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Bovine besnoitiosis: Assessment of the diagnostic accuracy of three different tests using a Bayesian latent class model approach and clinical characterization of the disease

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ABSTRACT

Bovine besnoitiosis, a disease caused by the tissue cyst-forming apicomplexan *Besnoitia besnoiti*, is re-emerging in Europe, leading to significant impairment of health and production, as well as economic losses. The early detection of the disease is of the utmost importance for the implementation of effective control measures, yet this is a challenge due to the lack of specific early clinical signs. The objectives of our study were 1) to estimate the diagnostic accuracy of three tests to detect *B. besnoiti* in naturally exposed cattle (histopathology-skin (HIS-SK); PCR-skin (PCR-SK); and parallel PCR of nasal and scleroconjunctival swabs (PCR-NS-SC)) using a Bayesian latent class model (BLCM) and 2) to describe the clinical presentation of besnoitiosis in the studied animals. The study involved 54 adult Limousin cattle. Biosecurity measures were assessed and scored as medium. At clinical examination, a sire was diagnosed with a form of besnoitiosis between the end of the acute phase and the beginning of the chronic phase. Furthermore, 29 animals displaying a subclinical infection, characterized by the presence of scleroconjunctival cysts, were identified. The PCR-SK and PCR-NS-SC were able to detect *B. besnoitia*. The diagnostic performance of PCR-SK, PCR-NS-SC and HIS-SK was evaluated. The BLCM indicated that HIS-SK had the highest specificity (99.1 %, 95 % posterior probability interval PI: 96–100 %), while PCR-SK and PCR-NS-SC demonstrated higher sensitivities (91.0 %, 95 % PI: 68–100 %, and 85.0 %, 95 % PI: 67–100 %, respectively). The study concludes that the use of a parallel PCR-NS-SC could represent a viable alternative for the early detection of *B. besnoiti*, providing a less invasive method to monitor and control bovine besnoitiosis at the herd level.

1. Introduction

Bovine besnoitiosis is a re-emerging disease in Europe and has a wide distribution including many countries in Europe, Asia, Africa and the Middle East (Gentile et al., 2012; Lee et al., 1970; Malatji et al., 2023). This disease mainly affects cattle and causes significant economic losses,

Abbreviations: SC, scleroconjunctival; NS, nasal; PCR-SK, PCR on skin biopsies; PCR-SC, PCR on scleroconjunctival swabs; PCR-NS, PCR on nasal swabs; PCR-NS-SC, parallel PCR on nasal swabs and scleroconjuctival swabs; PI, posterior probability interval; HIS, histopathology; BLCM, Bayesian latent class model.

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mostly associated with reduced health, production and reproduction (Cortes et al., 2014; Malatji et al., 2023). This parasitic disease is caused by the tissue cyst-forming apicomplexan *Besnoitia besnoiti* (Besnoit and Robin, 1912), which is genetically related to *Neospora caninum* and *Toxoplasma gondii*, but exhibits differences in life cycle and transmission. The life cycle of *Besnoitia besnoiti* is suspected to be heteroxenous, but the definitive host is still currently unknown (Basso et al., 2011a; Millán et al., 2012; Ramakrishnan et al., 2022). The primary suspected definitive host is the domestic cat. Nevertheless, the formation of intestinal sexual stages (oocysts) as the first stage of transmission has only been identified in the domestic cat (definitive host) for *B. darlingi*, *B. wallacei*, *B. oryctofelisi* and *B. neotomofelis* (Dubey and Lindsay, 2003; Olias et al., 2011; Smith and Frenkel, 1977; Wallace and Frenkel, 1975). Bovine species represent the most important intermediate hosts of *B. besnoiti*, exhibiting two distinct asexual parasitic stages: tachyzoites and bradyzoites (EFSA, 2010). Tachyzoites replicate rapidly, primarily in endothelial cells, and are responsible for the onset of acute clinical disease. During this phase, tachyzoites can also be detected extracellularly and intracellularly in blood, monocytes and neutrophils (Schares et al., 2009). The tachyzoites then undergo a transformation into bradyzoites, a process that is dependent on a mechanism that enables evasion of the immune response (Schares et al., 2009). Consequently, the bradyzoites form a thick-walled tissue cysts, which replicates at a slow rate and are typically found in subcutaneous tissue and mucous membranes, resulting in the chronic clinical disease (Olias et al., 2011). Transmission is known to occur by close contact (e.g. mating) or horizontally by mechanical transmission by biting flies (Álvarez-García et al., 2013).

During the acute phase, cattle may present pyrexia, nasal and ocular discharge, salivation, lameness and, in severe cases, clinically visible subcutaneous edema, which is most prevalent in the limbs. During the chronic phase, the skin may become severely hyperkeratotic and alopecic. Furthermore, bulls may develop orchitis, which may result in either temporary or permanent infertility (Bigalke, 1968; Cortes et al., 2014, Masebo et al., 2022). However, the majority of infected cattle are asymptomatic (Bigalke, 1968, Cortes et al., 2014). The detection of infected cattle in the acute phase is challenging due to the often unspecific clinical signs. In addition, the presence of antibodies and parasite stages in tissues may only be identified at a later stage of infection (Schares et al., 2013, 2011). Thus, early detection of infected cattle is important to prevent and control the spread of bovine besnoitiosis at the herd, national and international levels.

Prior to the development of PCR tests, early phases of infection were diagnosed by analysing blood and lymph node smears for *B. besnoiti* (Pols, 1960). More recently, PCR and real-time PCR assays for *B. besnoiti* have been developed using skin biopsies, which are reference standard techniques for diagnosing clinical cases (Cortes et al., 2007, 2006; Schares et al., 2013, 2011). However, skin biopsies are an invasive diagnostic method that involves the removal of a sample of tissue using a circular blade or punch tool. In the chronic phase, the presence of bradyzoite-containing tissue cysts in the scleral conjunctiva, vaginal mucosa, or skin, which can be identified macroscopically on clinical examination, allows the performance of an initial diagnosis (Cortes et al., 2006; Schares et al., 2009). The diagnosis can subsequently be confirmed by detecting specific antibodies using various serological tests or parasite's DNA by PCR or real-time PCR (Schares et al., 2013, 2011).

A popular approach to model test accuracy parameters, as well as dependence between tests, is Bayesian latent class analysis which is based on Bayes' theorem of conditional probability (Joseph et al., 1995; van Smeden et al., 2014). Bayesian latent class models (BLCMs) can include prior information on disease prevalence or test accuracy parameters, where available, and can therefore provide reliable estimates of disease prevalence and test parameters, facilitating the interpretation of the diagnostic tests considered (Branscum et al., 2005).

The first objective of this study was to evaluate the diagnostic accuracy of three tests for the detection of *B. besnoiti* in a naturally infected Limousine herd using a BLCM. The tests included skin histopathology (HIS-SK), skin PCR (PCR-SK), and a parallel PCR of nasal and scleroconjunctival swabs (PCR-NS-SC). The second objective was to provide a description of the clinical presentation of besnoitiosis in the animals under study.

2. Material and methods

2.1. Reporting standards

The study was conducted in accordance with the Standards for Reporting of Diagnostic Accuracy Studies that use Bayesian Latent Class Models (STARD-BLCM) (Kostoulas et al., 2017) (Supplementary Table S1).

2.2. Herd, housing and management

The study was conducted in a commercial Limousine cow-calf operation comprising 120 Limousine cows and three sires (one of which was imported from France) in the Apennine Mountains region of Italy. The present study was carried out between the months of November and December 2023.

From April to November, the animals had access to the pasture. During the remaining months, they were confined to an indoor freestall system. The farm consisted of three barns of similar size separated by a 10 m corridor (Fig. 1A). The barns were well ventilated and semi-closed with access to an external paddock. Each barn had 4 pens in a free-stall system. Feeders were placed along one side of the manger (Fig. 1B). The pens were built side by side and separated by iron bars allowing interaction of animals in adjacent pens. All pens had a deep bedding based on the repeated spreading of straw material.

Only natural mating was performed. All adult animals were treated with ivermectin (Ivomec®, Boehringer Ingelheim) twice a year. Animals were purchased within the last 12 months and were not tested for *B. besnoitia* on arrival. One of these was a sire imported from France that was referred to the Clinic for Ruminants of the University of Bologna on suspicion of clinical besnoitiosis in November 2023. Lameness, hyperkeratotic skin lesions, fever and swelling of the limbs were reported. The sire had been introduced to the herd two months previously and had been mounting cows since his arrival. The farm of origin of the sire was known to be endemic for besnoitiosis.

2.3. Biosecurity assessment

An adapted version of the Italian protocol for the biosecurity assessment beef cattle operations included in the ClassyFarm system (Bertocchi et al., 2020) was used as previously reported (Dini et al., 2024a; Masebo et al., 2023). The protocol used contained a list of 17 items: pests control measures, interaction with other animal species, general precautions to the entrance of occasional visitors, general precautions to the entrance of regular visitors, disinfection of vehicles upon entering the farm, possibility of contact between foreign vehicles and farmed animals (< 20 m), carcass collection (< 20 m), live animal loading, quarantine/housing management, control and prevention of most prevalent infectious diseases, health monitoring activities, control and prevention of endo/ectoparasites, control and analysis of water sources, cleaning of troughs/water point, storage buildings and rooms (hygiene, cleanliness and management of housing environment and bedding) and origin of the drinking water (Supplementary Table S2). For each item, a 2- or 3-point scale scoring system was applied (1 =insufficient; 2 =acceptable; 3 =optimal). A score for each section was calculated by summing the scores obtained for each item from each section. The obtained scores were then converted into percentages. A score below 59 % indicated a poor status (=low), a score between 60 % and 80 % indicated a medium status (=medium) and a score above 80 % indicated good status (=high).

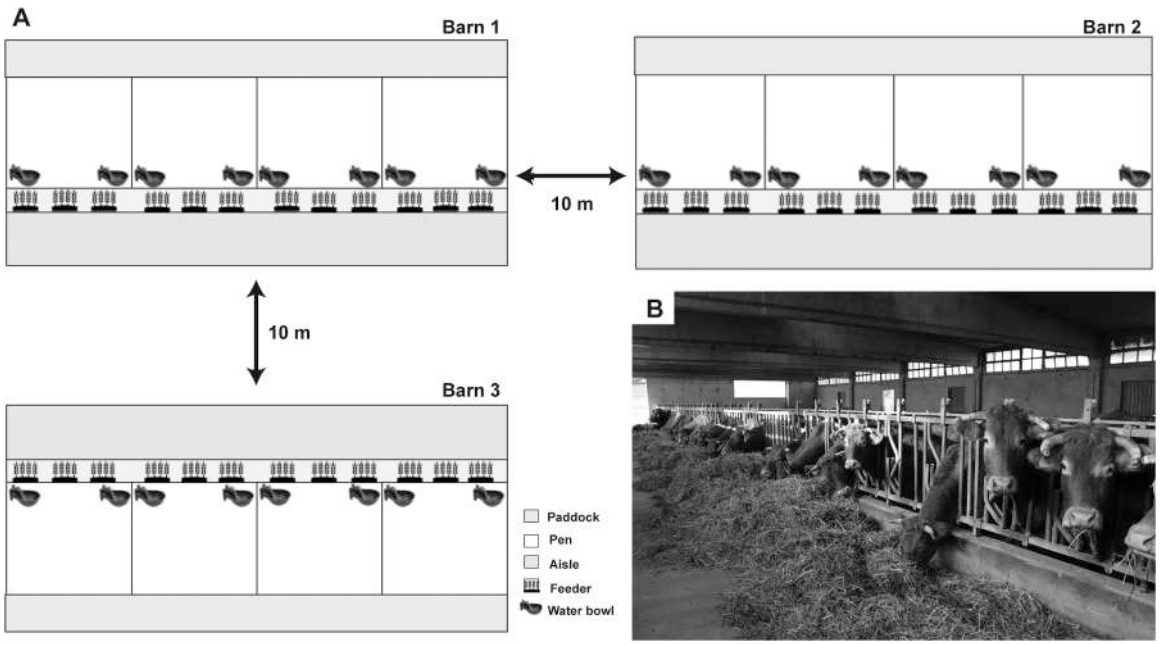

Fig. 1. Limousine cow-calf operation. (A) Schematic representation of the unit. (B) Image of one barn.

2.4. Study population, clinical investigation and samples

Considering an average within-herd prevalence of 50 % for *B. besnoiti* (Supplementary Table S3), a total of 54 adult Limousin cattle from the cow-calf herd described above were randomly selected and included in the study. All animals were clinically examined. Particular attention was given to the nasal and scleroconjunctival regions to detect any macroscopically visible bradyzoite cysts using an eye flashlight. The integumentary system was also carefully inspected for any lesions (e.g. hyperkeratosis, alopecia, scaling).

Skin biopsies (SK) from the neck and nasal and scleroconjunctival swabs were sampled (Fig. 2). SK for histopathology were obtained using an 8 mm sterile punch and fixed in formalin. SK for PCR were collected using a 5 mm sterile punch and stored in a sterile tube at - 20 °C until analysis.

Nasal and scleroconjunctival swabs for molecular diagnosis were taken using sterile dry swabs (MWE®, UK) stored in a sterile tube at −20 °C until analysis. To collect the nasal swabs (NS), animals were restrained and the nostrils cleaned with paper before performing swabbing to avoid contamination. To collect the scleroconjunctival swabs (SC), animals were restrained and the swab was placed in the inferior and superior medial canthus of one eye and in the superior and inferior sclera for 1 minute. The animals kept their eyes closed throughout the procedure. The study was conducted in a blinded

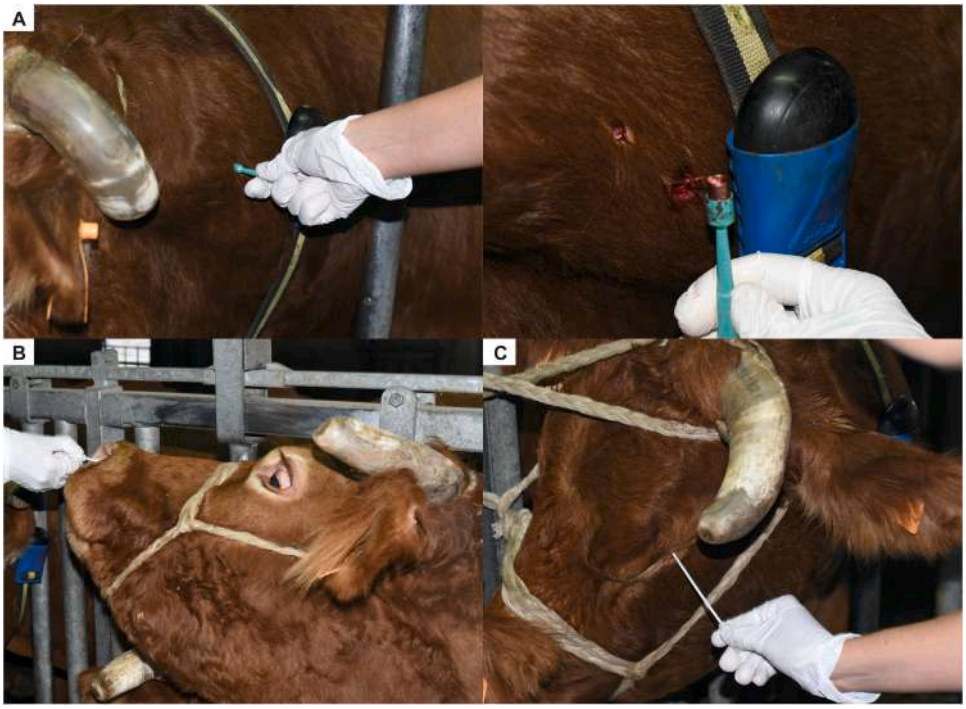

Fig. 2. Different sample methods and specimens used in the study. (A) and (B), skin biopsy. (C) Nasal swab. (D) Scleroconjunctival swab.

fashion; the laboratory technicians who examined the samples were unware of the disease status of the animals.

2.5. Clinical infection definition

Acute, sub-acute, and chronic phases of bovine besnoitiosis exhibit several specific and non-specific clinical signs as outlined in previous research (Álvarez-Garcia et al., 2014; Cortes et al., 2014). Here, we decided to use the following clinical classification to categorize animals into different disease phases: animals in the acute clinical phase of bovine besnoitiosis had to show fever (body temperature $> 39.0°C$) or a combination of clinical signs such as depression, conjunctivitis, subcutaneous oedema, lymphadenitis, and lameness; animals in the chronic clinical phase of bovine besnoitiosis had to show infected parasitic cysts visible in the scleral conjunctiva or mucous membranes. The period between the acute and chronic phases was defined as subacute phase of the disease. The estimated time of infection was considered to be 11–14 days before the onset of clinical symptoms, based on previous research (Bigalke, 1968).

2.6. Histological analysis and definition

The SKs were formalin-fixed and paraffine-wax embedded. Three-micron thick sections were cut and routinely stained with haematoxylin and eosin (H&E). An SK was considered positive for *B. besnoiti* if the tissue cyst was characterized by three layers: the outer layer with hyaline content, the middle layer with host cell cytoplasm and nuclei, and the inner membrane with the parasitophorous vacuole containing typical basophilic banana-shaped *Besnoitia* sp. bradyzoites (Fig. 3).

2.7. Molecular analysis

A single SK and mucosal swabs (NS and SC) for each animal underwent DNA extraction using the Pure Link® Genomic DNA Mini kit from Invitrogen by Thermo Fisher. The extraction process followed the manufacturer's protocol, with minor adjustments tailored for the swab DNA extraction. The terminal portion of the swab was placed directly into a 1.5 ml tube to which Lysis Buffer and Proteinase K were added. After vortexing the tubes, the samples were incubated at 56°C for 2 hours according to the manufacturer's instructions.

End-point PCR of the gene ITS-1 of the rDNA was performed according to Cortes et al. (2007) with minor modifications. Briefly, a reaction volume of 25 μL, containing 12.5 μL 2 × Dream Taq Hot Start Green PCR Master Mix (Thermo Scientific), 9.6 μL ddH2O, 0.2 μL (0.25 μM final concentration) of each *B. besnoitia*-specific forward ITS1F (TGACATTTAATAACAATCAACCCTT) and reverse ITS1R (GGTTTGTATTAACCAATCCGTGA) primers, and 2.5 μL template DNA were used. Subsequently, amplification was done in 45 cycles of denaturation at 94 °C for 30 s, annealing at 58 °C for 30 s, and extension at 72 °C for 1 min; this was followed by a final 15 min extension at 72 °C and a 10 °C hold at the completion of the profile. The amplification product of the conventional PCR had the expected size of 231 base pairs (bp). Amplifications were performed in a T-personal thermal cycler (Biometra, Goettingen, Germany). Water was used as a negative control, and *B. besnoiti* positive DNA extracted from biopsy was added as a positive control. PCR products were electrophoresed on 2 % agarose gel stained with SYBR Safe DNA Gel Stain (Thermo Fisher Scientific, Carlsbad, CA, USA) in 0.5 × TBE. For each PCR assay, one PCR product has been sequenced to confirm the specificity of the amplification. For sequencing, the amplicons were excised and purified by Nucleo-Spin Gel and PCR Clean-up (Mackerey-Nagel, Düren, Germany), and sequenced with an ABI 3730 DNA analyzer (StarSEQ, Mainz, Germany). The DNA trace files were assembled with Contig Express (VectorNTI Advance 11 software, Invitrogen, Carlsbad, CA, USA), and the consensus sequences were compared with published data by BLAST tools. The sequences generated in this study have been deposited in GenBank under accession numbers PP911643–5.

2.8. Diagnostic test evaluation

PCR-SK was considered the reference test for *B. besnoiti* despite its limitations in detecting acute and subacute infection (Cortes et al.,

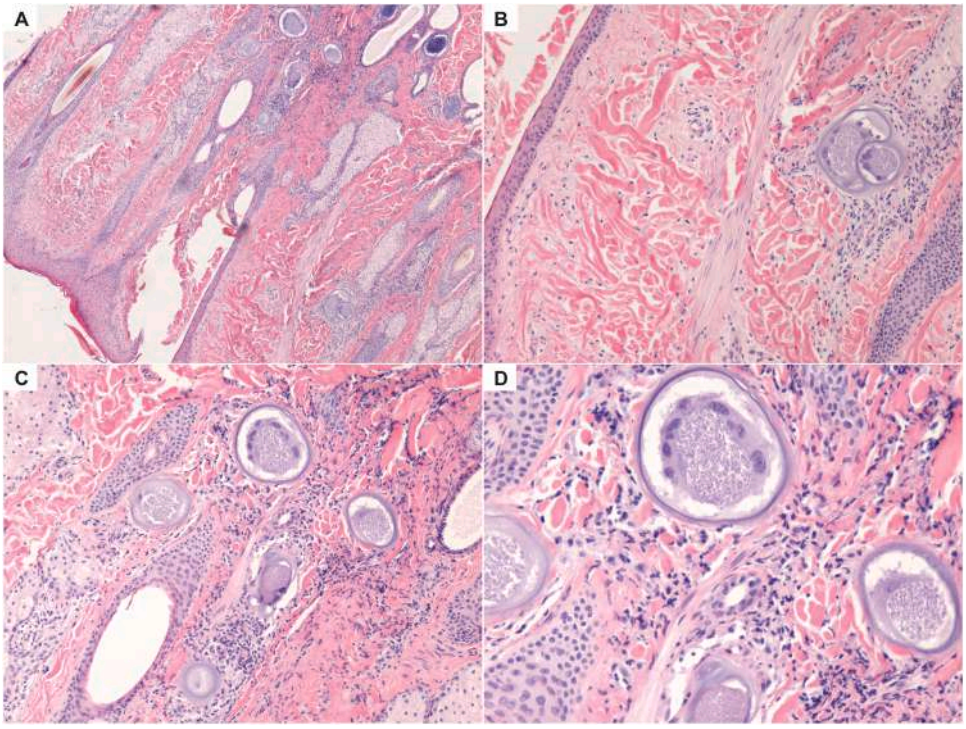

Fig. 3. Histology of typical *Besnoitia* spp. cysts. Note the presence of the characteristic three-layered cyst surrounded by inflammatory reaction. Magnification 10x (A), 25x (B, C), 40x (D); haematoxylin & eosin staining.

2007). Given this limitation in these phases of infection, PCR-NS-SC was considered a potentially suitable alternative. In addition, given the availability of HIS-SK, this test was also evaluated.

To overcome the lack of a gold standard for the diagnosis of *B. besnoiti*, we considered calculating the diagnostic sensitivity (Se) and specificity (Sp) of the three tests performed (HIS-SK, PCR-SK and PCR-NS-SC) using a BLCM.

2.9. Diagnostic sensitivity and specificity

Click or tap here to enter text.As a preliminary step, diagnostic Se and Sp of HIS-SK, PCR-SK, PCR-NS and PCR-SC were assessed using a BLCM (Hui and Walter, 1980) (Supplementary Material 1). Subsequently, a definitive BLCM including HIS-SK, PCR-SK and PCR-NS-SC was developed based on the individual test results, as outlined below.

2.10. Model construction, assumptions and prior specification

The diagnostic accuracy of three tests (HIS-SK; PCR-SK; PCR-NS-SC) was evaluated using a model containing three tests in one population (Toft et al., 2005). Considering that the same end-point PCR protocol was applied to detect rDNA of *B. besnoiti* in SK, NS, and SC, a potential conditional dependency (correlation) between the tests was hypothesized and further explored in the Se analysis (Gardner et al., 2000). Combinations of three test results were considered under a multinomial distribution (i.e. + + +; + + −; − + +; − + −; + − +; + − −; − − +; − − −).

Estimated parameters included prevalence, Se and Sp of PCR-SK, Se and Sp of PCR-NS-SC, and Se and Sp of HIS-SK, for a total of seven parameters ($K(2^P-1)$, K= 1 population; P = 3 tests) (Jones et al., 2010; Toft et al., 2007). The BLCM was implemented in Just Another Gibbs Sampler (JAGS) using the 'runjags' package in the R software (R Core Team, n. d.). The R code previously provided (Hartnack et al., 2021) was adapted and used for the Bayesian analysis hereby presented. The code to reproduce the analysis is available online (https://github.com/GiuliaGraziosi/BLCA_Jacinto_et_al). Priors on population prevalence and Sp of HIS-SK were derived using Beta (a,b) distributions, where "a" and "b" were obtained from the mode and 95th percentile of values using the epi.betabuster function of the 'epiR' package (Stevenson et al., 2013). Literature information was used to compute the above-mentioned priors (Supplementary Table S3). As the Se and Sp of the PCR-SK and PCR-NS-SC have not been previously assessed, an uninformative flat beta (1,1) prior was applied to simulate their performance. To examine the influence of different priors on the obtained results, a sensitivity analysis was conducted (Branscum et al., 2005; Monti et al., 2023). LCMs with different prior beta distributions and conditional dependence between PCR-SK and PCR-NS-OC were assessed. Flat beta (1, 1) prior distributions over the ranges of the two potential conditional dependencies (Se and Sp of PCR-SK and PCR-NS-OC) were used in the Se analysis, along with weakly informative and flat uninformative priors for the model parameters. The priors used for the BLCM and Se analysis are outlined in Table 1. Bayesian estimates of within-herd prevalence and Se and Sp of the diagnostic tests were generated using three Monte-Carlo Markov chains (MCMCs). Each MCMC was run with different initial values and 100,000 iterations, with a burn-in period of 10,000 iterations. The convergence of the MCMCs was assessed using trace plots, cumulative quantile plots, and autocorrelation plots of the MCMC samples. The medians of the posterior distributions were also reported, along with the 95 % posterior probability intervals (PI) based on the empirical distribution of the estimates. Convergence of the models was assessed using the Brooks-Gelman-Rubin (BGR) convergence statistic, with values less than 1.1 indicating convergence for the model parameters (Cheung et al., 2021; Gunn-Sandell et al., 2024). The deviance information criterion (DIC) was employed to evaluate the models, with the ultimate model selected based on having the lowest DIC value (Spiegelhalter et al., 2002).

Table 1
Parameter values for a prior distributions.

Bayesian latent class analysis			
Parameter	**A priori distribution**	**Mode**	**Minimum**
Prevalence	Beta (1.532, 1.532)	0.5	0.1
Sp of PCR-SK	Beta (58.404, 1.0)	1.0	0.95
Sensitivity analysis			
Weakly informative priors	Beta (2.0, 2.0)	0.4	0.2
Conditional correlation of PCR-SK and PCR-NS-SC in infected animals	Beta (1.0, 1.0)		
Conditional correlation of PCR-SK and PCR-NS-SC in non-infected animals	Beta (1.0, 1.0)		

3. Results

3.1. Biosecurity assessment

In the studied herd, biosecurity was scored as medium (67 %).

3.2. Clinical investigation

The sire suspected to present clinical besnoitiosis, at the general physical examination showed sensorial depression, a reduced nutritional status, an empty rumen, mild dehydration (5 %), and dull hair (Fig. 4A). Moderate oedema was observed in the hindlimbs (Fig. 4C), and the coronary bandage on both the fore- and hindlimbs exhibited moderate hyperemia. The scleroconjunctival mucosa was congested with episcleral vessels mildly injecteted. The explorable lymph nodes were slightly enlarged. The rectal temperature was recorded at 39.1°C, the respiratory rate at 25 respirations per minute and the pulse rate at 68 pulsations per minute. The appetite was reduced, the rumen was hypokinetic, and the feces were mushy. The skin exhibited multiple regions of hyperkeratosis, including in the scrotum (Fig. 4C and D), dewlap and sternum (Fig. 4B). The skin was observed to be fragile, with a propensity for detachment in areas of hyperkeratosis. The condition of the haircoat was observed to be poor and dull. Based on the history of recent introduction in the herd and clinical examination, the clinical presentation of the sire was consistent with a form of besnoitiosis between the end of the acute phase and the beginning of the chronic phase.

The remaining 53 examined animals were observed to display no systemic clinical signs, however, 29 animals showed scleroconjunctival cysts, a finding that is typically observed in chronic besnoitiosis (Fig. 5).

3.3. Test results

The PCR-SK, HIS-SK, PCR-NS, and PCR-SC assays were capable of detecting *B. besnoitia*. Table 2 provides a summary of the combinations of PCR-SK, PCR-NS-SC, and HIS-SK test results used to detect *B. besnoiti* in the cattle herd under study. Seven animals were positive for all the aforementioned combinations, 19 were positive for at least two tests, 17 were positive for at least one test and 11 were negative for all cases. Table M1 in the Supplementary Materials 1 provides the combinations of the four tests that were initially evaluated (PCR-SK, PCR-NS, PCR-SC, HIS-SK).

Furthermore, the combination of the aforementioned test results for the detection *B. besnoiti* and the clinical signs observed in the cattle herd under study are summarized in Table 3.

3.4. Bayesian estimates of test accuracy

The BLCM of the three tests in a single population, without any covariates, displayed the lowest DIC (178.9819). The medians of the

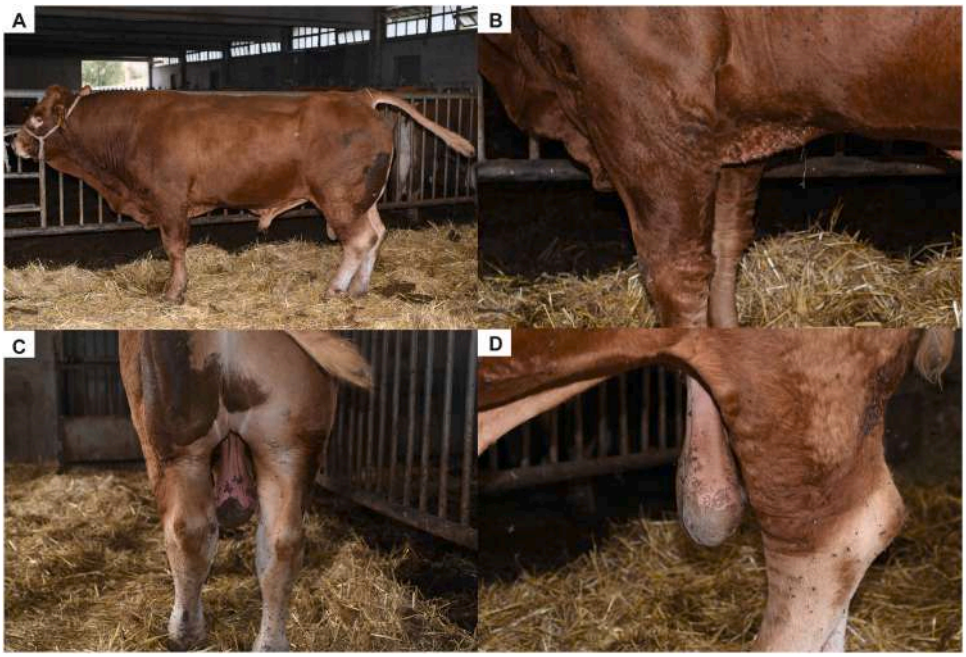

Fig. 4. Sire referred due to clinical besnoitiosis. (A) Note the moderate swelling of the hind limbs associated with lack of support of the left hind, note the poor appearance of the coat. (B) Note sternal crustal lesions. (C) Detail of swelling of hind limbs and lack of support of left hind; note scrotal hyperkeratosis. (D) Detail of scrotal hyperkeratosis.

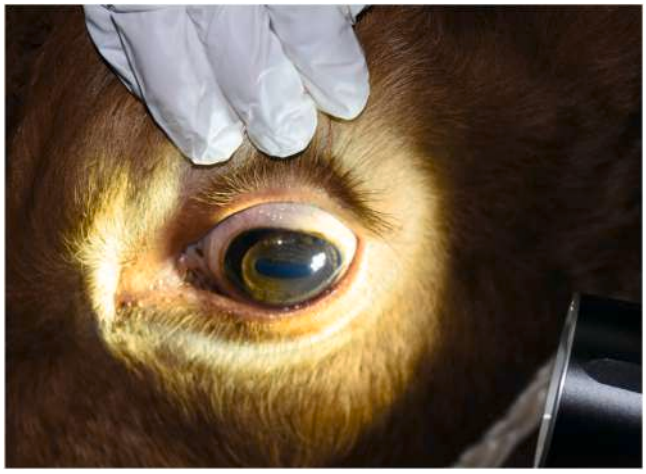

Fig. 5. Scleroconjunctival cysts typically observed in chronic besnoitiosis in one examined affected animal.

posterior distributions, along with 95 % PI for Se and Sp of the three tests evaluated, are presented in Table 4. The diagnostic parameters of MCMCs indicated that the models converged. The trace plots revealed convergence of the three MCMCs chains, while the BGR convergence statistic was close to 1. Furthermore, the autocorrelation plots revealed a rapid drop in autocorrelations, obviating the need for thinning (Supplementary Fig. S1). In the selected model, HIS-SK exhibited the highest Sp (99.1 % (95 % PI= 96.0; 100 %)), compared to the PCR-SK (Sp 71.6 % (95 % PI=47.4–99.0 %)) and PCR-NS-SC (47.6 % (95 % PI= 24.7; 75.3 %)). Regarding Se, PCR-SK and PCR-NS-SC showed similar results (PCR-SK = 91.0 % (95 % PI = 68.0–100 %), PCR-NS-SC= 85.0 % (95 % PI = 67.0; 100 %)), with overlapping 95 % PI, while the Se of HIS-SK resulted lower than that of both PCR-SK and PCR-NS-SC (33.0 % (95 % PI = 10.7–73.0 %)). Results of the sensitivity analysis using weakly informative and flat priors are reported in Supplementary Table S4. The model diagnostics of sensitivity analysis were all satisfactory. According to the obtained results, the Se and Sp of the two PCR tests would not appear to be conditionally dependent (Supplementary Table S4), as indicated by the low correlation (1.7 %) and the 95 % PI, which includes zero.

4. Discussion

In our study, we showed that it is possible to detect *B. besnoitia* DNA by PCR when using NS and SC swabs as diagnostic matrices. In addition, we found that the Se of the PCR-NS-SC was adequate to diagnose *B. besnoitia*. However, the abovementioned test lacked in Sp. Consequently, the use of a PCR-NS-SC could serve as a valuable screening test for detecting bovine besnoitiosis. In contrast, the HIS-SK showed high Sp and low Se, therefore, in the case of positive results it will have the highest reliability in stating that a certain test is really positive, making HIS-SK a good confirmatory test for the diagnosis of bovine besnoitiosis.

The previously reported diagnostic options to detect *B. besnoiti* are limited and often involve slow or invasive methods where SK-PCR is currently considered the reference direct diagnostic test for *B. besnoiti* (Cortes et al., 2014). However, it can be difficult to perform in a practical farm setting, both for management reasons and because of the invasive

Table 2
Combinations of positive and negative test results for end-point PCR of skin biopsies (PCR-SK), parallel end-point PCR testing of nasal swabs and scleroconjunctival swabs (PCR-NS-SC), and histological examination of skin biopsies (HIS-SK).

	Test results (PCR-SK, PCR-NS-SC, HIS-SK)								Total no. of cattle
	(+,+,+)	(+,+,-)	(+,-,+)	(+,-,-)	(-,+,+)	(-,+,-)	(-,-,+)	(-,-,-)	
No. of cattle	7	18	1	5	0	12	0	11	54

Table 3
Combinations of positive and negative test results for end-point PCR of skin biopsies (PCR-SK), parallel end-point PCR testing of nasal swabs and scleroconjunctival swabs (PCR-NS-SC), histological examination of skin biopsies (HIS-SK) and clinical signs.

Test	No.	Clinical signs												
		Eyes		Skin			Fever		Nasal discharge		Oedema		Lameness	
		C	N	A	H	N	Y	N	Y	N	Y	N	Y	N
PCR-SK (+)	31	24	7	0	1	30	2	29	1	30	1	30	1	30
PCR-SK (-)	23	5	18	0	0	23	0	23	0	23	0	23	0	23
PCR-NS-SC (+)	37	24	13	0	0	37	1	36	1	36	0	37	0	37
PCR-NS-SC (-)	17	5	12	0	1	16	1	16	0	16	1	16	1	16
HIS-SK (+)	8	7	1	0	1	7	1	7	0	8	0	7	0	7
HIS-SK (-)	46	22	24	0	0	46	1	45	1	45	0	46	0	46

No. = number of animals; N = absence of symptom; Y = presence of symptom; C = presence of scleroconjunctival cysts; A = alopecia in the integumentary system; H = hyperkeratosis in the integumentary system.

Table 4
Results from the Bayesian latent class model applied for estimating the diagnostic accuracy parameters (sensitivity and specificity) of the three diagnostic tests evaluated to diagnose *Besnoitia besnoiti,* and the estimated true prevalence using all samples (N = 216) originating from 54 cattle sampled in the studied herd.

Parameter	Posterior median (95 % probability interval)
Prevalence	46.9 (15.2–73.6)
PCR-SK Sensitivity	91.0 (68.0–100)
PCR-SK Specificity	71.6 (47.4–99.0)
PCR-NS-SC Sensitivity	85.0 (67.0–100)
PCR-NS-SC Specificity	47.6 (24.7–75.3)
HIS-SK Sensitivity	33.0 (10.7–73.0)
HIS-SK Specificity	99.1 (96.0–100)
DIC	178.9819

End-point PCR of skin biopsy (PCR-SK).
Parallel end-point PCR testing of nasal and scleroconjunctival swabs (PCR-NS-SC) Histological examination of skin biopsies (HIS-SK).
Deviance information criterion (DIC).

procedure required to obtain the skin biopsies. Therefore, the use of alternative less invasive specimens for *B. besnoiti* detection represents an interesting alternative (Cortes et al., 2014). Compared to skin biopsies, mucosal swabs are less invasive, faster and easier to perform, and require less instrumentation and operator expertize. In addition, the cost of a sterile dry swab is inferior to the cost of a biopsy punch.

Several risk factors previously reported for *B. besnoitia* infection were identified within the Limousin beef cow-calf operation under study. The use of a free-stall system alllowed continuous interaction among animals, increasing the likelihood of contact with asymptomatic chronically infected animals (Álvarez-García et al., 2013) and intermingling between animals of varying ages (Diezma-Díaz et al., 2017). Importing Limousin cattle from France, where besnoitiosis is endemic, posed an additional infection risk. Natural mating, and access to pasture during warmer months served as major risk factors for horizontal mechanical and vector borne transmission (Álvarez-García et al., 2013; Coelho et al., 2023). Regular parasite control and rodent management help prevent *B. besnoiti* spread. Although rodents' role in its life cycle is unclear, recent molecular evidence has identified *B. besnoiti* DNA in synanthropic rodents from central northern Italy (Dini et al., 2024b)

Diagnosing besnoitiosis in a herd is crucial, especially in non-infected herds where the primary goal is to prevent the introduction of the disease. Lack of besnoitiosis testing for purchased animals was observed in the studied herd and currently is still a common practice due to limited awareness and the disease's non-notifiable status, despite its significant health and economic impact (Cortes et al., 2014; Saegerman et al., 2022). According to a global consensus, the primary risk factor for besnoitiosis is the transportation of animals, which presents the potential for introducing subclinically and chronically infected individuals into the herd (Saegerman et al., 2022). In infected herds, strategic control programs focused on eliminating positive cattle should be implemented. This goal is currently achieved through serial serological diagnosis, primarily using ELISA and immunoblotting (Gutiérrez-Expósito et al., 2017). This approach is applied to bulls purchased from non-infected herds and to cattle displaying clinical signs of either the acute or chronic phases of infection in herds with a history of besnoitiosis. However, serology testing can lead to diagnostic challenges. Infected cattle in the acute phase may present negative serological tests due to the delayed production of IgG. Additionally, some chronically infected bovines with tissue cysts in their sclera or *vestibulum vaginae* might go undetected, as their antibody levels can fall below the detection limits of the tests (Álvarez-García et al., 2013; Schares et al., 2009). It is estimated that the number of these elusive animals can reach up to 17 % in endemic herds. This low antibody response is attributed to a small number of tissue cysts (Basso et al., 2011b; Gutiérrez-Expósito et al., 2017). In our study, the parallel utilization of PCR-NS-SC has revealed the presence of *B. besnoitia* DNA in animals that do not exhibit overt signs of chronic besnoitiosis. It is hypothesized that the presence of a low number of cysts in the examined mucosa does not result in macroscopically evident lesions, thereby complicating clinical detection of the disease. Given that these animals may lack detectable antibody titers against *Besnoitia* (Gutiérrez-Expósito et al., 2017), the application of this novel molecular diagnostic method is of critical importance. This is particularly relevant as such asymptomatic animals can readily transmit *B. besnoitia* during mating and through mechanical vectors (Álvarez-García et al., 2013). Given the limitations of serological diagnosis, such as reduced specificity due to cross-reactions with other Sarcocystidae common in cattle and poor sensitivity in acute and subclinical chronic forms of besnoitiosis (Álvarez-García et al., 2013), NS-SC-PCR can be considered a valuable alternative for screening. This approach is particularly important for endemic farms, where asymptomatic cattle could facilitate the spread of the disease throughout the herd.

As this dataset only assessed a single herd, the sample size limited the precision of the estimates (Sackett, 2002). Consequently, it is not possible to generalize the results of the BLCM beyond the specific study population. Several methods were used to test the robustness of our models. Prior estimations of HIS-SK test specificity and prevalence of *B. besnoitia* were based on available published literature. Since the Se of HIS-SK and the Se and Sp of the PCR-SK and PCR-NS-SC have not been previously evaluated, uninformative flat priors were applied to simulate their performance. To assess sensitivity in the choice of prior information, two alternative priors (flat priors and weakly informative priors) were used in the current study. The means of Se and Sp for the tests showed no significant difference between the selected model and the ones applied in the sensitivity analysis, showing that the estimates were stable and robust. However, we found that considering a conditional dependency between the PCRs greatly changed test outcomes, although we do not believe this is biologically sound, given that the type of biological sample tested (skin and swab samples) is different. Therefore, we

consider the model selected was the most suitable for our study design and accurate in predicting the diagnostic performance of the tests.

Future studies could explore the testing of vaginal swab specimens. This sampling method could be particularly useful in contexts where cows are routinely gynecologically examined, as it allows for quick and efficient swabbing during the same procedure.

5. Conclusion

Our study provides for the first-time insight into the potential of using NS and SC as valuable diagnostic matrices to detect *B. besnoiti* in cattle. The use of combined PCR-NS-SC is a viable alternative diagnostic tool for early detection and represents a potential useful screening test to detect the parasite because of its good Se, rapidity and economy. In addition, it represents a less invasive diagnostic test that could be easily used to monitor and control bovine besnoitiosis in cattle herds. In addition, we suggests that SK-HIS represents a good confirmatory test to detect *B. besnoiti*.

CRediT authorship contribution statement

Giulia Graziosi: Writing – original draft, Visualization, Methodology, Investigation, Formal analysis. **Anastasia Poluzzi:** Visualization, Methodology. **Roberta Galuppi:** Writing – review & editing, Visualization, Methodology. **Gianfranco Militerno:** Visualization, Methodology. **Tolulope Ogundipe:** Visualization, Methodology. **Arcangelo Gentile:** Visualization, Methodology, Formal analysis, Conceptualization. **Andrea Beltrame:** Visualization, Methodology. **Filippo Maria Dini:** Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis. **Joana Jacinto:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Conceptualization.

Ethics approval

This study did not necessitate official or institutional ethical approval because it was conducted as part of routine clinical veterinary diagnostics rather than as an experimental investigation. The multiple swab sampling was initially conducted for screening purposes to diagnose various pathogens, such as respiratory viruses. Consequently, these swab samples were also utilized for the diagnosis of *B. besnoiti*. All animals involved were examined with their owners' consent and handled in strict accordance with established ethical standards in Italy and European Union.

Ethical statement

The study did not required formal ethical approval, as it was conducted as part of routine clinical veterinary diagnostics rather than as an experimental investigation. All animals involved were examined with their owner's consent and handled in strict accordance with the established ethical standards.

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Data and model availability

All data are provided in the manuscript and in the supplemental materials. The sequences generated in this study have been deposited in GenBank under accession numbers PP911643–5.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.prevetmed.2024.106415.

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