15.5% and 18.5% in Farm 1 and Farm 2), with differences between the farms concerning the antibody titres (Chi-square, p-value = 0.04), particularly in cows (Chi-square, p-value = 0.018). Regarding the episodes of abortions, a different pattern was depicted in the two investigated farms. Data on fertility and production were considered. The number of insemination necessary to get an animal pregnant resulted higher in seropositive animals (2.4 and 2.9) than in seronegative animals (2.1 and 2.4 in Farm 1 and 2, respectively). Similarly, particularly in Farm 1, the number of days in lactation of not-pregnant cows resulted higher in seropositive (167.7) than seronegative animals (133.4). Moreover, although the association between *N. caninum* infection and milk production is still unclear, both the daily production and the mature equivalent milk yield were lower in seropositive (31.02 and 11838.94) than seronegative cows (33.59 and 12274.88) in Farms 1.

The study showed that even if *N. caninum* circulated equally in farms, the dynamics of the parasite infection and its outcome were different. Serological screening by IFAT proved to be a useful diagnostic tool to identify not only herd seroprevalence, but also to identify, through the determination of antibody titre, animals at higher risk of abortion. Further studies are needed to clarify the role of *N. caninum* in alterations of reproductive and productive parameters in cattle.

Keywords: Neosporosis; Dairy cows; Herd efficiency; Serology; Abortion.

Introduction

Neospora caninum, an obligate intracellular protozoan parasite, is the causative agent of neosporosis, primarily a clinical disease of cattle and dogs. Serological evidence in domestic and wild animals indicates that many species were exposed to this parasite (Almería and López-Gatius, 2013).

Domestic dogs and in wild canids (gray wolves, coyotes, and dingoes) are the definitive hosts; various species were reported as intermediate hosts of the parasite, including ruminants and equids (Dubey et al., 2017).

Ruminants get infected via the ingestion of oocysts (horizontal transmission) and transplacentally (vertical transmission) because of a primary infection by oocysts (exogenous transplacental transmission) or by recrudescence of a chronic infection (endogenous transplacental transmission) during pregnancy. *N. caninum* is one of the most efficiently transplacentally transmitted organisms in cattle: indeed, up to 95% of calves are born infected (Dubey et al., 2007). Infection in pregnant ruminants can induce damage to the fetus in the uterus and abortion or produce a still-born calf, a new-born calf with clinical signs or a clinically healthy but infected calf (Dubey et al., 2006).

N. caninum is a major cause of abortion, the main clinical manifestation of bovine neosporosis: worldwide, these abortions are a cause of economic loss (about 2-5% but also up to 20% annually) to both the dairy and beef industries (Goodswen et al., 2013). Cows of any age may abort from 3 months of gestation to term, with most abortions occurring at 5–7 months of gestation (Dubey et al., 2007). Moreover, the parasite also causes reproductive problems, such as stillbirths, early fetal death, and resorption, manifested as return to service, increased time to conception or infertility.

N. caninum infection in cattle was reported from most parts of the world (Dubey et al., 2017); a pooled prevalence of 13% was evidenced in Europe (Ribeiro et al., 2019). In Italy, only few epidemiological studies were conducted in cattle. Seroprevalence values between 16 (Otranto et al., 2003) and 24.3% (Magnino et al., 1999) and between 8.7 (Otranto et al., 2003) and 30% (Rinaldi et al., 2005) were reported in northern and southern Italy, respectively; besides, a serological screening on tank bulk milk reported a farm prevalence of 55% in Sardinia (Varcasia et al., 2006). Moreover, high seroprevalence values were reported in Lombardy region (Sala et al., 2018).

Regarding individual, reproductive, and productive data, it was reported that the risk of testing seropositive to *N. caninum* may increase with the age of cattle; besides, apart the obvious association with bovine abortion, it was suggested that *N. caninum* may cause adverse effects on

fertility in early pregnancy, increased calving interval, and a reduction of milk production, but data are contrasting (Dubey et al., 2017).

With the aim of evaluating the spread of neosporosis and the association between the parasite infection in the farm, the clinical outcome, and the effects on reproductive and productive performances, an epidemiological study was designed in two herds recruited as case-study. Both selected farms, located in Lombardy region, performed genetic improvement of Holstein Friesian, and reported cases of abortions ascribable to *N. caninum*.

Materials and methods

Farms description

2 dairy cattle farms with similar management were selected for the study. Farm 1 and Farm 2 host about 360 (170 lactating cows, 20 dry cows, and 170 heifers) and 530 animals (240 lactating cows, 40 dry cows, and 250 heifers), with a mean daily production at the time of sampling of 37 and 36.5 liters of milk, respectively. Both farms are family-run and grow their own crops to feed the cattle. Besides, both Farm 1 and Farm 2 performed embryo transfer for genetic improvement of Holstein Friesian cattle. Previous analyses evidenced that the selected farms reported cases of abortion due to *N. caninum*; however, no specific control plan for neosporosis was implemented. Both herds used the AfiFarm Software (AfiMilk Ltd., Kibbutz Afikim, Israel) for farm management, allowing a better comparison of data.

Area description

The farms were located in the provinces of Lodi (45°21′17″N 9°22′31″E) and Bergamo (45°38′23″N 9°30′23″E), respectively. Both sites belong geographically to the Po Valley, an area with a high density of cattle farms and one of the largest milk-producing areas in Italy, that stands out for the high zootechnical vocation. Here, dairy farming is mainly based on Italian Holstein Frisian cattle under the intensive production system.

The sites have an average altitude of about 110 m above sea level. The climate is continental, typical of the Po Valley, with hot muggy summer with a few thunderstorms and cold and foggy winter with some snow. It is characterized by a large annual thermal excursion with a mean maximum temperature of 25/28 °C and a mean minimum temperature of -1/-2°C. Rainfall is well distributed throughout the year, particularly in spring and autumn, with an average total annual rainfall of approximately 700-1200 mm.

Sample collection

Overall, 540 cattle, including lactating and dry cows and heifers above 12 months of age, were selected and included in the study. Sample collection was carried out in April 2018. Blood samples were collected in tubes without anticoagulants by puncturing of the tail vein using a Vacutainer® sterile collection system and preserved refrigerated during the transportation to the laboratory within a few hours. Once in the laboratory, sera were separated by centrifugation (2120 g, 15 min) and stored at − 20 °C until serological analysis.

Data collection

Epidemiological data, including individual data and information regarding reproductive and productive parameters, were noted. Data were collected from the farm managerial software. Individual data included breed (Italian Holstein Friesian), sex (only female), age, productive category (heifer and cow) and origin of the animals (born in the farm). Concerning reproductive performances, data on episodes of embryonal reabsorption and abortion, number of lactations, number of inseminations, and days in milking were recorded. Regarding productive parameters, daily kg of milk and 305-mature equivalent milk yield, were noted: this parameter adjusts all cows to the same age, season of calving and lactation length, and also to the different geographic area of the herd (Si@llEvA, Italian Breeder Association, www.sialleva.it).

Serology

Sera samples were analyzed for anti-*N. caninum* antibodies by a commercial immunofluorescence antibody test (MegaScreen® FLUO NEOSPORA caninum, Megacor, Austria), following the manufacturer's instruction. An initial screening dilution of 1:160 was used; then, seropositive samples were serially diluted to determine the end-point antibody titer.

Data analysis

The seroprevalence of *N. caninum* antibodies was calculated considering the farms and the productive categories (heifers and cows) of sampled animals. Chi-square test was used to infer the difference in *N. caninum* seroprevalence considering the two farms, the productive category, the age and the number of lactations, and the antibody titres (considering both the farms and the productive categories). Analysis of risk factors associated with the parasite infection in the two farms was also carried out. For each farm and productive category, univariate generalized linear models (GLMs) with binary logistic distribution were performed. The binary outcome (presence/absence of anti-*N. caninum* antibodies) on the basis of serology results was used as

dependent variable. The following independent variables were considered: reproductive (number of lactations, number of inseminations, days in milking) and productive parameters (daily kg of milk, mature equivalent milk yield). The models were developed through a backward selection procedure (significance level to remove variables from the model = 0.05), based on AIC values. Statistical analysis was performed using SPSS software (Statistical Package for Social Science, IBM SPSS Statistics for Windows, version 25.0., Chicago, IL, USA).

Results

Overall, 540 animals, 340 and 200 from Farm 1 and 2 respectively, were included in the study. All sampled animals were female Holstein Friesian cattle and all of them were born in the farm. Both heifers and cows were included. The mean age of sampled cattle was 42.3 months (SD=20.4, Min-Max=9.8-115.4): in particular, mean age of sampled animals was 39.8 (SD=14.5, Min-Max=22.0-110.1) and 43.1 months (SD=21.8, Min-Max=9.8-115.4) in Farm 1 and 2, respectively.
Detailed serological results and antibody titers of *N. caninum* infection in the sampled farms according to both immunofluorescence antibody test results and the considered categories of animals are reported in Tab. 1.

Tab. 1. Serological prevalence and antibody titers of *Neospora caninum* infection in sampled farms according to both immunofluorescence antibody test results and the considered categories of animals.

Farm	Category	Number	Positive	P %	CI 95%	Antibody titre 1:160	1:320	1:640	>1:1280
Farm 1	Cows	110	21	19.1	11.7-26.4	23.8% (5/21)	28.6% (6/21)	33.3% (7/21)	14.3% (3/21)
	Heifers	90	10	11.1	4.6-19.6	30.0% (3/10)	50.0% (5/10)	20.0% (2/10)	0.0% (0/10)
	Total	200	31	15.5	10.5-20.5	25.8% (8/31)	35.5% (11/31)	29% (9/31)	9.7% (3/31)
Farm 2	Cows	258	51	19.7	14.9-24.6	37.2% (19/51)	47.0% (24/51)	5.8% (3/51)	9.8% (5/51)
	Heifers	82	12	14.6	6.9-22.3	50.0% (6/12)	41.7% (5/12)	8.3% (1/12)	0.0% (0/12)
	Total	340	63	18.5	14.4-22.7	39.7% (25/63)	46.0% (29/63)	6.3% (4/63)	7.9% (5/63)
Total	Cows	368	72	19.6	15.5-23.6	33.3% (24/72)	41.7% (30/72)	13.9% (10/72)	1.1% (8/72)
	Heifers	172	22	12.8	7.8-17.8	40.9% (9/22)	45.5% (10/22)	13.6% (3/22)	0.0% (0/22)
	Total	540	94	17.4	14.2-20.6	35.1% (33/94)	42.5% (40/94)	13.8% (13/94)	8.5% (8/94)

Overall, 94 animals scored positive to *N. caninum* antibodies with a prevalence of 17.4%. Considering the two study farms, similar seroprevalence values were evidenced, with a slightly higher value in Farm 2 (P=18.5%) than in Farm 1 (P=15.5%). In both farms the presence of *N. caninum* antibodies was higher in cows than in heifers; besides, higher mean age (in months, 38.8 and 44.1 in Farm 1 and 43.0 and 45.1 in Farm 2 in seronegative and seropositive cows, respectively) and number of lactations (1.5 and 1.8 in Farm 1 and 2.1 and 2.2 in Farm 2 in seronegative and seropositive cows, respectively) was evidenced in infected animals. Serological prevalence results of parasite infection in sampled farms according to both age and number of lactations are reported in Tab. 2.

Tab. 2. Serological prevalence of *Neospora caninum* infection in sampled farms according to both age and number of lactations.

		Farm 1	Farm 2
Age	**< 2 years**	11.9% (11/92)	17.1% (12/70)
	2-4 years	15.5% (13/84)	15.9% (24/151)
	> 4 years	29.1% (7/24)	22.7% (27/119)
Lactations	**1**	17.7 % (11/62)	19.8% (19/96)
	2	20.0% (7/35)	16.0 (12/75)
	3	14.3% (1/7)	25.5% (12/47)
	4 and more	33.3% (2/6)	23.1% (9/39)

Moreover, the distribution of antibody titers was found to be different between the two study farms, with higher titres in Farm 1 than in Farm 2: indeed, 38.7% and 14.3% of seropositive animals showed an antibody titre higher than 1:640 titer in Farm 1 and Farm 2, respectively (Fig. 1).

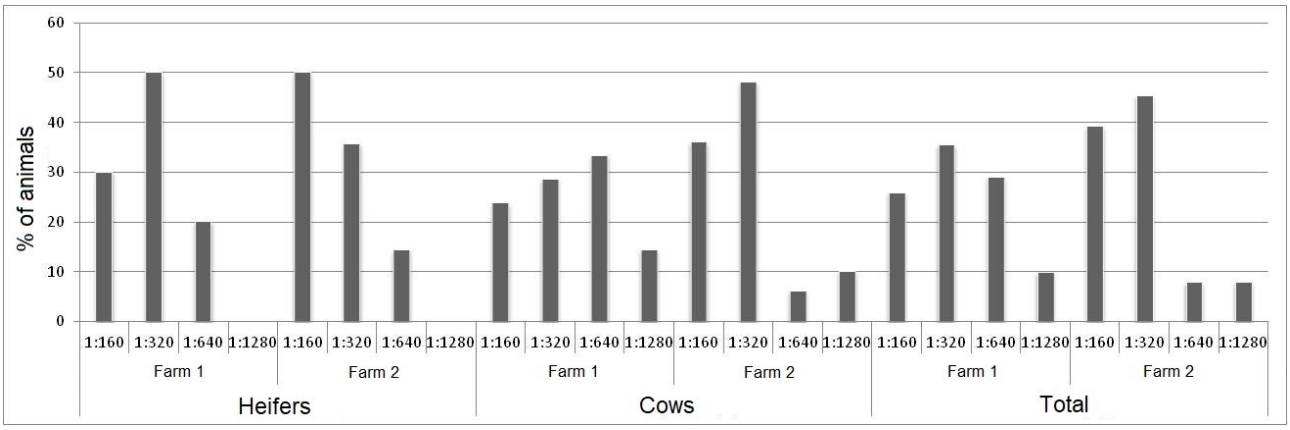

Fig. 1. Distribution of antibody titres in *N. caninum* seropositive animals of sample farm.

Regarding the episodes of abortions, a different pattern was depicted in the two investigated farms. Between January 2017 and April 2018, 14 abortions were recorded in Farm 1, 7 in the first and 7

in the second trimester of pregnancy, respectively, whereas in Farm 2 out of an overall of 26 abortions, 20 occurred in the first, 4 in the second and 2 in the third trimester of pregnancy. 4 and 5 abortions from seropositive cows were recorded in Farm 1 and Farm 2, respectively, corresponding to the 28.5% and 19.2% of all recorded abortions. In Farm 1 all the abortions from seropositive cows occurred in the second trimester of gestation and showed high antibody titers (1:320 and 1:640). Instead, in Farm 2, two cows aborting in the first trimester showed cut-off antibody titre, while the three cows that aborted in the second trimester evidenced antibody titers of 1:160 and 1:320 (Tab. 3).

Tab. 3. Individual, reproductive, and productive data of *N. caninum* seropositive aborting cows.

Farm	ID	Trimester of abortion	Antibody titre	Age	Number of lactations	Number of inseminations	Days in milking	Mature equivalent milk yield
1	**91**	II	1:640	41.0	2	n.d.	n.d.	11165
	122	II	1:320	49.9	2	5	380	12242
	126	II	1:640	38.3	1	2	313	12852
	136	II	1:320	39.9	1	3	321	8732
2	**22**	II	1:320	56.0	3	3	126	11938
	165	II	1:160	30.0	1	n.d.	174	n.d.
	176	I	1:160	97.9	5	1	n.d.	n.d.
	201	II	1:320	42.7	1	6	120	17802
	250	I	1:160	44.7	2	3	n.d.	10802

Finally, some considerations should be underlined regarding the reproductive and productive performances of the cows (Tab. 4).

Tab. 4. Reproductive and productive performances in seronegative versus *N. caninum* seropositive cows in the sampled farm.

	Farm 1		Farm 2	
	Seronegative	**Seropositive**	**Seronegative**	**Seropositive**
Number of inseminations	2.1±1.5	2.4±1.4	2.4±1.9	2.9±1.6
Days in milking	133.4±42	167.7±54.3	219.6±94	221.6±117.7
Daily milk production (Kg)	33.59±8.27	31.02±4.99	39.92±7.68	40.37±8.03
Mature Equivalent Milk Yield	12274.88 ±2434.806	11838.94 ±2337.747	12144.59 ±1851.022	12464.93 ±2029.262

For what concerns reproductive parameters, in both farms the number of insemination necessary to get an animal pregnant resulted higher in seropositive animals (2.4 and 2.9) than in seronegative animals (2.1 and 2.4 in Farm 1 and 2, respectively). Similarly, the number of days in milking of not-pregnant cows resulted higher in seropositive (167.7 and 221.6) than seronegative animals (133.4 and 219.6 in Farm 1 and 2, respectively), particularly in Farm 1. Concerning productive parameters, both the daily production and the mature equivalent milk yield were lower in seropositive (31.02 and 11838.94) than seronegative cows (33.59 and 12274.88) in Farms 1; instead, in Farm 2 both parameters were higher in seropositive animals (40.37 and 12464.93) if compared to seronegative ones (39.92 and 12144.59).

Data analysis revealed that no statistically significant difference was evidenced in the seroprevalence of infection between the two farms, between the production categories, or considering the age and number of lactations of sampled animals (Chi-square, p-value> 0.05). Instead, a statistical significant difference was underlined in the distribution of antibody titers was found between the two study farms, considering both the total number of animals (Chi-square, p-value = 0.04) and also only the cows (Chi-square, p-value = 0.018), whereas there was no significant difference between the only heifers (Chi-square, p-value> 0.05).

Statistical analysis by GLMs did not show any association between *N. caninum* infection and the considered risk factors.

Discussion

In the study, a serological screening of *N. caninum* was performed in two dairy farms in Lombardy. The results showed that the parasite circulates with similar prevalence values in both farms (17.4 and 15.5% in Farm 1 and 2, respectively), but with a different impact from a productive and reproductive point of view, and therefore also economic.

Even if with the limit of differences in sampling and serological techniques, the prevalence values of this study resulted in accordance both with other Italian studies and those of international literature (Dubey et al, 2017), and confirmed the diffusion of the parasite within dairy cattle bred in Italy. Indeed, in Europe a pooled prevalence of 13% was recently reported (Ribeiro et al., 2019), whereas previous studies conducted in northern Italy reported seroprevalence values between 16 (Otranto et al., 2003) and 24.3% (Magnino et al., 1999).

The serological screening of the herd is a valid method for estimating the prevalence of *N. caninum* at both individual and farm level; in addition, the evaluation of the serological status of cattle using IFAT also allows the antibody titration in seropositive animals. It was demonstrated that

seropositive cows are more likely to abort than seronegative cows (Dubey et al., 2017); besides, the risk of abortion increases to the increase in levels of specific anti-*N. caninum* antibodies: therefore, the information regarding the antibody titer of the cows could be a predictive tool to identify cows at increased risk of abortion in infected farms (Quintanilla-Gozalo et al., 2000). Thus, the determination of the antibody titer in the study farms could have an important practical application considering that both widely use embryo transfer: indeed, the antibody titre could help the breeder in the choice of the right animal to implant the embryo.

No differences were found in the seroprevalence values neither between the two farms nor considering the production categories, age, and lactation class; however, *N. caninum* seropositivity was more prevalent in cows than in heifers in both farms, in older animals, with higher mean number of lactations. Indeed, as previously demonstrated, the risk of testing seropositive to *N. caninum* may increase with the age or the number of lactations (Dubey et al., 2017). Moreover, a statistically significant difference in the distribution of antibody titers between the two farms, considering both the total number of animals and the cows only, was evidenced; indeed, in Farm 1 antibody titers resulted higher compared to Farm 2. The similar seroprevalence values indicate that the circulation of the parasite is probably comparable between the two farms, whereas the difference in antibody titres may be due to the existence of different objective conditions between two farms that negatively affect the immune status of cows in Farm 1 where highest titres were found. These conditions could be traced to stressful situations or to a different health status of the herd; it should be also noticed that in Farm 1 milk production was lower than in Farm 2, and in the same farm it was particularly decreased in seropositive cows. A further hypothesis could be the presence of different *N. caninum* strains in the two farms; in fact, differences in the pathogenicity and growth rate for different parasite isolates was demonstrated, influencing also the host's immune response and subsequently the outcome of the infection (Regidor-Cerrillo et al., 2014). Besides, a high level of antibodies could be indicative of high dose infection and/or efficient multiplication of the parasite in the infected host; in the case of a latent infection, a high level of antibodies could also reflect the presence or intensity of recurrence of a current chronic infection (Dubey et al., 2017).

Moreover, the different abortion pattern evidenced in the two study farms may be indicate of *N. caninum* etiology for these episodes particularly in Farm 1: indeed, in this farm, 4 abortions in the second trimester, the period in which most neosporosis abortions occur, were recorded in seropositive cows showing high antibody titres. Instead, in Farm 2, 3 abortions were evidenced in seropositive cows with low antibody titres in the second trimester.

Regarding reproductive performances, in both farms the mean number of inseminations was higher in seropositive cows; besides, an increase in milking days in not-pregnant cows was also evidenced, particularly in Farm 1. Some previous reports demonstrated that *N. caninum* could have an adverse effect on reproductive parameters, i.e., causing increased number of inseminations and increased calving interval; however, in contrast, others did not observe any influence of N. *caninum* infection in early pregnancy (Dubey et al., 2017). Concerning productive parameters, a different trend was evidenced: indeed, the daily milk production and the mature equivalent milk yield resulted lower in seropositive cows in Farm 1, but the same parameters were increased in seropositive animals in Farm 2. However, to date it is not clear whether *N. caninum* may be considered as a cause of decrease in milk production (Dubey et al., 2017).

The evidence of abortions in the second trimester in seropositive cows with high antibody titre and the effects on reproductive, i.e., increased number of inseminations and days in milking, and subsequently on productive parameters, suggest that in Farm 1 *N. caninum* infection may be endemic, leading problems to the herd economy. Instead, in Farm 2, the main problem seems to be linked to early fetal death, to which other infectious or managerial causes may contribute. In fact, the minor percentage of abortion episodes recorded in the second trimester was evidenced in seropositive cows with low antibody titres; besides, even if the number of inseminations resulted higher in seropositive cows, no alterations were detected in productive parameters. Other variables contributing to the differences in the two study farms may be the genetic characteristics of the parasite, the intensity of horizontal transmission, and all those managerial features (i.e., feeding, lame management, milking system) that could affected animal health and welfare.

Conclusions

In conclusion, the study showed that although *N. caninum* circulates equally in farms, the dynamics of the parasite infection and its outcome were different. Using serological methods, the prevalence of *N. caninum*, both at individual and farm level, was determined. Neosporosis should always be considered in dairy farms devoted to genetic improvement, regularly testing animals before fecundation or embryo transfer. Serological screening by IFAT proved to be a useful diagnostic tool to identify not only herd seroprevalence, but also to identify, through the determination of antibody titre, animals at higher risk of abortion. Direct and indirect costs due to reproductive failure represent the main economic impact of neosporosis in cattle farms. However, further studies conducted on more animals from different farms are needed to clarify the role of *N. caninum* in alterations of reproductive and productive parameters in cattle.

Spatial distance between sites of sampling associated with genetic variation among *Neospora caninum* in aborted bovine foetuses from northern Italy

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Abstract

Neospora caninum, a coccidian protozoan, represents an important cause of bovine abortion. While only a single genotype of *N. caninum* exists word-wide, available *N. caninum* strains show considerable variation *in vitro* and *in vivo*, including different virulence in cattle. To which extent sexual recombination, possible in the intestines of domestic dogs and closely related carnivores as definitive hosts, contribute to this variation is not clear, yet.

Aborted bovine foetuses were collected between 2015 and early 2019 from Italian Holstein Friesian dairy herds suffering from reproductive problems. A total of 198 samples were collected from 165 intensive farms located in Lombardy, northern Italy. *N. caninum* samples were subjected to multilocus-microsatellite genotyping (MLMG) using ten previously established microsatellite markers. In addition to own data, those from a recent study providing data on five markers from other northern Italian regions were included and analyzed.

Of the 55 samples finally subjected to MLMG, 35 were typed at all or 9 out of 10 loci. Linear regression revealed a statistically significant association between the spatial distance of the sampling sites with the genetic distance of *N. caninum* MLMGs ($P< 0.001$). Including data from a previous North Italian study (eBURST analysis) revealed that part of *N. caninum* MLMGs from northern Italy separate into four groups; most of the samples from Lombardy clustered in one of these groups. Principle component analysis revealed similar clusters and confirmed MLMG groups identified by eBURST. Variations observed between MLMGs were not equally distributed over all loci, but predominantly observed in MS7, MS6A, or MS10.

Our findings confirm the concept of local *N. caninum* subpopulations. The geographic distance of sampling was associated with the genetic distance as determined by MLMGs. Results suggest that multi-sexual recombination in *N. caninum* is a rare event, but does not exclude uniparental mating. More comprehensive studies on microsatellites in *N. caninum* and related species like *T. gondii* should be undertaken, not only to improve genotyping capabilities, but also to understand possible functions of these regions in the genomes of these parasites.

Keywords: Neospora caninum; Microsatellite typing; Multilocus genotyping; Bovine abortion; Holstein Friesian cattle; Italy.

Introduction

Neospora caninum is a protozoan coccidian parasite closely related to *Toxoplasma gondii* and *Besnoitia besnoiti*. It has a worldwide distribution and causes foetal losses or stillbirth in livestock, especially cattle (Dubey et al., 2017; Dubey et al., 2007). Domestic dogs and other phylogenetically closely related wild carnivores like coyotes, wolves, or dingoes are the only known definitive host of *N. caninum* (Dubey et al., 2017; Dubey et al., 2011; Gondim et al., 2004; King et al., 2010), i.e. hosts in which sexual recombination can take place. The main mode of transmission in cattle, the main intermediate host of the parasite, seems to be endogenous vertical transmission (Dubey et al., 2017; Dubey et al., 2007), i.e. transmission from a persistently or chronically infected dam to her offspring (Trees and Williams, 2005). Exogenous vertical transmission to a foetus or an unborn calf occurs in cattle that became infected during pregnancy after the ingestion of oocysts shed by a definitive host (McCann et al., 2007; Trees and Williams, 2005). Transmission via oocysts by definitive hosts, most likely farm dogs, seems to occur frequently in cattle populations. These infections are the likely cause of epidemic abortions in bovine herds (McAllister et al., 2005). Such abortion storms were reported to be associated with both (i) low avidity, i.e. a recently established antibody response against *N. caninum* (McAllister et al., 2005; Schares et al., 2002) and (ii) an identical microsatellite genotyping pattern in *N. caninum* detected in several foetuses of a single herd (Basso et al., 2010).

A recent study on the genomes of 50 *N. caninum* isolates collected worldwide from a wide range of hosts using 19 linked and unlinked genetic markers showed that there is only a single genotype of *N. caninum* worldwide (Khan et al., 2019). Moreover, whole genome sequencing of seven isolates from two continents revealed less than 10^4 bi-allelic single nucleotide polymorphisms (SNPs), which is very little compared to the situation in the genome of the closely related apicomplexan parasite *T. gondii* with more than 10^6 SNPs between the compared strains (Khan et al., 2019). Since more than 50% of the SNP clustered in 6 haploblocks which had been already partially observed in a former study (Calarco et al., 2018), it was concluded that unisexual reproduction together with a non-sexual expansion formed the actual world-wide *N. caninum* population rather than non-sexual expansion alone (Khan et al., 2019). The extremely limited genetic diversity was explained by genetic bottlenecks during the domestication of cattle in the Near East probably about 10 K years ago (Khan et al., 2019). Breeding and moving specific cattle breeds including the Holstein Friesian breed examined here during the following centuries may also have contributed (Felius et al., 2014). The domestication of dogs may have formed another bottleneck, which shaped the *N. caninum* population that exists today (Khan et al., 2019).

Nevertheless, *N. caninum* is less uniform than the previously mentioned study (Khan et al., 2019) suggests. In fact, different *N. caninum* isolates show large differences *in vitro* (Dellarupe et al., 2014b; Jimenez-Pelayo et al., 2017; Regidor-Cerrillo et al., 2011) and *in vivo* (Dellarupe et al., 2014a; Regidor-Cerrillo et al., 2010; Rojo-Montejo et al., 2009b), also in cattle (Chryssafidis et al., 2014; Jimenez-Pelayo et al., 2019; Regidor-Cerrillo et al., 2014; Rojo-Montejo et al., 2009a). The pioneering work of Regidor et al. (Regidor-Cerrillo et al., 2006) contributed a number of microsatellite markers that allow to fingerprint *N. caninum* isolates or DNAs and undertake population studies.

We aimed at genotyping *N. caninum* in aborted bovine foetuses from Lombardy, one of the most important dairy cattle production areas in Italy (Zucali et al., 2017). In this region, where the Italian Holstein Friesian breed prevails, *N. caninum* was recognized as an important cause of abortion; however, data are unpublished, yet (MTM, unpublished data). Moreover, a high *N. caninum* seroprevalence was revealed in cattle from northern Italy (Otranto et al., 2003; Sala et al., 2018). We therefore determined the proportion of *N. caninum* PCR-positive aborted foetuses in this area and characterised the available isolates by multi-locus microsatellite genotyping.

A previous world-wide study (Regidor-Cerrillo et al., 2013), a South-American study (Cabrera et al., 2019), a local study from Spain (Pedraza-Diaz et al., 2009), and a recent study conducted in northern Italy (Regidor-Cerrillo et al., 2020) suggested the existence of *N. caninum* sub-populations specific for particular countries or regions. The North-Italian study conducted predominantly in two areas (Piedmont and Veneto-Trento) close to our study area, suggested at least three sub-populations. We aimed to find out, how *N. caninum* isolates in aborted foetuses from Lombardy fit into the pattern of these sub-populations. To understand the reasons for the genetic differences, we used detailed geographic information on the sites, where *N. caninum*-positive foetal material had been sampled.

Materials and Methods

Study area

All farms, from which aborted foetuses had been sampled, were located in the “Bassa Padana” in the Po valley. This area comprises the territory of the provinces of Lodi, Cremona, Mantova, Pavia, and the south of the provinces of Milano and Brescia (Figure 1). This is one of the largest dairy production areas in Italy and stands out for the high density of cattle farms, mainly based on Italian Holstein Friesian under the intensive production system. In particular, the Italian National Zootechnical Registry counts 5446 dairy farms hosting 1,071,164 animals in the

Lombardy region, corresponding to the 20.7% and 40.8% of all farms and animals in Italy, with an annual production of 5,215,408 tons of milk (http://www.assolatte.it/zpublish/4/uploads/4/news_down/15641362883148578112_RAPPORTO%20ASSOLATTE%202018.pdf; last access 06.08.2020). Most farms in the area host between 100 and 500 animals, but several farms hold more than 500 cattle (National Zootechnical Database, https://www.vetinfo.sanita.it; last access 06.08.2020).

The sites have an average altitude of about 69 m above sea level. The climate is continental, typical of the Po valley, with hot muggy summers with a few thunderstorms and cold and foggy winters with some snow. There is a large annual thermal excursion with a mean maximum temperature of 25-28 °C and a mean minimum temperature of -1 - -2°C. Rainfall is distributed over the year with peaks in spring and autumn. The average total annual rainfall ranges between 700 and 1200 mm.

Sample collection

Aborted bovine foetuses were collected between 2015 and early 2019 from Italian Holstein Friesian dairy herds suffering from reproductive problems. Overall, 198 samples were collected from 165 intensive farms located in the Po valley (Provinces of Cremona, Lodi, Mantova, and Milano). Abortion events had been notified by the farm veterinarian, foetuses were maintained refrigerated and collected directly from the farms or submitted to the laboratory. In the laboratory, a pool of organs was prepared from each aborted foetus, which included brain, lung, and liver. This pool was mechanically homogenized and stored at -20°C until molecular analysis. The geographical localization (municipality and province as well as the geographic coordinates) of each farm with abortion episodes was recorded along with the data for each aborted foetus.

DNA extraction

Genomic DNA was extracted from tissue homogenates of pooled target organs (brain, lung, liver) of aborted fetuses using a commercial kit (NucleoSpin® Tissue, Macherey-Nagel, Düren, Germany), according to the manufacturer's instructions. To optimize DNA extraction from aborted fetuses, a 200 µl aliquot of the homogenate was pre-lysed with buffer T1 and proteinase K as recommended for the NucleoSpin Tissue Kit, by scaling up eight times the volumes used for the initial digestion. After digestion (56 °C, overnight), 230 µl of the final suspension (1840 µl) were taken and the protocol of the NucleoSpin Tissue kit followed as recommended. A negative extraction control was included in each batch of approximately twenty-four samples, which was subsequently tested by real-time PCR in the same way as the samples from aborted fetuses were analyzed.

Screening N. caninum *RT-PCR*

Fetal DNA samples were initially subjected to a *N. caninum*-specific RT-qPCR with the primers and probes targeting the Nc5 gene as previously described (Constantin et al., 2011; Legnani et al., 2016). Primers were used at a final concentration of 600 nM and the fluorogenic probe (NeoProbe) at 100 nM. Ten µl of 2 x iQ Supermix (Bio-Rad Laboratories GmbH, Feldkirchen, Germany) were added to each reaction. For PCR analysis, 5 µl of DNA from each sample was used in a 20 µl reaction on 96-wells reaction plate. The thermal cycling protocol consisted of an initial polymerase activation and DNA denaturation at 95 °C for 5 min, followed by 46 cycles of amplification including denaturation at 95 °C for 10 s and annealing/extension at 58 °C for 30 s. NC-1 (Dubey et al., 1988) was included as a positive control, while water PCR Reagent (Sigma–Aldrich) was used as a negative control; negative extraction controls were also included.

Microsatellite typing

Samples confirmed as positive for *N. caninum* were subjected to multilocus-microsatellite typing using ten microsatellite markers (MS1B, 2, 3, 5, 6A, 6B, 7, 10, 12, and 21). Nested-PCR (n-PCR) techniques were used for the amplification of microsatellite-containing regions *N. caninum* DNA from aborted fetuses. Microsatellites MS2 and MS10 were amplified and sequenced using primers and protocols previously described (Basso et al., 2009). The microsatellites MS1B, 3, 5, 6A, 6B, 7, 12, and 21 were analyzed by nested-PCR and fragment lengths determined by capillary electrophoresis (Basso et al., 2010). Forward primers of the internal PCRs were labeled with either 6-FAM, HEX, or NED dyes to allow the simultaneous length determination of three different microsatellites per capillary electrophoresis. In addition, previously sequenced microsatellite-containing fragments from the *in vitro*-grown *N. caninum* NC-1 strain (Dubey et al., 1998) were included in the amplifications, the sequencing, and sizing analyses as a positive control.

For all n-PCRs, primers were used at a final concentration of 0.5 µM, and dNTPs at 250 µM each (Amershan Biosciences, Piscataway, USA). DyNAzyme II DNA polymerase (Finnzymes, Espoo, Finland) was added at 1 U/25 µl with the provided buffer. The reaction mix was supplemented with bovine serum albumin at a concentration of 20 µg/ml. 1.5 µl of genomic DNA or 1 µl of amplification product were used as template for the external and internal PCR amplification steps, respectively. Water PCR Reagent (Sigma–Aldrich) was used as a negative control. DNA from cell culture-derived *N. caninum* tachyzoites (NC-1) was used as a positive control. The PCR reactions were performed in a thermal cycler (Eppendorf Mastercycler, Personal Thermal Cycler). The external PCR was performed with an initial denaturation step of 94 °C for 5 min, followed by 35 cycles of denaturation (1 min at 94 °C), annealing (1 min at 60 °C for all MS, except for MS2: 50

°C, and MS10: 54 °C) and extension (1 min at 72 °C), and a final extension step at 72 °C for 10 min. The internal PCR consisted of an initial denaturation of 94 °C for 5 min, followed by 35 cycles of denaturation: 1 min at 94 °C, annealing: 1 min at 60 °C (MS2, 10) or 30 s at 52 °C (MS1B, 3, 5, 6A, 21), 54 °C (MS6B, 12) or 60 °C (MS7) and extension: 1 min at 72 °C (MS2, 10), 30 s at 70 °C (MS1B, 3, 5, 6A, 6B, 12, 21) or 65 °C (MS7), and a final extension step at 72 ° C (MS2,10), 70 °C (MS1B, 3, 5, 6A, 6B, 12, 21) or 65 °C (MS7), for 10 min.

The amplification products were visualized after electrophoresis in 2% agarose gels stained with ethidium bromide using a 100 bp DNA ladder (Invitrogen GmbH, Karlsruhe, Germany) as a size standard.

For sequencing MS2 and MS10, bands of the expected size were excised from the agarose gels and purified with a commercial kit (NucleoSpin® Gel and PCR Clean-up, Macherey-Nagel, Düren, Germany), following the manufacturer's instructions. Purified amplification products were then cloned into a commercially available vector (pGEM®-T Easy Vector System I, Promega, Mannheim, Germany) and used to transform chemically competent *Escherichia coli* (OneShot TOP10, Thermo Fisher Scientific, Langenselbold, Germany). The transformed *E. coli* were cultivated, and the plasmid DNA was subsequently collected using a commercial kit (QIAprep Spin Miniprep Kit, Qiagen, Hilden, Germany), according to the manufacturer's instruction. Sequencing was finally performed using the BigDye Terminator v1.1 Cycle Seq. Kit (Thermo Fisher Scientific, Langenselbold, Germany) and passage with NucleoSEQ Columns (Macherey-Nagel, Düren, Germany) for cleaning up nucleic acids, in an ABI 3130 capillary sequencer (Thermo Fisher Scientific, Langenselbold, Germany).

For all other MS, *N. caninum*-positive samples were analyzed on an ABI 3130 capillary sequencer (Thermo Fisher Scientific, Langenselbold, Germany). A ROX dye-labeled standard (GeneScan™ 500 ROX™ dye Size Standard, Thermo Fisher Scientific, Langenselbold, Germany) was included in each analysis as a size reference. The results were analyzed using the Geneious 1.11.5 software (Biomatters, Inc., 2365 Northside Dr., Suite 560, San Diego, CA 92108, USA).

Statistical analysis

Differences in *N. caninum* specific RT-PCR results (Ct values) in relation to the number of microsatellite markers typed were assessed using the command "wilcox.test" from the package "stats" in R, version 3.5.3 (R Foundation for Statistical Computing, Vienna, Austria; http://www.R-project.org). Microsatellite marker-specific differences in the number of repeats between *N. caninum* isolates from different regions were assessed using the command

"pairwise.wilcox.test" from the package "stats" in R, version 3.5.3 "BH adjusted" option due to multiple testing (Benjamini and Hochberg, 1995).

Pairwise Bruvo's genetic distance was calculated using MLMG data of all DNAs from aborted fetuses in Lombardy, for which a complete MLMG pattern was obtained (n=20). Since MS10 and MS2 were combined microsatellite markers, which consist of three sub-loci (or motifs) each, these sub-loci were analyzed separately in a dendrogram. Calculations were done by R, version 3.5.3., employing Poppr, version 2, a R package designed for the analysis of populations with mixed modes of reproduction (Kamvar et al., 2014), by using the command "bruvo.dist". To obtain an overview of the relatedness of MLMGs a dendrogram was establishes using Poppr representing the results of K-means clustering using Bruvo's Distance with non-parametric bootstrapping.

For testing of a standardized index of association (I_A^S) by multilocus linkage disequilibrium (LD) among different genotypes including nine loci the LIAN v 3.7 web interface program (http://guanine.evolbio.mpg.de/cgi-bin/lian/lian.cgi.pl/query) was applied as described (Haubold and Hudson, 2000; Regidor-Cerrillo et al., 2013).

Testing population differentiation was done by likelihood ratio G-statistic (Goudet, 2005), using the function "test.between" from the R-package "HierFstat" (de Meeûs and Goudet, 2007).

Geographic distance based on the geographic coordinates of the farms were calculated (straight-line distance, accounting for curvature of the earth) using the command "spDists" of the package sp, version 1.4-2, in R. To determine the relatedness of Bruvo's distance, geographic distance and the time between samplings linear regression was performed using the "lm" command in R, version 3.5.3 from the package "stats".

The eBURST software (Feil et al., 2004) was used to generate networks based on MLMGs using the double-locus option (DVL – at least 7 shared loci of 9). Samples with missing data were excluded from the analysis. As the eBURST analysis was performed on samples from this study and those from a previous study, the analysis was restricted to those microsatellite loci that were used in both studies. Since MS10 is a combined microsatellite marker, which consists of three sub-loci (or motifs), these sub-loci were analysed separately. The same data (MLMGs data collected in the present study on foetuses from Lombardy and data from a previous study) were also analysed by a Principal Coordinates Analysis (PCoA) using the command "dudi.pco" employing the R package Poppr, version 2.

Figures were assembled using R, version 3.5.3 or 4.0.0 (packages ggplot2, reshape and scales).

Results

Overall N. caninum *DNA findings in aborted foetuses*

One hundred and ninety-eight aborted bovine fetuses from 165 farms located in the Po valley of Lombardy, i.e. the provinces of Cremona, Lodi, Mantova, and Milano, were included in the study. Out of 198 aborted fetuses 55 were positive for *N. caninum* by RT-PCR, yielding a prevalence of 27.8% (presence of the parasite in pooled tissue homogenates of brain, lung, and liver); 43 farms recorded at least one positive fetus (26.1%). Overall, 55 samples from 43 herds of fetuses collected from 2015 to early 2019 were subjected to microsatellite typing.

Relationship between real time PCR results and success in genotyping

The majority of the 55 DNA samples subjected to multilocus microsatellite typing were typed at all 10 (n=20), or at 9 (n=15) loci. Nine DNA samples could be typed at 8 (n=2), 7 (n=2), or 6 (n=6) loci. The remaining 10 DNAs could only be typed at 5 (n=3), 4 (n=3), 3 (n=2), 2 (n=1), or 1 (n=1) of the loci.

The numbers of genotyped loci were related to the amount of parasitic DNA in the sample as reflected by the Ct values determined by real time PCR (Table 1).

Table 1. *N. caninum* DNA content in samples of aborted foetuses and success in genotyping.

Typing success, # of loci typed	**Ct value, Mean +/- Standard deviation**	**Minimum – Maximum Ct**	***P* value, Wilcoxon Rank sum test to assess difference in Ct values**
10 or 9	30.4 +/- 3.2	26.2 – 42.4	Reference
8 – 1	33.0 +/- 1.8	29.2 – 36.5	$P = 0.00013$

Microsatellite typing on individual farms at different time points revealed differences in the MLMG results

In seven farms, more than one fetus had been sampled and detected *N. caninum* DNA typed in the study period from 2015 – 2018. Most of the differences in typing comprised of only one or two repeat units in MLMG (Table 2). However, in five farms, more prominent differences per locus (i.e. differences of 3 or even more repeat units per locus) were observed. In herd 6, i.e. the farm with the largest number of fetuses analysed, 48.1% of the comparisons of individual loci revealed differences (Table 2, Table 3). These more prominent differences affected the loci MS7 and MS21 (Chromosome 7a), MS10 (MS10.2, Chromosome 8), and MS6A (Chromosome 10) (Table 3).

Table 2. Comparison of the MLMG patterns in dairy cattle farms in northern Italy, where more than one *Neospora caninum* positive foetus had been sampled and genotyped during the study period.

Farm #	**Number of fetuses per farm (period)**	**Number of pairs of loci or sub-loci with differences of…**				**Differences/total number of comparisons (%)**
		1 repeat	2 repeats	3 repeats	>3 repeats	
4	2 (2015)	4	-	-	-	3/14 (21.4)
6[a]	4 (2015-16)	30	6	3	-	39/81 (48.1)
9	2 (2015)	1	1	-	-	2/3 (66.7)
10[a]	2 (2016)	2	-	-	1	3/10 (30.0)
15[a]	2 (2017)	2	-	-	1	3/11 (27.3)
17[a]	3 (2017-18)	4	-	-	2	6/13 (46.2)
25	2 (2017-18)	2	1	-	-	3/14 (21.4)
35[a]	2 (2018)	1	-	-	1	2/5 (40.0)

[a] Table 3 provides details on microsatellite typing results for farms, where loci with 3 or > 3 repeats were observed.

Table 3. MLMG patterns on dairy cattle farms from northern Italy where more than one *Neospora caninum*-positive foetus had been sampled during the study period, which showed differences (bold numbers) in length for ≥3 repeats at microsatellite loci on various chromosomes (Chr2, 7a, 8, 9, 10, 12).

Farm	**Date**	**Chr II**	**Chr VIIa**			**Chr VIII**	**Chr IX**	**Chr X**		**Chr XII**	
		MS2, x-y-z, *(AT)*x-TTGTATC-*(AT)*y-GT-*(AT)*z	**MS1B**, x=y+z, *(AT)*y-AC-*(AT)*z	**MS7,** x, AT-AA-$(TA)_X$-GG	**MS21,** x=y+4, TG-$(TACA)_3$-TACC-$(TACA)_y$TT	**MS10,** x-y-z, AGT-$(ACT)_X$-$(AGA)_Y$-$(TGA)_Z$-CAA	**MS5,** x, CG-$(TA)_X$-TGTAGG	**MS6A**, x, GC-$(TA)_X$-AC	**MS6B**, x, CC-$(AT)_X$-GT	**MS12,** x, GC-$(GT)_X$-GC	**MS3**, x, GC-*(AT)*x-AA
6	May 2015	6-8-2*	12	15	6	6-23-10	11	**22**	13	17	13
	February 2016	7-10-2	12	15	6	6-25-10	11	**25**	13	16	12
	April 2016	6-11-2	13	13	NA	5-25-10	11	**20**	14	16	11
	June 2016	6-11-2	12	14	6	6-24-9	11	**22**	13	16	12
10	January 2016	5-10-2	12	15	**3**	NA	11	21	13	16	12
	April 2016	6-11-2	12	15	**6**	6-27-10	11	21	13	NA	12
15	August 2017	6-11-2	12	15	6	6-**19**-10	NA	NA	NA	16	12
	August 2017	6-11-2	12	14	6	6-**28**-9	11	18	13	16	12
17	May 2017	6-10-2	NA	**14**	6	NA	11	NA	NA	17	12
	November 2017	6-9-2	12	**15**	6	7-24-12	11	**21**	13	16	12
	February 2018	NA	12	**10**	6	NA	NA	**13**	12	NA	NA
35	July 2018	6-11-2	12	15	6	NA	11	**19**	13	16	12
	October 2018	NA	NA	15	6	NA	12	**13**	NA	NA	12

Relationship between the spatial distance of sampling sites and the genetic distance of N. caninum isolates

For n=20 of the *N. caninum* positive samples of bovine foetuses from Lombardy, which had been typed at the complete set of microsatellites loci (MS1B, 2, 3, 5, 6A, 6B, 7, 10, 12 and 21, this study), pairwise Bruvo's genetic distances were calculated. Bruvo's genetic distance and the geographical distance between sampling sites of the individual *N. caninum*-positive foetuses (i.e. based on the geographical coordinates of 17 farms) were tested for a correlation. Linear regression revealed that genetic Bruvo's distance correlated statistically significantly with the geographical distance between the sampling sites (Fig. 1a; Table 4). The model (Model 1, Table 4) had an adjusted R^2 of 19.5%. Including the number of days between the sampling of foetuses into the model did not improve the model significantly (Model 2, Table 4). Bruvo's distance with non-parametric bootstrapping allowed the identification of at least four groups, separated by bootstrap values >50 (Fig. 1b). While the farms, from which the largest group of samples originated from (Fig. 1b , red), were located with two exceptions central-north in the Cremona district (Fig. 1c, red), the farms, where the remaining samples had been derived from, were located in the far north (Fig. 1 c, green), in the south of the Cremona district (Fig. 1c, blue), or in the neighbouring district of Mantova (Fig. 1c, yellow).

Table 4. A linear regression model to characterize the association between genetic distance (Bruvos's distance) of *N. caninum* isolates and the spatial distance of the sampling sites. The model had an adjusted R^2 of 19.8%.

Model (adjusted R^2)	**Variable**	**Estimate**	**Standard error**	**T value**	**Pr(>|t|)**
1 (19.5%)	Intercept	0.2378241	0.0100212	23.732	<2e-16 ***
	Geographical distance (km)	0.0025708	0.0002615	9.831	<2e-16 ***
2 (19.8%)	Intercept	0.2502685	0.0143581	17.43	<2e-16 ***
	Geographical distance (km)	0.0026139	0.0002638	9.91	<2e-16 ***
	Time between dates of sampling	0.0013088	0.0010820	-1.21	0.227

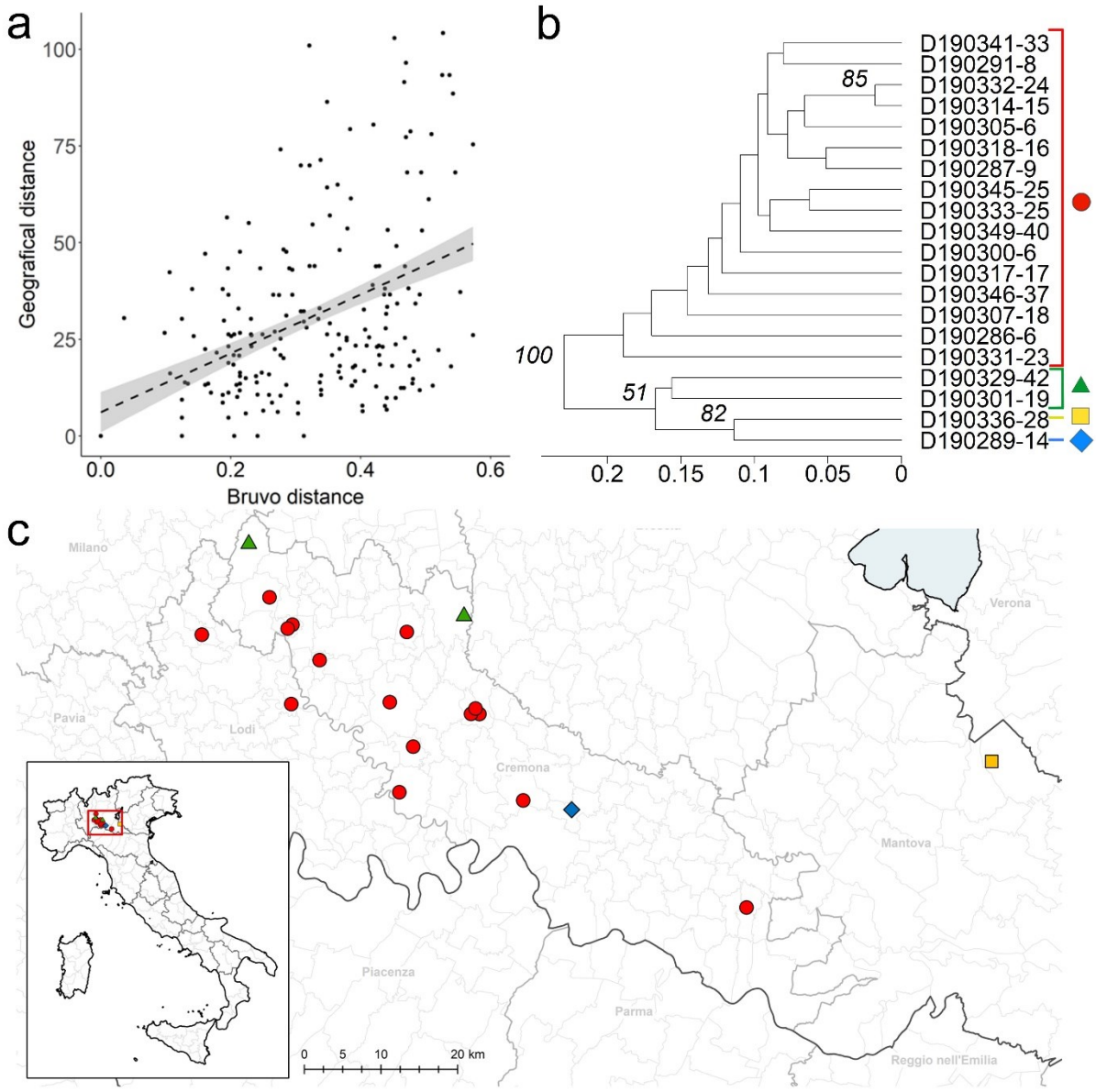

Fig 1. Genetic distance between *N. caninum* isolates from bovine foetuses collected in Lombardy, northern Italy, is associated with the geographical distance of the sampling sites. (a) Graphical representation of a linear regression model to associate genetic Bruvo's distance of *N. caninum* in bovine foetuses and spatial distance of sampling sites (km). (b) Dendrogram representing the results of K-means clustering using Bruvo's distance with non-parametric bootstrapping; the analysis identified at least four groups (different colours of sample designation, consisting of a string of sample ID, village name, and farm number), separated by bootstrap values > 50. (c) Map showing farm locations, using the same colours as for the groups in the dendrogram.

An eBURST and PCoA analysis reveal different groups or clusters of N. caninum *MLMGs in northern Italy*

In addition to the microsatellite data established in this study (Lombardy; n=25), further data on n=50 *N. caninum isolates* from northern Italy covering mainly other regions than Lombardy (i.e., Piedmont n=17, Veneto-Trento n=6, including also one goat isolate), Lombardy (n=1), and a further North-Italian bovine *N. caninum* isolate (n=1) were analysed. These additional data were available from a recent study (Regidor-Cerrillo et al., 2020). Since the latter study had employed a set of microsatellite markers that overlapped only partially with the one we used, our analysis was restricted to MS6A, MS6B, MS10, MS12 and MS21, for which data were available from both groups. Linkage disequilibrium (LD) was assessed for the entire population excluding the goat sample; results (I_A^S=0.0411, V_D=1.8249, L=15508, *P*=0.0411) indicated LD because V_D > L (Haubold and Hudson, 2000).

Network analysis using eBURST with double locus variation (DLV) among 6 loci and 3 MS10 motifs or sub-loci (MS10.1-3) revealed that many of *N. caninum* MLMGs from Lombardy (n=11/25; this study) clustered separately (eBURST G4) from those obtained from Piedmont (n=10/17, eBURST G1, G2, G3) or Veneto-Trento (n=3/6, eBURST G1, G2) (Fig. 2a). The grouping of isolates from Piedmont and Veneto-Trento had already been assessed by others (Regidor-Cerrillo et al., 2020). This previous grouping of particular *N. caninum* isolates into the eBURST groups G1, G2 and G3 matched perfectly (n=10/10) with our grouping (Table S1). Genotyping of an Italian isolate, for which the province of origin was not known (violet dot) revealed results close to most isolates from Lombardy (group G4). The full representation of the MLMST network using the MST option in eBURST, including the complete data set (n=49 *N. caninum* samples), shows the G4 samples in the centre with most of the Veneto-Trento samples on the right side (G1) and the Piedmont samples both left (G2, G3) and right (G1) in the network (Fig. 2b).

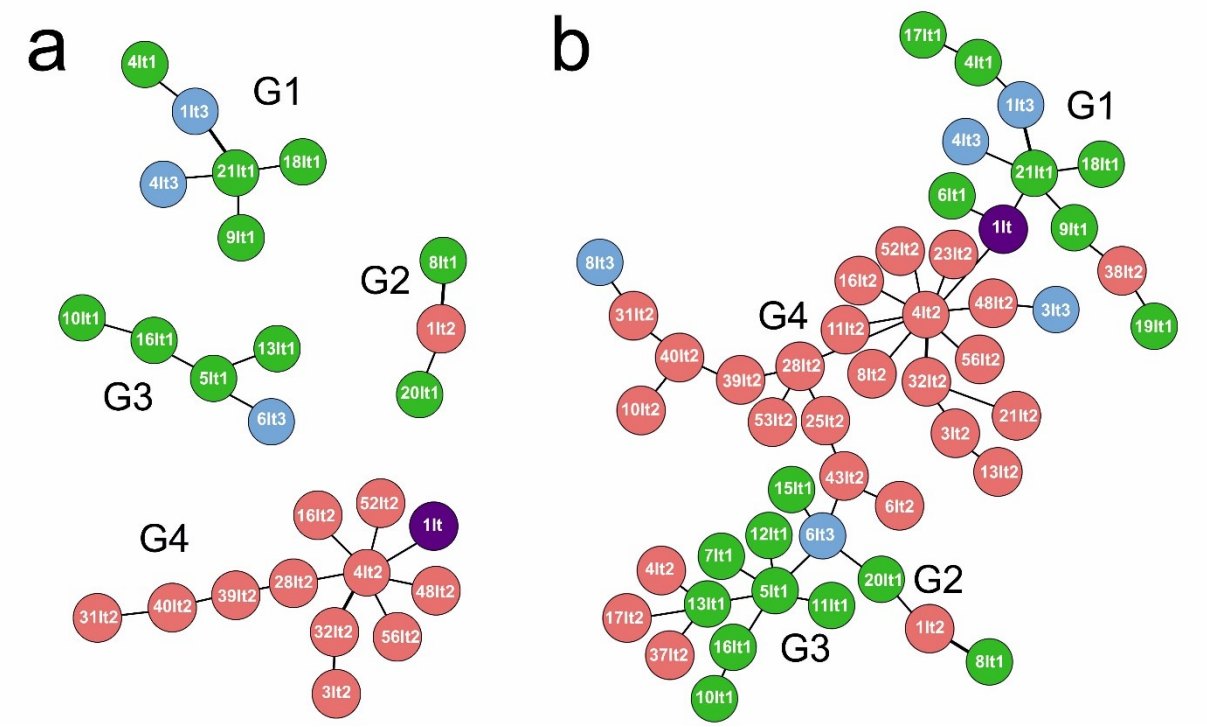

Fig. 2. eBURST analysis of 49 northern Italian *Neospora caninum* samples. Specimens from Lombardy (red dots) tend to cluster separately from those from Piedmont (green dots) or Veneto-Trento (blue dots). (a) Analysis using the Double Locus Variant (eBURST DLV) option shows that *N. caninum* multilocus genotypes can be separated into four groups (G1-4). Genotyping of an Italian isolate, for which the province of origin was not known (violet dot) revealed results close to most isolates from Lombardy (group G4). (b) The full MST option in eBURST, including the complete data set (n=50 *N. caninum* samples) shows the location of the individual groups within a network of all samples. Note: The analysis was restricted to microsatellite markers available from this and a previous study (Regidor-Cerrillo et al., 2020). Only samples that could be typed for all markers (i.e. MS5, 6A, 6B, 7, 10, 12, 21) were included. Moreover, MS10, which combines variation in three separate motifs (sub-loci), was analysed per each motif individually. Groups G1-G3 resemble the grouping reported in (Regidor-Cerrillo et al., 2020), while G4 represents a new group including 11 of 25 bovine *N. caninum* samples from Lombardy that were added to the analysis by this study.

Investigating *N. caninum* positive samples (n=48, excluding a sample, for which the province of origin was unknown, and the goat sample) by PCoA revealed a clear axis #1 separation of the samples from Piedmont (Fig. 3) and Lombardy (Fig. 3). While n=17/17 samples from Piedmont were located on the left side, all from Lombardy (n=17/25) were on the right side (Fig. 3). The majority (n=3/5) of samples from Veneto-Trent (Fig. 3) were left or right in the PCoA graph.

When the eBURST grouping (DLV option) was compared to the PCoA result, eBURST G1 and eBURST G3 was located separately in the upper left quarter of the PCoA graph, while eBURST G2 was located in the lower left part (Fig. 3). *Neospora caninum* samples that had been separated by eBURST into G4 (exclusively Lombardy samples) clustered in both the upper and lower corners on the right side of the PCoA graph (Fig. 3).

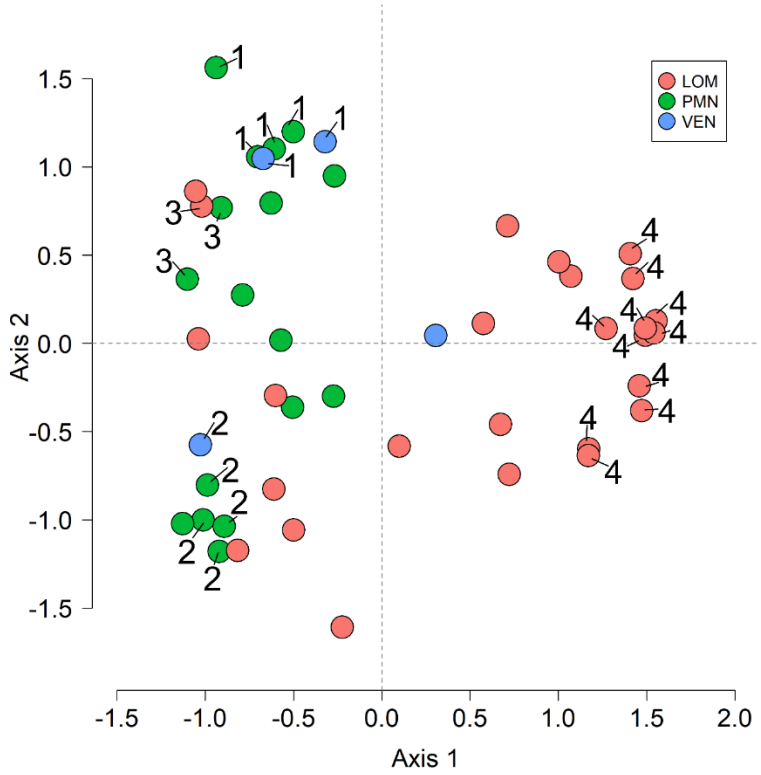

Fig. 3. Principle coordinate analysis (PCoA) separates north Italian *N. caninum* based on their MLMGs into clusters. Data from the present and a previous study conducted in cattle from northern Italy (Regidor-Cerrillo et al., 2020) were used for this analysis. The two dominant Eigenvalues of a PCoA based on the allele-sharing coefficient separated all *N. caninum* positive samples isolated from Piedmont and most from Veneto-Trento so that they clustered on the left side of the PCoA graph, while most of the samples from Lombardy were located on the right side of the PCoA graph. Groups G1-4 (represented by circles with numbers 1-4), as determined by eBURST, were located clearly in separate positions. While G1-3 was found on the left side, G4 (exclusively Lombardy samples) was localised on the right side of the PCoA graph.

Differences between the *N. caninum* populations in northern Italy were assessed F_{ST} analysis values (F) and Nei's unbiased genetic distance (D) (Table 5). Both, results for F and D suggest that there is a statistically significant genetic difference between *N. caninum* from Piedmont and Lombardy as well as between Lombardy and Veneto-Trento (Table 5).

Table 5. Genetic differences between northern Italian *N. caninum*. Pairwise population matrix of F_{ST} analysis values (F) and Nei's unbiased genetic distance (D). Values written in italic style indicate statistical significance based on of goodness-of-fit test (G-test) assessing the significance of the effect of region on genetic differentiation.

		F_{ST} analysis values		
	Region	Piedmont	Lombardy	Veneto-Trento
Nei's genetic distance (D)	Piedmont	NA	*0.1559***	0.0279
	Lombardy	*0.1561***	NA	*0.0973**
	Veneto-Trento	0.0331	*0.1082**	NA

**P* = 0.012; ***P* = 0.001

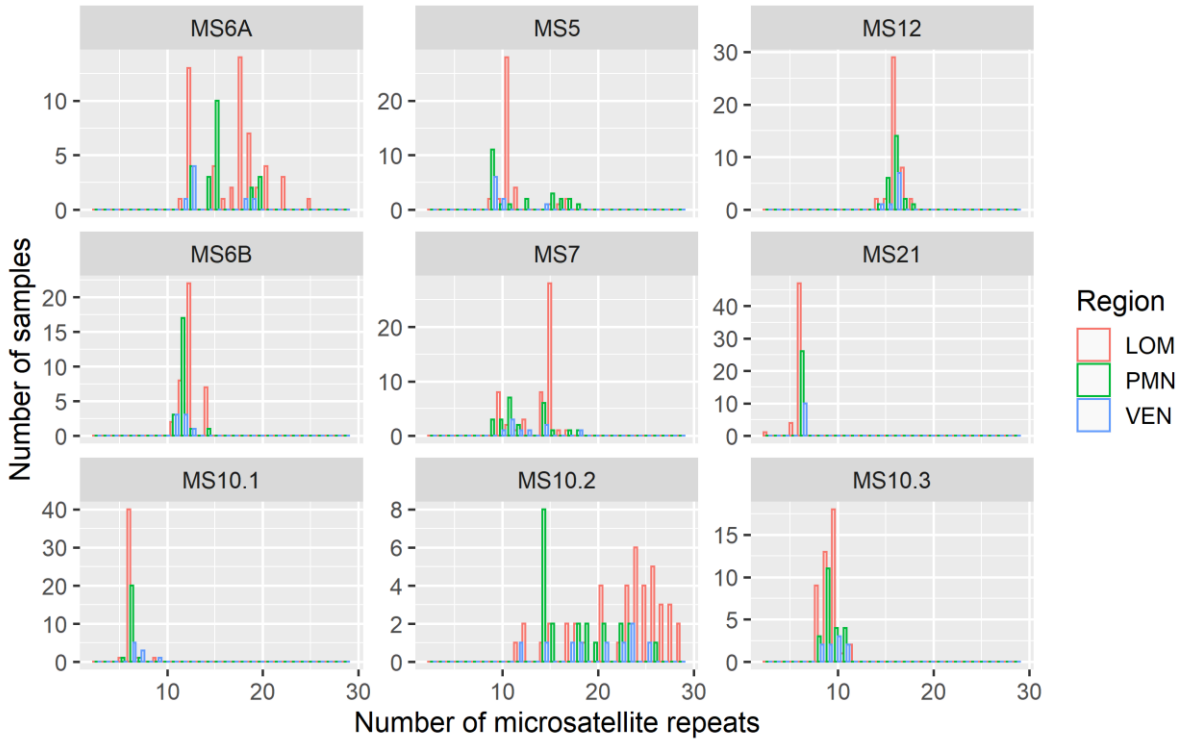

Fig. 4. Differences in the heterogeneity of the numbers of repeat units characteristic for *Neospora caninum* microsatellite loci examined in the present and a previous study conducted in cattle from northern Italy (Regidor-Cerrillo et al., 2020), i.e. in the regions Lombardy (LOM), Piedmont (PMN), and Veneto-Trento (VEN). The range between the lowest and highest number of repeats was largest for MS10.2 (17 repeats), followed by MS6A (13), MS5 (9) and MS7 (9). For the remaining repeats, the range was 4 (MS12, MS21, MS10.1, MS10.3) or 3 repeats (MS6B). Pairwise Wilcoxon test (BH adjusted) revealed statistically significant differences in MS5 (LOM vs VEN), MS6B (LOM vs PMN, LOM vs VEN), MS7 (LOM vs PMN), MS10.1 (LOM vs VEN, PMN vs VEN) and MS10.2 (LOM vs PMN, LOM vs VEN).

The graphical representation of the heterogeneity in numbers of repeat units, stratified for various loci, suggested differences between bovine *N. caninum* sampled in the North Italian regions of Lombardy, Piedmont, and Veneto-Trento (Fig. 4). The range between the lowest and highest number of repeats was largest for MS10.2, followed by MS6A, MS5 and MS7. However, the pairwise Wilcoxon test (BH adjusted) revealed statistically significant differences between regions only for MS5, MS6B, MS7, MS10.1, and MS10.2 (Fig. 4).

Discussion

This study focussed on microsatellite multilocus genotyping of *N. caninum* in bovine foetuses sampled in Lombardy, northern Italy, between 2015 and early 2019 from dairy herds suffering

from reproductive problems. 27.8% of aborted foetuses (55/198) sampled in 43/165 farms tested positive for *N. caninum,* which shows that this parasite is present in many herds of this region.

A similar study conducted had also focussed on MLMGs in *N. caninum* in cattle and a goat from northern Italy (Regidor-Cerrillo et al., 2020). Both beef (Piedmontese breed) and dairy (Italian Holstein Friesian breed) as well as crossbreeds or local cattle breeds were included, whereas our work was focussed on Italian Holstein Friesian cattle under intensive farming conditions. Regarding the geographical area, this previous study had concentrated on bovine foetuses from the regions of Piedmont and Veneto-Trento (n=38) and included only a few samples (n=2) from Lombardy. In addition, the previous study lacked detailed data on the geographic origin of samples. The regions of Piedmont and Veneto-Trento neighbour our study area; Piedmont is located west and Veneto-Trento east from Lombardy. On the basis of microsatellite typing results, it has been hypothesized that local *N. caninum* subpopulations exist in Europe (Constantin et al., 2011; Pedraza-Diaz et al., 2009). We therefore aimed at confirming these findings and finding reasons for genotypic differences between *N. caninum*, even if the isolates or specimens had been sampled from neighbouring areas.

Entirely unexpected was our finding that geographic distance in such a small area as Lombardy (less than 30,000 sqkm size) may at least partially explain the MLMGs genetic distance of *N. caninum* isolates. This suggests that MLMGs pattern remains relatively constant over time in particular herds and only slowly disperse in an area, probably due to animal trade. As it can be assumed that trade between herds was mostly local, this may have resulted in the observed correlation of the geographic distance with the MLMGs-based genetic distance between *N. caninum* isolates. Within herds, *N. caninum* seems to be transmitted endogenously mainly, i.e. from latently infected dams to their offspring. However, even an exogenous transmission via oocysts shedding definitive hosts (e.g. farm dogs or wolves) could explain the conserved microsatellite patterns we observed, if the definitive hosts were infected only with a single strain, i.e. the local one, which results in a so-called uniparental mating of *N. caninum* (Khan et al., 2019). Both endogenous and exogenous transmission without or only limited contact to neighbouring herds or infectious material might have resulted in local *N. caninum* sub-populations.

All cattle analysed in our study in Lombardy belonged to the Italian Holstein Friesian breed, which has been founded in the late 20th century by the importation of cattle from the Netherlands and North America (Buchanan, 2016; Mancini et al., 2014). This breed originates from the north of the Netherlands and Germany and became the predominant dairy breed world-wide; present Holstein Friesian cattle represent crosses of the Dutch Friesian and North-American Holstein lines (Buchanan, 2016; Mancini et al., 2014). It was hypothesized that individual *N. caninum* strains

may have been imported together with the Holstein Friesian cattle breed into Italy (Regidor-Cerrillo et al., 2020). Although MLMGs of *N. caninum* from northern Italy showed a relationship to those from Germany (Regidor-Cerrillo et al., 2020), it is almost impossible to clarify, whether the *N. caninum* genotypes now observed in Italian Holstein Friesian originated from Italy (i.e. from local cattle breeds) or were introduced by the importation of cattle from abroad. Attempts to find genetic differences between *N. caninum* isolates from Holstein Friesian and from local breeds (i.e. the Piedmontese breed), which could have supported the hypothesis of importation, failed (Regidor-Cerrillo et al., 2020). Interestingly, it has been observed that Argentinian *N. caninum* isolates are still related to those from Spain, even long after the importation of the first Iberian cattle into South America in 15th century (Regidor-Cerrillo et al., 2013).

In a few herds, we were able to sample more than a single foetus, and in four of these herds, several months or even a period of years separated the sampling dates. In contrast to findings in bovine herds with epidemic abortions (i.e. in herds, where a point source exposure to *N. caninum* oocysts shed by a dog was assumed (Basso et al., 2010)), particular MLMGs loci differed strongly (i.e. by 3 or even more repeats). It may be discussed, if these larger differences in single loci (MS7, MS6A) or sub-loci (MS10.2) might be an indication for sexual recombination in a definitive host (probably by a farm dog) in the past. However, typing details (Table 2) revealed that related loci on the same chromosome (like MS1B, MS6B) or even sub-loci (e.g. MS10.1 or MS10.2) were not affected. Thus, these differences are best explained by the loss or gain of repeat units, which is typical for microsatellites and caused by point or polymerase template-slippage mutations (Schlötterer and Tautz, 1992; Viguera et al., 2001). Nevertheless, sexual recombination events in these herds cannot completely ruled out, if this recombination had occurred with two or more separate strains with very similar microsatellite patterns, e.g. the microsatellite pattern of the local *N. caninum* population. Our findings in individual herds with more than one *N. caninum*-MLMG result, but also the overall analysis of the *N. caninum* MLMG results in northern Italy, suggests that particular microsatellite loci may be more prone to variation than others. In our study, especially the sub-locus MS10.2, followed by the loci MS6A, MS5 and MS7 showed the strongest variation in the number of repeats. It is well known that several factors contribute to these differences between microsatellite loci, including repeat number, the sequence of the repeat motif, the length of the repeat unit, and flanking sequences (reviewed in (Ellegren, 2000; Estoup et al., 2002; Schlötterer, 2000)). Thus, high variability in particular microsatellite loci may indicate that these loci are of limited importance for the parasite. On the other hand, the observation of changes in particular microsatellite loci raises questions regarding the possible effects of such variations on the affected parasite. Microsatellites are highly abundant in the noncoding DNA of all eukaryotic genomes

(Bagshaw, 2017) and changes in these loci might be "neutral", but microsatellites may also locate in coding regions and variation can be associated with an altered phenotype (reviewed (Schlötterer, 2000)). A recent study on different *Plasmodium* spp. affecting humans suggested that a significant proportion (one fifth to one third) of microsatellite-related sequences are related to coding sequences. Based on gene ontology, the respective coding sequences can be involved in molecular functions like binding or in biologic processes such as metabolism or reproduction (Mathema et al., 2020). Although, we are far from understanding the biological relevance of microsatellites for *N. caninum*; it would be intriguing, if future studies could comprehensively address the microsatellites of *N. caninum* to gain more knowledge on their function. It has so far not been possible to link particular microsatellite patterns to particular traits, e.g. virulence for foetuses (Dubey and Schares, 2011). Nevertheless, virulence differences have been observed among *N. caninum* strains also in its main intermediate host, the cattle (Chryssafidis et al., 2014; Dellarupe et al., 2014b; Jimenez-Pelayo et al., 2019; Regidor-Cerrillo et al., 2014; Regidor-Cerrillo et al., 2010; Rojo-Montejo et al., 2009a; Rojo-Montejo et al., 2009b), and it would be fascinating to know, to which extent differences in microsatellite loci contribute to virulence. Recently, highly virulent isolates of *N. caninum* were shown to express a subset of particular secreted proteins in more abundance (Roman et al., 2020). The reasons for differential expression between strains could at least in part be due to differences in microsatellite loci as shown for other eukaryotic cells and organisms (Bagshaw, 2017). In *T. gondii*, an apicomplexan parasite closely related to *N. caninum*, the situation is similar. Currently, relatively conserved microsatellite loci are used to differentiate dominant clonal lineages of *T. gondii*, while also a number of other microsatellite loci are known, which were called finger-printing microsatellite loci. They can be used to differentiate strains of a single clonal type on a local level (Ajzenberg et al., 2015).

Conclusions

Our findings confirm the concept of local *N. caninum* sub-populations. For the first time, we could show a correlation between the genetic distance of *N. caninum* isolates based on MLMGs and the geographic distance of the places, where the isolates had been obtained. Our results confirm that sexual recombination in *N. caninum* is a rare event. Possible reasons for this might be that endogenous vertical transmission is dominating and that the chance for a definitive host to feed on intermediate hosts with a mixed infection of viable and non-related *N. caninum* strains is extremely low. More comprehensive studies on microsatellites in *N. caninum* and on related species like *T. gondii* should be undertaken, not only to improve genotyping capabilities, but also to understand the possible function of these regions in the genomes of these important parasites.

Declarations

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Ethics approval and consent to participate

All procedures were approved by the Institutional Animal Care and Use Committee of Università degli Studi di Milano ("Organismo Preposto al Benessere degli Animali," Prot. no. OPBA_34_2017).

Competing interests

The authors declare that they have no competing interests.

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Research Line 3:

Evaluation of *Toxoplasma gondii* infection in beef cattle raised in Italy

Toxoplasma gondii seroprevalence in beef cattle raised in Italy: a multicenter study

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Abstract

Toxoplasmosis represents an important public health issue, with the consumption of raw or undercooked meat being a major way of human infection. The role of beef in the transmission of the parasite to humans is questioned due to lower quantity of tissue cysts compared with other meat-producing species. However, the habit of consuming raw beef is regionally diffused, and the risk posed by *Toxoplasma gondii* infection in cattle should not be overlooked. Therefore, to update information on *T. gondii* in cattle reared in Italy, a multicentric seroepidemiological survey was designed and implemented in four Northern regions (Liguria, Lombardy, Piedmont, and Trentino Alto Adige) and Sardinia. Overall, a convenience sampling was performed, collecting 1444 serum samples from 57 beef cattle herds. Thirteen beef breeds were sampled, besides cross-breed; bovines age varied from 3 months to over 12 years. Sera were tested with a commercial ELISA for the detection of anti-*T. gondii* antibodies. Individual and herd data were analyzed by binary logistic regression analysis. A *T. gondii* seroprevalence of 10.2% was recorded, with differences among regions and values ranging from 5.3% in Liguria to 18.6% in the Piedmont region (p value = 0.0001). Both young and adult animals and males and females tested positive, without any significant difference (age and gender: p value > 0.05). Lower seroprevalence values were recorded in cattle born in Italy (8.7%) if compared with animals imported from abroad (13.4%) (p value = 0.046). The spread of *T. gondii* in beef cattle destined to Italian consumers is confirmed, suggesting the need of continuous monitoring of the infection.

Keywords: Serology; Food-borne parasite; Toxoplasmosis; Zoonoses; Beef cattle.

Introduction

Toxoplasmosis is a zoonotic parasitic disease with a worldwide distribution. Though usually asymptomatic, toxoplasmosis can seriously impact on the health of immunocompromised patients or if acquired during pregnancy (Weiss and Dubey, 2009). Furthermore, chronic infection can be reactivated following immunosuppression due to organ transplants or HIV infection (Weiss and Dubey, 2009).

In humans, a part the vertical transmission (congenital infection, from mother to fetus), the horizontal transmission may occur through the ingestion of oocysts from environmental contamination of water, soil, or food and, in less extent, through the ingestion of tissue cysts of infected intermediate hosts (Pinto-Ferreira et al., 2019).

Food-producing animals have a different significance in the transmission of *Toxoplasma gondii* to humans, due to species-related differences in prevalence and intensity of tissue cysts in the edible parts (Stelzer et al., 2019). Compared with other species (i.e., game, sheep, goats, pigs), cattle are deemed to play a limited role in the transmission of *T. gondii* to humans (Guo et al., 2015).

However, a number of surveys have shown that the risk for humans is also a function of the consumers' eating habits, which largely vary between regions or countries due to different culinary traditions (Guo et al., 2015; Belluco et al., 2018). In Italy, the annual per person consumption of beef has increased slightly in recent years (17.2 kg in 2018, + 2% compared with the previous year), although lower than that of pork (38.2 kg), probably also due to the higher cost of beef (10.86–11.59 €/kg, depending on the cuts) compared with pork (7.39 €/kg) (http://www.ismeamercati.it). The consumption of raw bovine meat is part of the cultural and traditional heritage in many countries and also characterizes the new food trends. In Italy, consumers' preferences set beef at a putatively high risk of transmitting the infection, as beef is more often consumed raw or undercooked compared with other animal species (Belluco et al., 2018). Recently, the European Authority for Food Safety (EFSA) reconsidered the hierarchical scale of the risk deriving from the consumption of raw or undercooked meat and stated that beef represents a health risk more than what was believed in the past (EFSA, 2018).

Hence, a large-scale seroepidemiological serosurvey was planned, targeting beef cattle raised in Italy, with the aim of obtaining information on their exposure to *T. gondii* infection in two study areas, Northern Italy and Sardinia Island, which differ geographically and for cultural and food habits.

In fact, the consumption of beef meat is higher in Northern regions (58.6% of the population consume beef several times a week) compared with Sardinia (53.3%)

(http://dati.istat.it/Index.aspx?QueryId=16813). In addition, traditional cuisine includes various raw beef preparations in many regions of Northern Italy: tartare, carpaccio, and cured beef meat, some of which with protected geographical indication (e.g., bresaola, carne salada, slinzega, mocetta).

Since the correlation between the serological data and the presence of cysts in the edible parts in cattle is poor (Opsteegh et al., 2019), the detection of antibodies does not reflect the infectivity of the meat but gives an indication of the population exposure to the infection. In parallel, individual and herd risk factors were analyzed.

Material and methods

Ethics statement

No animals were sampled for the purposes of the present study; aliquots from serum samples previously taken for unrelated national surveillance program were used. Serum samples and farms were coded, and serological and data analyses were performed blinded.

Sample and data collection

A convenience sampling was carried out including beef cattle from farms located in two separate Italian macro-areas: mainland Northern Italy (Lombardy, Piedmont, Liguria, and Trentino Alto Adige regions) and Insular Italy (Sardinia Island), representative of a high variety of management systems and of differences in landscape and climate. Overall, 1444 serum samples (Lombardy: 267, 8 farms; Piedmont: 226, 8 farms; Liguria: 434, 19 farms; Trentino Alto Adige: 157, 7 farms; Sardinia: 360, 15 farms) were selected among those collected between October 2016 and May 2017 by local veterinarians in conjunction with routine sampling for regional sanitary controls. For farms with fewer than 50 animals, all animals were included in the study; for farms with more than 50 animals, a representative animal sample of approximately 10% (10–18%, average 12%) of the herd was included in the study. A mean of 24.9 animals was sampled per farm. Serum samples were stored at − 20 °C until analyzed.

Both young and adult animals were included in the survey, with age varying from 3 months to over 12 years (mean in months ± standard deviation: 63.2 ± 53.2). Besides cross-breed, both purely beef breeds (Piedmontese, Sardo-Bruna, Podolica, Charolaise, Limousine, Aubrac, Blonde D'Aquitaine, Gasconne) and dual-purpose breeds (Alpine Gray, Alpine Brown, Pezzata Rossa Italiana, Salers, and Valdostana Pezzata Rossa) were sampled.

Individual data (gender, age, and breed) were collected. GPS (Global Positioning System) coordinates of each farm were gathered to map its location. Furthermore, the altitude of municipalities where the farms were located was recorded (https://www.istat.it/it/archivio/156224).

Serological analysis

Sera were analyzed with a commercial ELISA (ID Screen® Toxoplasmosis Indirect Multi-Species, IDVET, Montpellier, France) for the detection of anti-*T. gondii* antibodies, performed according to the manufacturer's instruction. Positive and negative control sera provided with the kit were used as controls. For each sample, the resulting values were calculated by applying the formula supplied in the kit: S/P% = OD sample − OD-negative control/OD-positive control − OD-negative control) × 100. Samples with SP% ≥ 50% were considered positive.

Statistical analysis

The seroprevalence at individual level was computed with the associated 95% confidence interval. A herd was considered positive if at least one seropositive animal was found. For descriptive statistics, age and altitude, respectively computed in months and meters above sea level (m a.s.l.), were categorized.

Univariate binary logistic regression analysis was performed to determine factors that could be considered predictors of seropositivity to *T. gondii* (dichotomous animal-level outcome: seropositive or seronegative). Both individual and herd data were considered: gender, age (continuous variable), breed, breed purpose, region, origin. The Wald test was used to assess the association of significant variable to *T. gondii* seropositivity. Subsequently, all the variables showing a p value < 0.1 and their two-way interactions were entered in a multivariate model, developed by backward elimination until all remaining variables were significant (p value < 0.05). Confounding was evaluated as > 20% change in the odds ratio. Given a possible clustering of the serological status of animals from the same farm, the variable "farm" was entered as a random factor. Statistical analysis was performed with SPSS (version 19.0; SPSS, Chicago, IL, USA).

Results

T. gondii seroprevalence was 10.2% (95% CI: 8.79–11.92), with 148 seropositive animals out of 1444 tested. S/P% values of positive samples ranged from 50.0 to 266.1 (mean ± standard deviation: 87.8 ± 37.3). The majority of the herds (39/57, 68.4%, 95% CI 55.52–78.99) had at least

one *T. gondii* seropositive animal. The proportion of seropositive cattle tested within each herd ranged between 0 and 42.9%, being ≥ 30% in four farms. Both young and adult animals tested positive and similar prevalence values were obtained in males (12.3%) and females (10%). Particularly, an increase of seroprevalence was evidenced in females until the age of 3 years, whereas males scored positive only until 2 years of age.

A higher number of seroreactors were detected below 200 and between 200 and 400 m a.s.l. (34/278, 12.2% and 36/233, 15.5%), compared with those from municipalities located at 400–600 and > 600 m a.s.l. (28/405, 6.9% and 13/160, 8.1%). Descriptive statistics and results from statistical analysis are reported in Table 1.

Statistical analysis performed by univariate logistic regression analysis showed that the origin of the animals was associated with a higher infection risk. In particular, the seropositivity values differed significantly according to region and animals imported from abroad were at higher risk of infection than cattle from Italy. Seropositivity was also associated to purpose. Any of the variables and of their two-way interactions entered in the multivariate model were not significantly associated to *T. gondii* infection, not even when the variable "farm" was included as a random factor.

Table 1 – Serological data related to *Toxoplasma gondii* in beef cattle from Italy: descriptive statistics, and results obtained in the univariate regression analysis.

Variable category	Seropositive/examined	P% (95% CI)	β±s.e.	Wald's Chi-square	OR (95% CI)	p-value	AIC
Region				30.102		**0.0001**	35.372
Lombardy	24/267	8.9 (6.12-13.03)	-0.838±0.2739		0.433 (0.253-0.740)	**0.002**	
Liguria	23/434	5.3 (3.56-7.83)	-1.406±0.2741		0.245 (0.143-0.420)	**0.0001**	
Sardinia	36/360	10 (7.31-13.53)	-0.720±0.2452		0.473 (0.301-0.787)	**0.003**	
Trentino Alto Adige	23/157	14.6 (9.96-21.02)	-0.285+0.2832		0.752 (0.432-1.310)	0.314	
Piedmont (reference)	42/226	18.6 (14.05-24.16)	0		1		
Age				0.105		>0.1	
≤12 months	35/297	11.8 (8.59-15.94)					
13-36 months	43/334	12.9 (9.7-16.89)					
37-60 months	7/175	4 (1.95-8.03)					
61-84 months	16/218	7.3 (4.57-11.59)					
>84 months	48/419	11.5 (8.75-14.87)					
Gender				1.152		>0.1	
F	121/1224	9.9 (8.34-11.69)					
M	27/220	12.3 (8.57-17.26)					
Breed				16.779		>0.1	
Alpine Brown	0/1	0 (0-79.35)					
Alpine Grey	1/12	8.3 (1.49-35.38)					
Aubrac	1/18	5.6 (0.99-25.76)					
Blonde D'Aquitaine	0/3	0 (0-56.15)					
Charolaise	13/126	10.3 (6.13-16.86)					
Gasconne	0/3	0 (0-56.15)					
Limousine	22/167	13.2 (8.86-19.14)					
Pezzata Rossa Italiana	7/18	38.9 (20.31-61.38)					
Piemontese	50/558	9 (6.86-11.62)					
Podolica	0/1	0 (0-79.35)					
Salers	2/15	13.3 (3.73-37.88)					
Sardo-Bruna	10/74	13.5 (7.51-23.12)					
Valdostana Pezzata Rossa	1/8	12.5 (2.24-47.09)					
Crossbreed	41/440	9.3 (6.94-12.4)					
Purpose				6.109			21.779
Beef	96/950	10.1 (8.35-12.19)	0.822-0.3546		2.276 (1.136-4.560)	**0.02**	
Dual-purpose	11/54	20.4 (11.77-32.9)	0		1		
Origin				3.996			14.743
Imported from abroad	42/314	13.4 (10.05-17.59)	0.485±0.2424		1.624 (1.009-2.611)	**0.046**	
Born in Italy (reference)	35/403	8.7 (6.31-11.83)	0		1		

Discussion

The present study is evidence that *T. gondii* infection is widespread among beef cattle in Italy, with 10.2% of the samples testing seropositive and 68.4% of seropositive farms. The exposure to the pathogen is thus confirmed, as suggested by previous serosurveys conducted in the same study area in other domestic animals (Gazzonis et al., 2015; 2018a; 2020; Villa et al., 2018) and wildlife (Zanzani et al., 2016; Gazzonis et al., 2018b; 2018c). The obtained results are in line with other similar surveys in Europe, in which seroprevalence values ranged from 7.8 up to 76.3% (Klun et al., 2006; Gilot-Fromont et al., 2009; Berger-Schoch et al., 2011; Jokelainen et al., 2017; Blaga et al., 2019).

A significant difference was observed between dual-purpose breeds and purely beef breeds or crossbreeds, probably for differences in breeding systems, as reported also for other species including goats (Gazzonis et al., 2015): breeds with higher production are usually raised with more intensive systems, while more rustic breeds can be adapted to more mountainous territories and extensively bred. Moreover, a possible influence of genetic differences existing among breed on the susceptibility or in the antibody response to *T. gondii* should be taken into account (Jokelainen et al., 2017).

Age was not associated with differences in seropositivity between young and adult animals, as previously reported (Gilot-Fromont et al., 2009) but in contrast to other studies (Berger-Schoch et al., 2011; Blaga et al., 2019). Interestingly, in our study, female and male animals differed in age-related seroprevalence values. In males, anti-*T. gondii* antibodies were only found in animals younger than 2 years, while in females the percentage of seropositive animals increased with age up to 3 years and a clear pattern was no longer evident in older animals. Possibly, unlike females, most males under 2 years of age include the category of young animals imported from abroad, extensively reared until being imported in Italy and thus highly exposed to the risk of acquired *T. gondii* infection at pasture. In a study by Jokelainen et al. (2017), a trend to higher seropositivity values was found up to 5 years of age, while a higher variability in seroprevalence values was pictured in older animals.

The lack of age differences is further evidence that anti-*Toxoplasma* antibody response is not lifelong in cattle: according to Opsteegh et al. (2011), anti-*Toxoplasma* antibodies persist shorter than lifelong in cattle, implying a mismatch between the serological data and the presence of tissue cysts. In cattle, seronegativity does not rule out the presence of the parasite DNA in tissues including meat and other edible organs (Opsteegh et al., 2019). On the other hand, seropositive

cattle indicate that cattle are exposed to the risk of infection and that consumers will be potentially at risk by eating their meat and derived products.

The origin of *T. gondii* infection in humans is often unknown. The way of infection through the ingestion of oocysts in the environment, water, or contaminated food seems to be more efficient than the way of infection through the consumption of meat containing tissue cysts (Pinto-Ferreira et al., 2019). Nevertheless, undercooked beef is considered a source of *T. gondii* for humans, especially in countries and regions where beef is often eaten raw, cured, or under-cooked (EFSA and ECDC 2018). The thermal treatments the meat is subjected to during cooking (at temperatures above 60 °C) or freezing (at − 20 °C for 3 days) are instead able to inactivate the tissue cysts, as demonstrated by several experimental studies recently reviewed (Alizadeh et al., 2018). Considering the Italian settings, and particularly in Piedmont, many traditional dishes are based on raw, such as tartare and carpaccio, and cured beef meat. Furthermore, in Italy, the sale of fresh beef is far higher (817,305 tons sold) than the sale of frozen beef (15,065 and 38,128 tons sold of Italian and imported frozen meat, respectively). In particular, the import of live animals destined for slaughter (about 79 thousand animals imported in 2019) mainly takes place from countries belonging to the European Union (especially Poland, France, and the Netherlands). Only to a lesser extent import occurs from non-European countries (e.g., Argentina) (http://www.ismeamercati.it), which could represent a risk due to the possible presence of atypical genotypes of *T. gondii*, potentially more virulent than those present in Europe (Shwab et al., 2014).

The serosurvey performed in Italy demonstrated the largest number of seroreactors in Piedmont. It should be noted that in the present study the prevalence was calculated at the individual level, and therefore the data interpretations at the herd level should be taken with caution. A possible explanation could be linked to the breeding system in this region: farms are often extensive, small family-run farms, with the possibility for animals to graze, thus contributing to the completion of the *T. gondii* cycle, as previously highlighted (Klun et al., 2006; Gilot-Fromont et al., 2009). Another possible reason for this result lies in the geographical and environmental characteristics of Piedmont, a region with a strong hilly and mountainous component. The altitude was found to be strongly associated to *T. gondii* seropositivity, with the greatest number of positive samples was found at altitudes up to 400 m a.s.l., where the climate conditions (humid and temperate) are more favorable for the survival and sporulation of *T. gondii* oocysts in the environment (Dubey, 2010), as previously demonstrated (Alvarado-Esquivel et al., 2013; Gazzonis et al., 2015).

The same regional differences were reported in *T. gondii* human infection. The incidence rates of hospitalizations due to toxoplasmosis are higher in the Northern provinces (Graziani et al., 2016), with 34 notified cases of toxoplasmosis in Piedmont from 2010 to 2018 (https://www.seremi.it).

Considering the data obtained from serological screening in pregnant women, the highest seroprevalence value (35.3%) was reported in Piedmont (https://www.ceirsa.org/).

Conclusions

Although seropositivity does not necessarily correspond to the infectivity of the meat and in general of the edible parts, the seroepidemiological data obtained from the present study confirmed the wide diffusion of *T. gondii* in the study area among beef cattle destined to human consumption. However, regulation at both national and European level concerning the control of *T. gondii* infection both in terms of animal handling and of inspection at slaughterhouses is still lacking today. Therefore, the application of hygienic-sanitary measures at farm level for the achievement and maintenance of an adequate biosecurity to contrast the spread of the pathogen in the beef cattle breeding sector is of fundamental importance. In parallel, the risk for human infection can be reduced through information and education of consumers on the correct procedures of meat preparation and handling, with a special focus on the appropriate heat treatments (both freezing and cooking) of beef.

Toxoplasma gondii infection in slaughtered cattle from Italy: meat-juice serology and molecular detection

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Abstract

To obtain epidemiological and molecular data on *T. gondii* infection in cattle slaughtered in Italy, 80 animals were sampled from one of the biggest Italian slaughterhouses. Dairy (Holstein Friesian) or dual purpose (Alpine Brown and Pezzata Rossa Italiana) breeds or crossbreeds from 15 farms located in northern Italy were sampled; age of animals varied from six months to three years. Approximately 50 g of diaphragm was collected to obtain meat juice and muscle homogenate samples. Individual data were noted. Meat juice samples were analyzed with a commercial ELISA to detect anti-*T. gondii* antibodies. DNAs extracted from muscle homogenate samples were subjected to B1 real-time PCR and sequencing. Anti-*T. gondii* antibodies were found in 10 (12.5%) out of 80 examined animals, whereas parasitic DNA was detected in 26 diaphragm muscle samples (32.5%). Only seven samples scored positive in both test: a fair agreement between ELISA and B1 real-time PCR results was achieved (κ value = 0.254). Nevertheless, higher ELISA S/P% values were recorded in diaphragm samples scoring positive to PCR. Higher number of positive samples were found in younger than older animals considering both ELISA and B1 real-time PCR results. Similarly, considering the provenience, animals that have been acquired from other holdings scored more frequently positive to both ELISA and B1 real-time PCR compared to animals that have never left the holding of origin until slaughter. Statistical analysis showed an effect of ELISA S/P% values on B1 real-time PCR results, increasing the risk of parasitic DNA detection when increasing the S/P% values. The other considered variables (age and provenience) did not show any effect on neither ELISA nor B1 real-time PCR. The study confirmed the role of beef meat as a potential source of *T. gondii* infection for humans. Considering the consumption of raw beef preparations in many regions of northern Italy, the zoonotic importance of *T. gondii* from beef should not be neglected.

Keywords: Toxoplasmosis; Foodborne zoonosis; Cattle; Serology; PCR; B1 gene.

Introduction

T. gondii is a protozoan parasite infecting a wide range of warm-blood intermediate hosts, including humans (Dubey, 2010). Toxoplasmosis is a widespread zoonotic disease with huge medical importance, particularly if acquired during pregnancy and in immunocompromised individuals (Weiss and Dubey, 2009). In humans, apart from the vertical congenital transmission, the parasite may be horizontally transmitted through the ingestion of oocysts from environmental contamination of water, soil, or food, but also the consumption of raw or undercooked meat containing tissue cysts (Pinto-Ferreira et al., 2019). Animal species destined to human consumption were shown to have a different significance in the transmission of *T. gondii* to humans, due to species-related differences in prevalence and intensity of tissue cysts in the edible parts (Stelzer et al., 2019). Indeed, among livestock parasite tissue cysts are most frequently observed in pigs, sheep, and goats, whereas tissue cysts are found only rarely in beef meat (Tenter et al., 2000). However, even if cattle are considered a poor host for *T. gondii* (Dubey and Jonas, 2008), a systematic review and meta-analysis of case-controlled studies demonstrated that consumption of raw/undercooked beef is an important risk factors for *T. gondii* infection in humans (Belluco et al., 2018). The risk of *T. gondii* infection from bovine meat is still higher in those countries or regions where traditional cuisine includes raw beef preparations.

However, data on the spread of *T. gondii* infection in cattle are often limited to seroepidemiological studies (Klun et al., 2006; Gilot-Fromont et al., 2009; Berger-Schoch et al., 2011; Garcia-Bocanegra et al., 2013; Jokelainen et al., 2017; Blaga et al., 2019; Sroka et al., 2020; Gazzonis et al., 2020), whereas prevalence data on the presence of the parasite in beef meat are scarce (Hosein et al., 2016; Opsteegh et al., 2019).

It was recently demonstrated that *T. gondii* antibody detection in cattle does not strictly correspond to the presence of parasite cysts in their tissues (Opsteegh et al., 2019). However, it was suggested that the use of both serology and molecular methods may allow to better assess the risk of *T. gondii* infection transmission from the consumption of meat originating from infected animals (Sroka et al., 2020).

Therefore, the aim of this study was to obtain both seroprevalence and molecular data on *T. gondii* infection in cattle slaughtered in northern Italy. With respect to potential threats to public health, only steers were selected for the study: indeed, their meat, in contrast with those from cows that are normally commercialized in meat preparations and consumed cooked, are more frequently commercialized fresh and potentially consumed raw from the consumer.

Material and Methods

Sampling

A convenience sampling was carried out from November 2019 to January 2020. Overall, 80 bovines were sampled from one of the biggest slaughterhouse in northern Italy (Lombardy). Dairy (Holstein Friesian) or dual purpose (Alpine Brown and Pezzata Rossa Italiana) breeds or crossbreeds from 15 farms located in northern Italy were sampled. Age of animals varied from six months to three years (mean±standard deviation, in months: 22.3±6.2).

During the slaughtering operations, approximately 50 g of diaphragm was obtained and stored in individual plastic bags. Tissue samples were kept refrigerated during transportation to the laboratory within a few hours. Part of tissue samples (25 gr) was frost in plastic bag overnight, then defrost to obtain meat juice (Nockler et al., 2005). A second aliquot (25 gr) was mechanically homogenized. Meat juice and muscle homogenate samples were then preserved at − 20 °C until immunological and molecular analysis, respectively.

Ear tag numbers were annotated and used to obtain individual data (gender, age, and breed) and the movement history using the National Cattle Registry Cattle Registry (https://www.vetinfo.it).

Anti-T. gondii *antibodies detection*

Meat juice samples were analyzed with a commercial ELISA (ID Screen® Toxoplasmosis Indirect Multi-Species, IDVET, Montpellier, France), validated for the detection of anti-*T. gondii* antibodies in meat juice, using 1:2 dilution according to the manufacturer's instruction. Positive and negative control sera provided with the kit were used as controls. For each sample, the resulting values were calculated by applying the formula supplied in the kit: S/P%= OD sample − OD-negative control/OD-positive control – OD-negative control) × 100. Samples with SP% ≥ 50% were considered positive.

Molecular analysis

Muscle homogenate samples were processed for DNA extraction using a commercial kit (Nucleospin tissue, Macherey-Nagel GmbH and Co., Germany), following manufacturer's instruction. Genomic DNA was stored at −20 °C until analyzed.

All DNA samples were subjected to a real-time PCR (B1 real-time PCR) targeting a region of about 129 bp within the 35-fold repetitive B1 gene (AF179871) (Burg et al. 1989), as described by (Gazzonis et al. 2018c) with slightly modifications. B1 real-time PCR was performed in a final volume of 20 µl, containing PowerUp™ SYBR® Green Master Mix (Thermo Fisher Scientific)

2×, 0.5 µM of each primer [ToxB41f (5′-TCGAAGCTGAGATGCTCAAAGTC-3′) and ToxB169r (5′-AATCCACGTCTGGGAAGAACTC-3′)], and 5 µl of DNA samples (approximately 250-500 ng of genomic DNA). Amplification and melting analysis were performed in a QuantStudio™ 3 Real-Time PCR System with a QuantStudio™ 3 software system (Applied Biosystems™ LSA28137) with the following cycling profile: incubation at 50°C for 2 min, denaturation at 95°C for 2 min, amplification for 40 cycles at 95°C for 15 s, and 60°C for 60 s, and a final step of melting analysis. Positive (Zanzani et al., 2016) and negative controls (no template DNA) were included in each run. The melting program, consisting in temperature increases from 60°C to 95°C at intervals of 0.15 °C/s, was performed at the end of each cycle. Each sample was analyzed in duplicate, and the mean Ct and melting temperature (Tm) values were recorded. A sample was defined as positive when a detectable amplification curve and a Tm value of ± 0.5 °C *vs.* Tm value of positive control were recorded.

Statistical analysis

Prevalence of infected cattle was calculated based on antibodies in meat juice samples and of parasitic DNA in muscle samples considering ELISA and B1 real-time PCR results, respectively, according to the animal categories (Bush et al., 1997). The agreement between results obtained with ELISA and B1 real-time PCR by Cohen's kappa statistic (κ) was calculated.

General linear models (GLMs) with binomial distribution and logit link function were performed to determine factors that could be considered predictors of the presence of i) anti-*T. gondii* antibodies in meat juice samples and ii) of *T. gondii* DNA in diaphragm samples. Results obtained in ELISA and in B1 real-time PCR (presence/absence, dichotomous variable) were used as dependent variables, respectively. The variables "age" (continuous variable) and "provenience" (dichotomous variable: born in farm, acquired from another Italian farm) were entered in the model. In addition, it was also verified if ELISA S/P% values could be predictor of the presence of *T. gondii* DNA, by entering the variable "ELISA S/P% values" (continuous variable) in the second model. The models were developed using a backward procedure until retained variables were significant (p-value < 0.05). Statistical analysis was performed using SPSS (version 19.0; SPSS, Chicago, Illinois).

Results

Antibodies anti-*T. gondii* were found in 10 (12.5%) out of 80 examined animals, while parasitic DNA was detected by B1 real-time PCR in 26 muscle samples (32.5%). Only seven samples scored

positive in both test: a fair agreement between ELISA and B1 real-time PCR results was achieved, with a κ value of 0.254 (Thrusfield, 2007). Nevertheless, higher ELISA S/P% values were recorded in diaphragm samples scoring positive to PCR (ELISA S/P%, mean±s.d: 85.4±32.817 and 55.7±1.528, respectively).

A higher number of positive samples were found in younger (≤18 months of age, 21 animals) than older animals (>18 months of age, 59 animals) considering both ELISA and B1 real-time PCR results. Particularly, five (23.8%) and nine (42.9%) younger animals scored positive to ELISA and B1 real-time PCR, while five (8.5%) and 17 (28.8%) older animals scored positive to ELISA and B1 real-time PCR, respectively.

Similarly, considering the provenience, animals that have been acquired from other holdings scored more frequently positive to both ELISA and B1 real-time PCR (5/46, 10.9% and 13/46, 28.3%, respectively) compared to animals that have never left the holding of origin until slaughter (5/34, 14.7% and 13/34, 38.2%, respectively).

Statistical analysis showed an effect of ELISA S/P% values on B1 real-time PCR results, increasing the risk of parasitic DNA detection when increasing the S/P% values (β±standard error: 0.031±0.0136; Wald's Chi-square: 5.242; OR (95% CI): 1.032 (1.004-1.059); *p*-value: 0.022). The other considered variables (age and provenience) did not show any effect on neither ELISA nor B1 real-time PCR (p-value >0.05).

Discussion

This study confirmed the spread of *T. gondii* infection among cattle bred and slaughtered in northern Italy destined to human consumption. Anti-*T. gondii* specific antibodies were revealed in 12.5% of meat juice samples: these results are in line with a recent study conducted on beef cattle in the same study area (Gazzonis et al., 2020) and with other European studies, in which seroprevalence values ranged from 7.8 up to 83.3% (Klun et al., 2006; Gilot-Fromont et al., 2009; Berger-Schoch et al., 2011; Garcia-Bocanegra et al., 2013; Jokelainen et al., 2017; Blaga et al., 2019; Sroka et al., 2020).

On these same samples, molecular analysis by B1 real-time PCR was performed: parasitic DNA was confirmed in 32.5% of diaphragm muscle samples.

The agreement between the seropositivity to anti-*T. gondii* antibodies and the presence of the parasite in host tissues resulted low (k=0.253), as previously demonstrated in bovine species (Opsteegh et al., 2019). Interestingly, in spite of the lack of agreement between serological and molecular results, statistical analysis showed that ELISA S/P% values were predictive variables

for positivity in real-time PCR. Indeed, positive samples by both ELISA and real-time PCR showed higher S/P% values if compared to seropositive samples that resulted negative for the presence of *T. gondii* DNA. Probably, to a higher presence of antibodies as detected by ELISA may correspond a higher parasite load to which the host immune system is subjected. However, this results contrasts with those recently reported in the study by Opsteegh et al. (2019), in which an higher antibody titre determined by MAT is not related neither with the probability of detection in seropositive samples nor with the concordance between serological and molecular techniques. Therefore, further studies would be advisable, also considering the potential application of serological patterns as predictors for the presence of *T. gondii* tissue cysts in bovine edible muscles. Apart from ELISA S/P% values, risk factors analysis did not show any other significative association between neither the seropositivity to *T. gondii* antibodies in meat juice nor the presence of parasitic DNA in diaphragm muscle and the considered variables, i.e. age and provenience.

In particular, the risk for *T. gondii* infection does not seem to increase with host age, as already evidenced in other studies (Gilot-Fromont et al., 2009; Jokelainen et al., 2017, Gazzonis et al., 2020). However, a higher percentage of animals under 18 months of age tested positive if compared to the proportion of older animals, thus confirming that antibody immunity does not persist lifelong in cattle (Dubet, 2010).

The movement history of the animals included in the study was also considered. Even if with no statistical significative difference, cattle that experienced one or more movements (with purchase by other farms) before being slaughtered showed higher values in terms of both antibody prevalence and the presence of *T. gondii* DNA in meat. The exchange of animals at national level, especially regarding cattle of high genetic value or breeders, may also represent a risk for the spread of pathogens. For *T. gondii*, animal trade among countries has not been demonstrated as a risk factor, and often cases of human infection are linked to the consumption of locally produced meat; nevertheless, as in general for foodborne diseases, the trade of animals or products of animal origin could be associated with the spread of pathogens, especially if meat inspection is lacking or not mandatory as in the case of *T. gondii* (Robertson et al., 2015).

Conclusions

The role of beef meat as a potential source of *T. gondii* human infection is therefore confirmed. Indeed, considering eating habits, since traditional cuisine includes various raw beef preparations in many regions of northern Italy, the zoonotic importance of *T. gondii* from beef should always be carefully monitored, and prioritized at meat inspection.

CONCLUSIONS

The studies conducted for my doctoral project were focused on three Apicomplexa protozoa of relevance for human health and animal health, welfare, and productivity in domestic ruminants, i.e., *Besnoitia besnoiti, Neospora caninum* and *Toxoplasma gondii* in cattle, posing major threats to world health and global economy.

B. besnoiti causes besnoitiosis, an (re)emerging disease of cattle in Europe, with an increasing geographical distribution and the number of cases of infection, compromising animal health and welfare, and responsible for economic losses on affected farms. *N. caninum* is a major cause of abortion in cattle, causing economic loss to both the dairy and beef industries. Among foodborne parasites, *T. gondii* is one of the most common parasitic zoonoses worldwide, with consequences on public health and food safety.

Moreover, due to the sanitary and veterinary importance, the exposure to these Apicomplexa parasites was also investigated in Italian equids. Indeed, besnoitiosis could compromise animal health and welfare in equids, and may be an emerging disease of donkeys also in Europe. *Neospora hughesi* is recognized as an etiological agent of the equine protozoal myeloencephalitis, an important neurological disease of horses; besides, *T. gondii* in horse meat could represent a risk of parasite infection for humans.

Therefore, the aim of my doctoral thesis project was to investigate on the epidemiology and molecular characterization of protozoa parasites of medical and veterinary importance: *Besnoitia* spp., *Neospora* spp. and *T. gondii* both in cattle and in equids in Italy. A multidisciplinary approach based on clinical features, laboratory tests including serological and molecular techniques, was applied throughout the research project, to achieve a multi-level comprehension of the epidemiology of these parasites.

A bovine besnoitiosis endemically infected dairy herd was identified in northern Italy. High intra-herd seroprevalence, clinical signs of the disease in a part of the seropositive animals, and a case of systemic besnoitiosis in a chronically affected cow were reported. Alterations in laboratory parameters were investigated: only mild changes were revealed in hematology, biochemistry, and enzyme activities; besides, increased cortisol levels in seropositive and clinically affected animals. The co-infection of *B. besnoiti* and *Demodex bovis* was diagnosed in two dairy cows from the same farm, reporting the first detection of demodectic mange in bovine besnoitiosis infected cattle.

Bovine besnoitiosis continues to spread in the Italian cattle population. However, besnoitiosis can be considered a neglected parasitic disease of cattle and effective knowledge through dissemination plans among breeders and veterinarians is needed to implement specific control programs. Breeders and veterinarians should be aware of this parasitic disease with consequences on the health and well-being of infected animals, as well as on the economy of affected farms. The surveillance of bovine besnoitiosis should be based on a standardized diagnostic procedure including both clinical and laboratory tests. Moreover, since *B. besnoiti* could have a role in alterations of laboratory parameters, these analyses could aid veterinarians in the diagnosis of bovine besnoitiosis with a view to optimizing a control plan for the disease in the infected herds.

Regarding equids, the first detection of *Besnoitia* spp. specific antibodies in Italian horses and donkeys and the first evidence of clinical besnoitiosis in two donkeys in Italy confirmed the circulation of *Besnoitia* spp. among equids in Europe. Equine besnoitiosis could be almost as spread as bovine besnoitiosis in Europe; however, due to difficulties in the diagnosis, besnoitiosis could be underdiagnosed and underreported, and it may be a neglected disease of donkeys in Europe. An early and accurate diagnosis is fundamental to implement adequate control measures to prevent a "silent" spread of *Besnoitia* spp. infection in equids populations.

N. caninum infection was investigated in two dairy herd performing genetic improvement of Holstein Friesian cattle in Lombardy region to evaluate the spread of neosporosis and its association with the clinical outcome and the effects on reproductive and productive performances. The study demonstrated that although *N. caninum* circulated equally in farms, the dynamics of the parasite infection and its outcome were different.
Moreover, *N. caninum* positive aborted bovine foetuses from Italian Holstein Friesian dairy herds suffering from reproductive problems were subjected to multilocus-microsatellite genotyping (MLMG). The results confirmed the concept of local *N. caninum* sub-populations. Besides, for the first time, it was showed a correlation between the genetic distance of *N. caninum* isolates based on MLMGs and the geographic distance of the places, where the isolates had been obtained.
Regarding *T. gondii*, apart from these two studies conducted on beef cattle and the serological study on horses, included within my doctoral thesis, it is worthy to mention that during my PhD project I actively collaborated to other studies conducted both on livestock and on wildlife. Obtained results confirmed both the exposure and the presence of *T. gondii* tissue cysts in meat of domestic (Gazzonis et al., 2018a) and wild pigs (Gazzonis et al., 2018b), and both in meat (Gazzonis et al., 2019) and milk (Gazzonis et al., 2020) for sheep and goats. In these mentioned

studies genotyping revealed that Type II was the most prevalent in Italian livestock and wildlife, even if also Type I and III were reported, particularly in domestic pigs. Genotyping of *T. gondii* DNA found in beef cattle samples will be further performed.

Regarding the exposure of Italian equids to these protozoa infections, low prevalence of *T. gondii* and *Neospora* spp. in horses raised in Italy was recorded; nevertheless, horse meat could represent a source for human toxoplasmosis.

In conclusion, obtained results from the studies of my PhD project allowed to update information on protozoa of medical and veterinary importance both in domestic ruminants and in equids.
It was demonstrated that bovine besnoitiosis continues to spread in Italy: both clinical and laboratory tests are needed for an accurate diagnosis, and thus to implement plans for the control of the disease. Moreover, *Besnoitia* spp. infection in equids may be considered an emerging disease of donkeys in Italy and also in Europe.
Serological screening on cows and molecular analysis of aborted foetuses confirmed the role of *N. caninum* in abortion and reproductive failure in dairy herds in northern Italy; besides, multilocus microsatellite genotyping confirmed the concept of local *N. caninum* subpopulations.
The zoonotic importance of *T. gondii* should not be underestimated in animal species destined to human consumption, including cattle and horses. Serological data are useful to give an indication of the population exposure to the parasite, whereas molecular methods allow to detect tissue cysts in the edible parts reflecting the risk for humans.

Regarding *Besnoitia* spp. infection, further perspectives include multilocus microsatellite genotyping and whole parasite sequencing, to genetically characterized the parasite species infecting Italian cattle and equids. Moreover, a better understanding of the epidemiology of besnoitiosis in Italian and European donkey populations would be advisable, to discern if the recent case reports represent unusual clusters of infection or may reflect a wider distribution of subclinical infections, largely undetected to date. Besides, more comprehensive studies on microsatellites in *N. caninum* should be undertaken, not only to improve genotyping capabilities, but also to understand the biological relevance and possible functions of these regions in the genome. Finally, the molecular identification and genotyping of *T. gondii* in edible muscle from both cattle and horses should be further performed.

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