Original Paper

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%, that 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 ~~*Besnoitia*~~ *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~~s~~ 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 ~~a~~ surveillance through a diagnostic procedure including both clinical examination and laboratory tests.

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

1. 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 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; Lienard et al. 2011; Gutierrez-Exposito et al. 2017; Gollnick et al., 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.

2. Materials and Methods

2.1 Herd study

2.1.1 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 3 heifers~~, respectively~~. 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.

2.1.2 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 one 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 ~~alimented~~ fed with hay supplemented with an 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 ~~and~~ muggy summer with a few thunderstorms and cold and foggy winter with some snow. The mean annual temperature is ~~of~~ 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.

2.1.3 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~~s~~ 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).

2.1.4 Serology

A serological screening was performed on the whole herd. According to international recommendations (Gutierrez-Exposito et al. 2017), an ELISA test and a subsequent confirmatory Western Blot were employed to detect the presence of anti-*B. besnoiti* specific antibodies. ~~According to international recommendations (Gutierrez-Exposito et al. 2017), with the aim to detect the presence of anti *B. besnoiti* specific antibodies, an ELISA test and a subsequent confirmatory Western Blot were employed to perform a serological screening on the whole herd.~~ 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% = net OD $_{sample}$ / net OD $_{positive\ control}$) × 100. Both samples considered doubtful (25% < S/P% < 30%) and positive (S/P% ≥ 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).

2.1.5 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 ~~were~~ was ~~noted~~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 light~~e~~ning 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.

2.1.6 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, 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).

2.2 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 ~~(Fig. 1)~~. Tissue sample from several organs, including skin of neck, rump and hind legs, eyelid, muzzle, scleral conjunctiva, masseter~~s~~ 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.

2.2.1 Histology and molecular analysis

Tissues samples submitted for histological analysis were embedded in paraffin wax, sectioned at 5 µm, stained with haematoxylin 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/).

3. Results

3.1 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) (Tab. 1).

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, fifteen 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) (Tab. 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 (Tab. 3). 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, reproductive and productive parameters was not detected ($p>0.05$), even considering the subgroup of the cows with *B. besnoiti* tissue cysts in skin.

3.2 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 ~~of~~ 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~~s~~ muscles, tonsils, mediastinal lymph nodes, liver, cardiac muscle, aorta wall, ovaries, uterus and vulva (both in skin and mucosa) (Tab. 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.

4. Discussion

The study confirms the circulation of *B. besnoiti* infection among cattle in Italy ~~(Gentile et al. 2012; Gazzonis et al. 2014; 2017)~~, 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 (Lienard 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 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 one 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-Diaz 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, twelve 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 (Lienard et al. 2011; Alvarez-Garcia et al. 2014), also in this case study the animals of the farm can be stratified in different categories according to both ~~to~~ 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 acquire~~e~~ing 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 (Tab. 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 ~~a~~ statistical evidence was lacking, it is reasonable to consider that a decrease in ~~the~~ 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 ~~to~~with debilitating factors or ~~to~~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 ~~the~~ mucous membranes of the upper respiratory tract, as also pointed out by

previous studies (Alvarez-Garcia 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 (Alvarez-Garcia 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-Diaz 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 (Alvarez-Garcia 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 organisation 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 ~~in regard to~~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). ~~In conclusion, t~~The presence of *B. besnoiti* in muscle should be further investigated ~~in order~~ to clarify if the parasite is commonly found in the cattle muscles and if it should be considered as a novel food-borne parasite.

5. 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 2016; Gutierrez-Exposito et al. 2017), 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 ~~an~~ effective knowledge through dissemination plans among breeders and veterinarians is needed to implement specific control programs.

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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. n° 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.

References

Alvarez-Garcia G, Frey CF, Ortega-Mora LM, Schares G (2013) A century of bovine besnoitiosis: an unknown disease re-emerging in Europe. Trends Parasitol 29:407-415. https://doi.org/10.1016/j.pt.2013.06.002

Álvarez-García G, García-Lunar P, Gutiérrez-Expósito D, Shkap V, Ortega-Mora LM (2014) Dynamics of *Besnoitia besnoiti* infection in cattle. Parasitology 141:1419-1435. https://doi.org/10.1017/S0031182014000729

Arnal M, Gutiérrez-Expósito D, Martínez-Durán D, Regidor-Cerrillo J, Revilla M, Fernández de Luco D, Jiménez-Meléndez A, Ortega-Mora LM, Álvarez-García G (2017) Systemic besnoitiosis in a juvenile roe deer (*Capreolus capreolus*). Transboud Emerg Dis 64:8-14. https://doi.org/10.1111/tbed.12514

Basso W, Lesser M, Grimm F, Hilbe M, Sydler T, Trösch L, Ochs H, Braun U, Deplazes P (2013) Bovine besnoitiosis in Switzerland: imported cases and local transmission. Vet Parasitol 198:265-73. https://doi.org/10.1016/j.vetpar.2013.09.013

Ciurnelli M, Ciarlantini A (1975) Un caso di rogna demodettica nel bovino. Atti SIB VII:290-295.

Cortes H, Leitao A, Gottstein B, Hemphill A (2014) A review on bovine besnoitiosis: a disease with economic impact in herd health management, caused by *Besnoitia besnoiti*. Parasitology 141:1406-1417. https://doi.org/10.1017/S0031182014000262

Diezma-Díaz C, Jiménez-Meléndez A, Fernández M, Gutiérrez-Expósito D, García-Lunar P, Ortega-Mora LM, Pérez-Salas JA, Blanco-Murcia J, Ferre I, Álvarez-García G (2017) Bovine chronic besnoitiosis in a calf: Characterization of a novel *B. besnoiti* isolate from an unusual case report. Vet Parasitol 247:10-18. https://doi.org/10.1016/j.vetpar.2017.09.017

Dubey JP, Sreekumar C, Rosenthal BM, Vianna MCB, Nylund M, Nikander S, Oksanen A (2004) Redescription of *Besnoitia tarandi* (Protozoa: Apicomplexa) from the reindeer (*Rangifer tarandus*). Int J Parasitol 34:1273-1287. https://doi.org/10.1016/j.ijpara.2004.07.002

EFSA (2010) Bovine Besnoitiosis: an emerging disease in Europe. EFSA J 8:1499-1514.

Fernandez-Garcia A, Alvarez-Garcia G, Risco-Castillo V, Aguado-Martinez A, Marcen JM, Rojo-Montejo S, Castillo JA, Ortega-Mora LM (2010) Development and use of an indirect ELISA in an outbreak of bovine besnoitiosis in Spain. Vet Rec 166:818–822. https://doi.org/10.1136/vr.b4874

Fernandez-Garcia A, Alvarez-Garcia G, Risco-Castillo V, Aguado-Martinez A, Marugan-Hernandez V, Ortega-Mora LM (2009) Pattern of recognition of *Besnoitia besnoiti* tachyzoite and bradyzoite antigens by naturally infected cattle. Vet Parasitol 164:104-110. https://doi.org/10.1016/j.vetpar.2009.06.020

Fernandez-Garcia A, Risco-Castillo V, Pedraza-Diaz S, Aguado-Martinez A, Alvarez-Garcia G, Gomez Bautista M, Collantes-Fernandez E, Ortega-Mora LM (2009) First isolation of *Besnoitia besnoiti* from a chronically infected cow in Spain. J Parasitol 95:474-476. https://doi.org/10.1645/GE-1772.1

Frey CF, Gutierrez-Exposito D, Ortega-Mora LM, Benavides J, Marcen JM, Castillo JA, Casasus I, Sanz A, Garcia-Lunar P, Esteban-Gil A, Alvarez-Garcia G (2013) Chronic bovine besnoitiosis: intra-organ parasite distribution, parasite loads and parasite-associated lesions in subclinical cases. Vet Parasitol 197:95-103. https://doi.org/10.1016/j.vetpar.2013.04.023

Garcia-Lunar P, More G, Campero L, Ortega-Mora LM, Alvarez-Garcia G (2015) Anti-*Neospora caninum* and anti-*Sarcocystis* spp. specific antibodies cross-react with *Besnoitia besnoiti* and influence the serological diagnosis of bovine besnoitiosis. Vet Parasitol 214:49-54. https://doi.org/10.1016/j.vetpar.2015.09.011

Garcia-Lunar P, Ortega-Mora LM, Schares G, Gollnick NS, Jacquiet P, Grisez C, Prevot F, Frey CF, Gottstein B, Alvarez-Garcia G (2013) An inter-laboratory comparative study of serological tools employed in the diagnosis of

Besnoitia besnoiti infection in bovines. Transboud Emerg Dis 60:59-68. https://doi.org/10.1111/j.1865-1682.2012.01318.x

Gazzonis AL, Alvarez-Garcia G, Zanzani SA, Garippa G, Rossi L, Maggiora M, Dini V, Invernizzi A, Luini M, Tranquillo VM, Ortega-Mora LM, Manfredi MT (2014) *Besnoitia besnoiti* among cattle in insular and northwestern Italy: endemic infection or isolated outbreaks? Parasit Vectors 7:585. https://doi.org/10.1186/s13071-014-0585-4

Gazzonis AL, Alvarez-Garcia G, Maggioni A, Zanzani SA, Olivieri E, Compiani R, Sironi G, Ortega-Mora LM, Manfredi MT (2017) Serological dynamics and risk factors of *Besnoitia besnoiti* infection in breeding bulls from an endemically infected purebred beef herd. Parasitol Res 116:1383-1393. https://doi.org/10.1007/s00436-017-5418-x

Gentile A, Militerno G, Schares G, Nanni A, Testoni S, Bassi P, Gollnick NS (2012) Evidence for bovine besnoitiosis being endemic in Italy: first *in vitro* isolation of *Besnoitia besnoiti* from cattle born in Italy. Vet Parasitol 184:108-115. https://doi.org/10.1016/j.vetpar.2011.09.014

Gollnick NS, Gentile A, Schares G (2010) Diagnosis of bovine besnoitiosis in a bull born in Italy. Vet Rec 166:599. https://doi.org/10.1136/vr.c2314

Gollnick NS, Scharr JC, Schares G, Langenmayer MC (2015) Natural *Besnoitia besnoiti* infections in cattle: chronology of disease progression. BMC Vet Res 11:35. https://doi.org/10.1186/s12917-015-0344-6

Gollnick NS, Scharr JC, Schares S, Barwald A, Schares G, Langenmayer MC (2018) Naturally acquired bovine besnoitiosis: Disease frequency, risk and outcome in an endemically infected beef herd. Transboud Emerg Dis 65:833-843. https://doi.org/10.1111/tbed.12810

Gutiérrez-Expósito D, Ferre I, Ortega-Mora LM, Álvarez-García G (2017) Advances in the diagnosis of bovine besnoitiosis: current options and applications for control. Int J Parasitol 47:737-751. https://doi.org/10.1016/j.ijpara.2017.08.003

Gutiérrez-Expósito D, Ortega-Mora LM, García-Lunar P, Rojo-Montejo S, Zabala J, Serrano M, Álvarez-Garcia G (2017) Clinical and serological dynamics of *Besnoitia besnoiti* infection in three endemically infected beef cattle herds. Transboud Emerg Dis 64:538-546. https://doi.org/10.1111/tbed.12402

Hornok S, Fedak A, Baska F, Basso W, Dencso L, Toth G, Szeredi L, Abonyi T, Denes B (2015) Vector-borne transmission of *Besnoitia besnoiti* by bloodsucking and secretophagous flies: Epidemiological and clinicopathological implications. Parasit Vectors 8:450. https://doi.org/10.1186/s13071-015-1058-0

Jacquiet P, Liénard E, Franc M (2010) Bovine besnoitiosis: Epidemiological and clinical aspects. Vet Parasitol 174:30-36. https://doi.org/10.1016/j.vetpar.2010.08.013

Langenmayer MC, Majzoub M, Gollnick NS, Scharr J, Schares G, Hermanns W (2012) Histological findings in bovine skin after natural infection with *Besnoitia besnoiti*. J Comp Pathol 146:48. https://doi.org/10.1016/j.jcpa.2011.11.012

Langenmayer MC, Gollnick N, Scharr J, Schares G, Herrmann D, Majzoub-Altweck M, Hermanns W (2015) *Besnoitia besnoiti* infection in cattle and mice: ultrastructural pathology in acute and chronic besnoitiosis. Parasitol Res 114:955-963. https://doi.org/10.1007/s00436-014-4261-6

Langenmayer MC, Gollnick NS, Majzoub-Altweck M, Scharr JC, Schares G, Hermanns W (2015) Naturally acquired bovine besnoitiosis: histological and immunohistochemical findings in acute, subacute, and chronic disease. Vet Pathol 52:476-488. https://doi.org/10.1177/0300985814541705

Liénard E, Salem A, Grisez C, Prévot F, Bergeaud JP, Franc M, Gottstein B, Alzieu JP, Lagalisse Y, Jacquiet P (2011) A longitudinal study of *Besnoitia besnoiti* infections and seasonal abundance of *Stomoxys calcitrans* in a dairy cattle farm of southwest France. Vet Parasitol 177:20-27. https://doi.org/10.1016/j.vetpar.2010.11.030

Lopez J, Grau-Roma L, Martinez J, Majo N, Marco A, Castillo A, Domingo M (2012) Histopathological findings in animals affected by bovine besnoitiosis, with special focus on male genital organs. J Comp Pathol 146:48. https://doi.org/10.1016/j.jcpa.2011.11.013

Manfredini L, Marangon S, Zucchetta S, Scarpa P, Arcangeli G (1994) La rogna demodettica nei bovini. Osservazioni cliniche in allevamenti della provincia di Venezia. Doc Vet 9:37-41.

Manuali E, Lepri E, Salamida S, D'Avino N, Mangili P, Vitellozzi G, Grelloni V, Filippini G (2011) An outbreak of bovine besnoitiosis in beef cattle born in Central Italy. Transboud Emerg Dis 58:464-467. https://doi.org/10.1111/j.1865-1682.2011.01220.x

Mutinelli F, Schiavon E, Ceglie L, Fasolato M, Natale A, Rampin F, Carminato A (2011) Bovine besnoitiosis in imported cattle in Italy. Vet Parasitol 178:198. https://doi.org/10.1016/j.vetpar.2010.12.019

Nobel TA, Neumann M, Klopfer U, Perl S (1977) Kystes de *Besnoîtia besnoîti* dans les organes génitaux de la vache. B Acad Vét France 50:569-574. https://doi.org/10.4267/2042/66051

Nobel TA, Klopfer U, Perl S, Nyska A, Neumann M, Brenner G (1981) Histopathology of genital besnoitiosis of cows in Israel. Vet Parasitol 8:271-276. https://doi.org/10.1016/0304-4017(81)90059-5

Olias P, Schade B, Mehlhorn H (2011) Molecular pathology, taxonomy and epidemiology of *Besnoitia* species (Protozoa: Sarcocystidae). Infect Genet Evol 11:1564-1576. https://doi.org/10.1016/j.meegid.2011.08.006

Pisoni G, Serotti L, Ottavis G, Pavone S, D'Avino N, Toni F (2017) Focolaio di besnoitiosi associato a Febbre Q in un allevamento di bovine da latte in Lombardia. Atti SIB XLIX:60-66.

Rinaldi L, Maurelli MP, Musella V, Bosco A, Cortes H, Cringoli G (2013) First cross-sectional serological survey on *Besnoitia besnoiti* in cattle in Italy. Parasitol Res 112:1805-1807. https://doi.org/10.1007/s00436-012-3241-y

Ryan EG, Lee A, Carty C, O'Shaughnessy J, Kelly P, Cassidy JP, Sheehan M, Johnson A, de Waal T (2016) Bovine besnoitiosis (*Besnoitia besnoiti*) in an Irish dairy herd. Vet Rec 178:608. https://doi.org/10.1136/vr.103683

Schares G, Langenmayer MC, Majzoub-Altvveck M, Scharr JC, Gentile A, Maksimov A, Schares S, Conraths FJ, Gollnick NS (2016) Naturally acquired bovine besnoitiosis: differential distribution of parasites in the skin of chronically infected cattle. Vet Parasitol 216:101-107. https://doi.org/10.1016/j.vetpar.2015.12.016

Vanhoudt A, Pardon B, De Schutter P, Bosseler T, Sarre C, Vercruysse J, Deprez P (2015) First confirmed case of bovine besnoitiosis in an imported bull in Belgium. Vlaams Diergeneeskund Tijdschr 84:205-211.

Villa L, Gazzonis AL, Alvarez-Garcia G, Diezma-Diaz C, Zanzani SA, Manfredi MT (2018) First detection of anti-*Besnoitia* spp specific antibodies in horses and donkeys in Italy. Parasitol Int 67:640-643. https://doi.org/10.1016/j.parint.2018.06.008

Tab. 1. Serological prevalence (P) of *Besnoitia besnoiti* infection in an infected dairy cattle herd in Italy according to both ~~to~~ 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%

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

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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.

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

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Figure Legends

Fig. 1. Cow affected by the chronic phase of bovine besnoitiosis.

Original Paper

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%, that 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.

1. 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 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; Lienard et al. 2011; Gutierrez-Exposito et al. 2017; Gollnick et al., 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.

2. Materials and Methods

2.1 Herd study

2.1.1 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 3 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.

2.1.2 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 one 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 an 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.

2.1.3 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).

2.1.4 Serology

A serological screening was performed on the whole herd. According to international recommendations (Gutierrez-Exposito et al. 2017), 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).

2.1.5 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.

2.1.6 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, reproductive (number of parturitions, number of inseminations, days between calving) and

138 productive parameters (daily Kg milk, % fat, % protein, somatic cell count, Mature Equivalent Milk Yield) on *B. besnoiti*
139 infection; the binary outcome (presence/absence of anti-*B. besnoiti* antibodies) on the basis of Western Blot results was
140 used as dependent variable. Furthermore, a second model was run considering the same independent variables and as
141 dependent variable the presence of *B. besnoiti* tissue cysts in the skin, i.e. affected by chronic besnoitiosis (binary
142 outcome) demonstrated at the clinical examination in seropositive animals. In both models, among individual
143 characteristics, only age was considered, since sex, breed and origin were the same for all the sampled animals. Besides,
144 GLMs were carried out considering only the productive category of cows. The models were developed through a
145 backward selection procedure (significance level to remove variables from the model = 0.05), based on AIC values.
146 Statistical analysis was performed using SPSS software (Statistical Package for Social Science, IBM SPSS Statistics for
147 Windows, Version 25.0., Chicago, IL, USA).
148
149 2.2 Case report
150
151 Among clinically examined animals, a form of chronic besnoitiosis was diagnosed in a cow that was regularly
152 slaughtered being in poor conditions and with severe skin lesions. Tissue sample from several organs, including skin of
153 neck, rump and hind legs, eyelid, muzzle, scleral conjunctiva, masseter muscles, mucous membranes of the upper
154 respiratory tract, tonsils, mediastinal lymph nodes, lungs, liver, cardiac muscle, aorta wall, spleen, ovaries, uterus and
155 vulva, were collected at slaughterhouse and transported refrigerated to the laboratory. An aliquot of these tissues was
156 fixed in 10% buffered formalin for histological examination; another part was mechanically homogenized and stored at -
157 20°C for molecular analyses.
158
159 *2.2.1 Histology and molecular analysis*
160 Tissues samples submitted for histological analysis were embedded in paraffin wax, sectioned at 5 μm, stained with
161 haematoxylin and eosin (HE) and microscopically examined.
162 Tissue sample homogenates were processed to extract genomic DNA using a commercial kit (NucleoSpin® Tissue,
163 Macherey-Nagel, Germany), following the manufacturer's instructions. DNA samples were analyzed using a
164 conventional PCR targeting a region of 231 bp of the ITS-1 region as described by Cortes et al (2007). Positive
165 (Gazzonis et al. 2017) and negative (non-template) controls were inserted in each run. PCR products were run on 1.5%
166 agarose gel containing 0.05% ethidium bromide in TBE buffer electrophoresis and visualized under UV light on a
167 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/).

3. Results

3.1 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) (Tab. 1).

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, fifteen 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) (Tab. 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 (Tab. 3). 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, reproductive and productive parameters was not detected ($p>0.05$), even considering the subgroup of the cows with *B. besnoiti* tissue cysts in skin.

198 3.2 Case report

199

200 Concerning the slaughtered cow chronically infected by besnoitiosis, histology carried out on tissue samples confirmed
201 the highest concentration of *Besnoitia* cysts in the skin of the neck, rump, hind legs, eyelid and vulva, in muzzle and in
202 mucosal membranes of the upper respiratory tract. Fewer and smaller dimension cysts were also seen in the lung. No
203 *Besnoitia* cysts were detected in the liver, heart, aorta wall, tonsils, mediastinal lymph nodes, spleen, ovaries, uterus and
204 vulvar mucosa. In the skin, hyperkeratosis and dermal inflammation with infiltration of macrophages, plasma cells,
205 eosinophils and lymphocytes were also present.
206 The presence of parasite DNA was confirmed in tissues where *B. besnoiti* cysts were evidenced by histological
207 examination and also in other organs, i.e. masseter muscles, tonsils, mediastinal lymph nodes, liver, cardiac muscle, aorta
208 wall, ovaries, uterus and vulva (both in skin and mucosa) (Tab. 4). Sequencing of 231-bp PCR fragments from all
209 examined tissues confirmed that they belonged to *B. besnoiti* with a homology of 100%. One of the obtained sequences
210 was submitted to GenBank under accession number MN104147.

211

212

213 **4. Discussion**

214

215 The study confirms the circulation of *B. besnoiti* infection among cattle in Italy, reporting a case of bovine besnoitiosis in
216 a dairy farm in Northern Italy. High seroprevalence of antibodies against *B. besnoiti* with a part of the seropositive
217 animals showing clinical signs, but only a few animals affected by a severe form of the disease, suggests that the
218 infection might have been undetected in the herd since years. Indeed, in this herd, an overall seroprevalence of 23.5%
219 was recorded. The percentage results higher when compared to a previous study conducted in a dairy farm in Central
220 Italy reporting an overall seroprevalence of 9.7% and of 17% if only lactating cows were considered (Gentile et al. 2012).
221 Previously, seropositivity to *B. besnoiti* in dairy cows was also detected in the Lombardy region, where Gazzonis et al
222 (2014) recorded an intra-herd prevalence of 5 and 5.2% in two dairy farms. Studies concerning *B. besnoiti* infection in
223 dairy cattle in Europe are limited; moreover, higher prevalence values in dairy cattle farms were reported in Ireland
224 (68%) and in France (Lienard et al. 2011; Ryan et al. 2016). Concerning Northern Italy, similar values of *B. besnoiti*
225 infection were reported in a serological survey conducted in a beef herd (36.5%) (Gazzonis et al. 2017), suggesting a
226 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 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 one 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-Diaz 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, twelve 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 (Lienard et al. 2011; Alvarez-Garcia 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 (Tab. 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 (Alvarez-Garcia 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 (Alvarez-Garcia 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-Diaz 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 (Alvarez-Garcia 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 organisation 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.

5. 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 2016; Gutierrez-Exposito et al. 2017), 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.

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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. n° 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.

References

Alvarez-Garcia G, Frey CF, Ortega-Mora LM, Schares G (2013) A century of bovine besnoitiosis: an unknown disease re-emerging in Europe. Trends Parasitol 29:407-415. https://doi.org/10.1016/j.pt.2013.06.002

Álvarez-García G, García-Lunar P, Gutiérrez-Expósito D, Shkap V, Ortega-Mora LM (2014) Dynamics of *Besnoitia besnoiti* infection in cattle. Parasitology 141:1419-1435. https://doi.org/10.1017/S0031182014000729

Arnal M, Gutiérrez-Expósito D, Martínez-Durán D, Regidor-Cerrillo J, Revilla M, Fernández de Luco D, Jiménez-Meléndez A, Ortega-Mora LM, Álvarez-García G (2017) Systemic besnoitiosis in a juvenile roe deer (*Capreolus capreolus*). Transboud Emerg Dis 64:8-14. https://doi.org/10.1111/tbed.12514

Basso W, Lesser M, Grimm F, Hilbe M, Sydler T, Trösch L, Ochs H, Braun U, Deplazes P (2013) Bovine besnoitiosis in Switzerland: imported cases and local transmission. Vet Parasitol 198:265-73. https://doi.org/10.1016/j.vetpar.2013.09.013

Ciurnelli M, Ciarlantini A (1975) Un caso di rogna demodettica nel bovino. Atti SIB VII:290-295.

Cortes H, Leitao A, Gottstein B, Hemphill A (2014) A review on bovine besnoitiosis: a disease with economic impact in herd health management, caused by *Besnoitia besnoiti*. Parasitology 141:1406-1417. https://doi.org/10.1017/S0031182014000262

Diezma-Díaz C, Jiménez-Meléndez A, Fernández M, Gutiérrez-Expósito D, García-Lunar P, Ortega-Mora LM, Pérez-Salas JA, Blanco-Murcia J, Ferre I, Álvarez-García G (2017) Bovine chronic besnoitiosis in a calf: Characterization of a novel *B. besnoiti* isolate from an unusual case report. Vet Parasitol 247:10-18. https://doi.org/10.1016/j.vetpar.2017.09.017

Dubey JP, Sreekumar C, Rosenthal BM, Vianna MCB, Nylund M, Nikander S, Oksanen A (2004) Redescription of *Besnoitia tarandi* (Protozoa: Apicomplexa) from the reindeer (*Rangifer tarandus*). Int J Parasitol 34:1273-1287. https://doi.org/10.1016/j.ijpara.2004.07.002

EFSA (2010) Bovine Besnoitiosis: an emerging disease in Europe. EFSA J 8:1499-1514.

Fernandez-Garcia A, Alvarez-Garcia G, Risco-Castillo V, Aguado-Martinez A, Marcen JM, Rojo-Montejo S, Castillo JA, Ortega-Mora LM (2010) Development and use of an indirect ELISA in an outbreak of bovine besnoitiosis in Spain. Vet Rec 166:818–822. https://doi.org/10.1136/vr.b4874

Fernandez-Garcia A, Alvarez-Garcia G, Risco-Castillo V, Aguado-Martinez A, Marugan-Hernandez V, Ortega-Mora LM (2009) Pattern of recognition of *Besnoitia besnoiti* tachyzoite and bradyzoite antigens by naturally infected cattle. Vet Parasitol 164:104-110. https://doi.org/10.1016/j.vetpar.2009.06.020

Fernandez-Garcia A, Risco-Castillo V, Pedraza-Diaz S, Aguado-Martinez A, Alvarez-Garcia G, Gomez Bautista M, Collantes-Fernandez E, Ortega-Mora LM (2009) First isolation of *Besnoitia besnoiti* from a chronically infected cow in Spain. J Parasitol 95:474-476. https://doi.org/10.1645/GE-1772.1

Frey CF, Gutierrez-Exposito D, Ortega-Mora LM, Benavides J, Marcen JM, Castillo JA, Casasus I, Sanz A, Garcia-Lunar P, Esteban-Gil A, Alvarez-Garcia G (2013) Chronic bovine besnoitiosis: intra-organ parasite distribution, parasite loads and parasite-associated lesions in subclinical cases. Vet Parasitol 197:95-103. https://doi.org/10.1016/j.vetpar.2013.04.023

Garcia-Lunar P, More G, Campero L, Ortega-Mora LM, Alvarez-Garcia G (2015) Anti-*Neospora caninum* and anti-*Sarcocystis* spp. specific antibodies cross-react with *Besnoitia besnoiti* and influence the serological diagnosis of bovine besnoitiosis. Vet Parasitol 214:49-54. https://doi.org/10.1016/j.vetpar.2015.09.011

Garcia-Lunar P, Ortega-Mora LM, Schares G, Gollnick NS, Jacquiet P, Grisez C, Prevot F, Frey CF, Gottstein B, Alvarez-Garcia G (2013) An inter-laboratory comparative study of serological tools employed in the diagnosis of *Besnoitia besnoiti* infection in bovines. Transboud Emerg Dis 60:59-68. https://doi.org/10.1111/j.1865-1682.2012.01318.x

Gazzonis AL, Alvarez-Garcia G, Zanzani SA, Garippa G, Rossi L, Maggiora M, Dini V, Invernizzi A, Luini M, Tranquillo VM, Ortega-Mora LM, Manfredi MT (2014) *Besnoitia besnoiti* among cattle in insular and northwestern Italy: endemic infection or isolated outbreaks? Parasit Vectors 7:585. https://doi.org/10.1186/s13071-014-0585-4

Gazzonis AL, Alvarez-Garcia G, Maggioni A, Zanzani SA, Olivieri E, Compiani R, Sironi G, Ortega-Mora LM, Manfredi MT (2017) Serological dynamics and risk factors of *Besnoitia besnoiti* infection in breeding bulls from an endemically infected purebred beef herd. Parasitol Res 116:1383-1393. https://doi.org/10.1007/s00436-017-5418-x

Gentile A, Militerno G, Schares G, Nanni A, Testoni S, Bassi P, Gollnick NS (2012) Evidence for bovine besnoitiosis being endemic in Italy: first *in vitro* isolation of *Besnoitia besnoiti* from cattle born in Italy. Vet Parasitol 184:108-115. https://doi.org/10.1016/j.vetpar.2011.09.014

Gollnick NS, Gentile A, Schares G (2010) Diagnosis of bovine besnoitiosis in a bull born in Italy. Vet Rec 166:599. https://doi.org/10.1136/vr.c2314

Gollnick NS, Scharr JC, Schares G, Langenmayer MC (2015) Natural *Besnoitia besnoiti* infections in cattle: chronology of disease progression. BMC Vet Res 11:35. https://doi.org/10.1186/s12917-015-0344-6

Gollnick NS, Scharr JC, Schares S, Barwald A, Schares G, Langenmayer MC (2018) Naturally acquired bovine besnoitiosis: Disease frequency, risk and outcome in an endemically infected beef herd. Transboud Emerg Dis 65:833-843. https://doi.org/10.1111/tbed.12810

Gutiérrez-Expósito D, Ferre I, Ortega-Mora LM, Álvarez-García G (2017) Advances in the diagnosis of bovine besnoitiosis: current options and applications for control. Int J Parasitol 47:737-751. https://doi.org/10.1016/j.ijpara.2017.08.003

Gutiérrez-Expósito D, Ortega-Mora LM, García-Lunar P, Rojo-Montejo S, Zabala J, Serrano M, Álvarez-Garcia G (2017) Clinical and serological dynamics of *Besnoitia besnoiti* infection in three endemically infected beef cattle herds. Transboud Emerg Dis 64:538-546. https://doi.org/10.1111/tbed.12402

Hornok S, Fedak A, Baska F, Basso W, Dencso L, Toth G, Szeredi L, Abonyi T, Denes B (2015) Vector-borne transmission of *Besnoitia besnoiti* by bloodsucking and secretophagous flies: Epidemiological and clinicopathological implications. Parasit Vectors 8:450. https://doi.org/10.1186/s13071-015-1058-0

Jacquiet P, Liénard E, Franc M (2010) Bovine besnoitiosis: Epidemiological and clinical aspects. Vet Parasitol 174:30-36. https://doi.org/10.1016/j.vetpar.2010.08.013

Langenmayer MC, Majzoub M, Gollnick NS, Scharr J, Schares G, Hermanns W (2012) Histological findings in bovine skin after natural infection with *Besnoitia besnoiti*. J Comp Pathol 146:48. https://doi.org/10.1016/j.jcpa.2011.11.012

Langenmayer MC, Gollnick N, Scharr J, Schares G, Herrmann D, Majzoub-Altweck M, Hermanns W (2015) *Besnoitia besnoiti* infection in cattle and mice: ultrastructural pathology in acute and chronic besnoitiosis. Parasitol Res 114:955-963. https://doi.org/10.1007/s00436-014-4261-6

Langenmayer MC, Gollnick NS, Majzoub-Altweck M, Scharr JC, Schares G, Hermanns W (2015) Naturally acquired bovine besnoitiosis: histological and immunohistochemical findings in acute, subacute, and chronic disease. Vet Pathol 52:476-488. https://doi.org/10.1177/0300985814541705

Liénard E, Salem A, Grisez C, Prévot F, Bergeaud JP, Franc M, Gottstein B, Alzieu JP, Lagalisse Y, Jacquiet P (2011) A longitudinal study of *Besnoitia besnoiti* infections and seasonal abundance of *Stomoxys calcitrans* in a dairy cattle farm of southwest France. Vet Parasitol 177:20-27. https://doi.org/10.1016/j.vetpar.2010.11.030

Lopez J, Grau-Roma L, Martinez J, Majo N, Marco A, Castillo A, Domingo M (2012) Histopathological findings in animals affected by bovine besnoitiosis, with special focus on male genital organs. J Comp Pathol 146:48. https://doi.org/10.1016/j.jcpa.2011.11.013

Manfredini L, Marangon S, Zucchetta S, Scarpa P, Arcangeli G (1994) La rogna demodettica nei bovini. Osservazioni cliniche in allevamenti della provincia di Venezia. Doc Vet 9:37-41.

Manuali E, Lepri E, Salamida S, D'Avino N, Mangili P, Vitellozzi G, Grelloni V, Filippini G (2011) An outbreak of bovine besnoitiosis in beef cattle born in Central Italy. Transboud Emerg Dis 58:464-467. https://doi.org/10.1111/j.1865-1682.2011.01220.x

Mutinelli F, Schiavon E, Ceglie L, Fasolato M, Natale A, Rampin F, Carminato A (2011) Bovine besnoitiosis in imported cattle in Italy. Vet Parasitol 178:198. https://doi.org/10.1016/j.vetpar.2010.12.019

Nobel TA, Neumann M, Klopfer U, Perl S (1977) Kystes de *Besnoîtia besnoîti* dans les organes génitaux de la vache. B Acad Vét France 50:569-574. https://doi.org/10.4267/2042/66051

Nobel TA, Klopfer U, Perl S, Nyska A, Neumann M, Brenner G (1981) Histopathology of genital besnoitiosis of cows in Israel. Vet Parasitol 8:271-276. https://doi.org/10.1016/0304-4017(81)90059-5

Olias P, Schade B, Mehlhorn H (2011) Molecular pathology, taxonomy and epidemiology of *Besnoitia* species (Protozoa: Sarcocystidae). Infect Genet Evol 11:1564-1576. https://doi.org/10.1016/j.meegid.2011.08.006

Pisoni G, Serotti L, Ottavis G, Pavone S, D'Avino N, Toni F (2017) Focolaio di besnoitiosi associato a Febbre Q in un allevamento di bovine da latte in Lombardia. Atti SIB XLIX:60-66.

Rinaldi L, Maurelli MP, Musella V, Bosco A, Cortes H, Cringoli G (2013) First cross-sectional serological survey on *Besnoitia besnoiti* in cattle in Italy. Parasitol Res 112:1805-1807. https://doi.org/10.1007/s00436-012-3241-y

Ryan EG, Lee A, Carty C, O'Shaughnessy J, Kelly P, Cassidy JP, Sheehan M, Johnson A, de Waal T (2016) Bovine besnoitiosis (*Besnoitia besnoiti*) in an Irish dairy herd. Vet Rec 178:608. https://doi.org/10.1136/vr.103683

Schares G, Langenmayer MC, Majzoub-Altvveck M, Scharr JC, Gentile A, Maksimov A, Schares S, Conraths FJ, Gollnick NS (2016) Naturally acquired bovine besnoitiosis: differential distribution of parasites in the skin of chronically infected cattle. Vet Parasitol 216:101-107. https://doi.org/10.1016/j.vetpar.2015.12.016

492 Vanhoudt A, Pardon B, De Schutter P, Bosseler T, Sarre C, Vercruysse J, Deprez P (2015) First confirmed case of
493 bovine besnoitiosis in an imported bull in Belgium. Vlaams Diergeneeskund Tijdschr 84:205-211.
494
495 Villa L, Gazzonis AL, Alvarez-Garcia G, Diezma-Diaz C, Zanzani SA, Manfredi MT (2018) First detection of anti-
496 *Besnoitia* spp specific antibodies in horses and donkeys in Italy. Parasitol Int 67:640-643.
497 https://doi.org/10.1016/j.parint.2018.06.008

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%

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

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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	54	Seronegative	31.43 (8.96)	16.3-53.2
(in kg)	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.

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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

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