Super-spreaders: detection and culling in the control of bovine besnoitiosis.

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Abstract

Background

Bovine besnoitiosis is an emerging vector-borne disease in Europe caused by the Apicomplexa *Besnoitia besnoiti*. The mechanical transmission from infected to naïve hosts is permitted by horse flies and stable flies. Bovine besnoitiosis is difficult to control due to the complexity of its diagnosis in the acute stage of the disease, poor treatment success and chronically asymptomatic cattle acting as parasite reservoirs. When serological prevalence is low, detection and specific culling of seropositive cattle is feasible; however, economic considerations preclude this approach when serological prevalence is high. The aims of this study were to evaluate the accuracy of detection of super-spreaders in highly infected herds and to test their selective elimination as a new control strategy for bovine besnoitiosis.

Methods

Real-time PCR analyses performed on skin tissues from 160 asymptomatic animals sampled at slaughterhouses showed that the tail base was the best location to evaluate the dermal parasite DNA load. All seropositive animals (N = 518) from eight dairy or beef cattle farms facing a high serological prevalence of besnoitiosis were sampled and their skin analysed by real-time PCR. A recommendation of rapid and selective culling of super-spreaders was formulated and provided to the cattle breeders. Subsequent serological monitoring of naïve animals was used to evaluate the interest of this control strategy over time.

Results

Among the 518 seropositive animals, a low proportion of individuals (13%) showed Ct values below 36, 17% had doubtful results (36 < Ct ≤ 40) and 70% had negative PCR results. These proportions were grossly similar on the eight farms, regardless of their production type (beef or dairy cattle), size, geographic location or history of besnoitiosis. Within two weeks of the biopsy, the rapid culling of super-spreaders was implemented on only three farms. The numbers of newly infected animals were lower on these farms compared to those where super-spreaders were maintained in the herd.

Conclusions

Real-time PCR analyses performed on skin biopsies of seropositive cattle showed huge individual variabilities in parasite DNA load. The rapid culling of individuals considered as super-spreaders seems to be a new and encouraging strategy for bovine besnoitiosis control.

Background
Bovine besnoitiosis is a re-emerging protozoan disease in Europe caused by the cyst-forming apicomplexan parasite *Besnoitia besnoiti* [1, 2]. The tachyzoite and bradyzoite stages of the parasite develop in the cattle host and are respectively responsible for the acute and chronic phases of this disease. During the acute stage of the disease, tachyzoites multiply quickly within bovine macrophages and endothelial cells of blood vessels [3]; cattle may present fever, photophobia, epiphora, ocular and nasal discharge, anorexia and depression. The time interval between the acute and the chronic stages of the disease is referred to as the subacute stage, with cattle presenting normal or slightly increased temperature, enlargement of lymph nodes and edematous swellings of head, limbs and lower parts of the body [4]. The subsequent chronic phase, which can last several years, is characterized by massive skin alterations resulting from the formation of numerous cysts containing thousands of bradyzoites in various tissues including the derma (scleroderma). This chronic and debilitating disease is responsible for heavy economic losses in cattle, and sometimes death. However, in most infected and seropositive cattle, no obvious clinical signs are observed.

The transmission routes of *B. besnoiti* have long been the subject of debate. No definitive hosts have been identified in Europe [5] and no parasite DNA has been detected in semen of *B. besnoiti* infected bulls [6], suggesting that cattle are not infected via the ingestion of oocysts or a venereal route. The presence of parasite cysts in the skin suggests that the main mode of transmission is likely mechanical, with hematophagous flies transmitting the parasite as experimentally demonstrated by Bigalke [7] and Sharif et al. [8]. Both acute and chronic cases of besnoitiosis served as sources of infection for susceptible cattle; however, Bigalke [7] regarded chronic cases as being of greater importance in this respect and Sharif et al. [8], demonstrated that a low number of stable flies (N = 300) were able to transmit bradyzoites from a chronically infected cow to a susceptible recipient host. Moreover, many field observations [4] reported that the introduction of chronic cases into non-infected herds led to disease outbreaks. Indeed, the mouthparts of these biting flies are able to penetrate bradyzoite-filled cysts. When blood feeding is interrupted by a host’s movements and then completed on a non-infected host, the flies may introduce bradyzoites into the tegument of the latter host [7, 8, 9, 10]. The experiments of Bigalke [7] have clearly shown that clinically and even some asymptomatic long-lasting infected cases of besnoitiosis act as efficient sources of infection for susceptible animals. It stands to reason that the greater the number of cysts in the dermis, the greater the odds in favour of one or more being penetrated by mouthparts, and the more suitable such an animal would be as a reservoir. We can thus hypothesize that the selective elimination of these super-spreaders from farms where the disease is enzootic could lead to a marked reduction in the incidence of bovine besnoitiosis.

It is difficult to control bovine besnoitiosis. Vaccines are available in some countries [11] but are not registered in Europe. Sulfonamides have been proposed to diminish the clinical signs during the acute stage of the disease [4] but are totally ineffective in the chronic phase when the parasite is protected within the cysts. Resistance to insecticides has been increasingly detected in the field in France [12] and Germany [13], making the control of vector transmission highly hypothetical. Finally, the control of bovine besnoitiosis relies entirely on flock management measures implemented after diagnostic test results are known. Biosecurity and biocontainment management measures, such as the rigorous serological testing
of new animals prior to entering a herd, is a key point. In an infected herd, the seroprevalence should be evaluated rapidly after the initial diagnosis [14]. Severe clinical cases must be systematically and rapidly eliminated from the herd; such animals only represent a small proportion of seropositive animals in both endemic and epidemic areas. Recommendations differ sharply depending on the intra-herd seroprevalence level. When the seroprevalence is low, i.e., below 10%, the rapid and selective culling of seropositive animals is considered as the best option to avoid the spread of the disease within the herd. However, in herds with high seroprevalence, very few options remain available because the rapid elimination of the entire group of seropositive animals is not possible for economic reasons. Keeping seropositive animals separate from non-infected ones until they are gradually replaced by healthy and seronegative animals is a feasible and successful approach [15]. However, not all farms can manage two separate herds over several years. This is especially true for dairy cattle farms, where non-infected and infected animals are brought together during milking. In this context, the rapid detection and culling of super-spreaders identified by Bigalke [7] could be a realistic option for bovine besnoitiosis control.

However, the proportion of asymptomatic infected animals which show high numbers of parasite cysts in their dermis compared to the total number of infected and seropositive animals is not known. Cysts in the dermis could be detected using histology and immunohistochemistry [16], but this method is time-consuming and probably lacks sensitivity. Cortes et al. [17] described a sensitive and specific real-time ITS1 rDNA PCR test which allows the detection of B. besnoiti infections in bovine skin biopsies. The sensitivity of the amplification reaction was high in that this test allowed the detection of DNA equivalent to one B. besnoiti cell. In this study, a immunofluorescence antibody test, histopathological analysis and real-time PCR were performed on 20 asymptomatic animals. Four groups of animals were identified: i) 12 animals showing negative results in an indirect fluorescent antibody test (IFAT), histology and PCR, ii) three animals with positive results for the three tests, iii) three animals with positive IFAT and PCR results and negative histology results, and iv) two animals with positive IFAT results and negative histology and PCR results. These results suggest that parasite DNA could be detected in the skin of some asymptomatic and infected animals and not in others. Therefore, real-time PCR on skin biopsies could identify super-spreaders, i.e., cattle whose dermis contains a high level of parasite DNA load. Nevertheless, it is a prerequisite to know the optimal sites for sampling skin biopsies, i.e., with the highest probability of finding tissue cysts and parasite DNA. In a study performed on a very low number of chronically and clinically infected cattle, Schares et al. [18] demonstrated that B. besnoiti cysts were not equally distributed in the skin, with the highest parasite DNA concentrations in the rump and in the distal parts of hind legs, and the lowest parasite DNA concentrations in the ventral, head and neck regions of the body. The reasons for this heterogeneous distribution of cysts are so far unknown.

This study aimed to i) assess the distribution of parasite DNA in the severe chronic stage of the disease, ii) compare the parasite DNA concentrations between three skin locations of asymptomatic infected cattle (base of the tail, neck and ear), iii) evaluate the proportion of individuals showing high parasite DNA concentrations in their skin in herds with high seroprevalence, and iv) assess the feasibility and the efficacy of a new control strategy for bovine besnoitiosis based on the rapid detection and culling of individuals considered to be super-spreaders.
Methods

Animals studied and collection of samples

Cattle in chronic phase of the disease

Fourteen adult cattle (11 females and three males) of various breeds, including Salers, Limousine, Charolaise, Gasconne des Pyrénées and Blonde d’Aquitaine, showing advanced clinical signs of the chronic phase of the disease were sent to the National Veterinary School of Toulouse by cattle breeders located within a 200 km radius around Toulouse. These animals were humanly euthanized by a veterinary surgeon using a lethal intra-venous injection of embutramide (T61, Intervet) and immediately necropsied.

For each animal, skin samples (1 cm²) were collected immediately after death from several sites: right foreleg, right hind leg, udder for females, right inner thigh, backline, right flank, right shoulder, right eyelid, dewlap, umbilicus area, tail base and ocular sclera for external zones. Tissue samples (1 cm² or 1 cm³) from internal organs (lung, spleen, liver, heart, right kidney, diaphragm, subcutaneous connective tissue, skin muscle, nasal and tracheal mucosa) also were collected.

All samples were transported individually in an identified dry tube (Corning MCT-150-G) and stored at 4 °C before analysis the following day.

Asymptomatic cattle from slaughterhouses

One hundred and sixty adult cattle were sampled in three slaughterhouses located in endemic areas: Ariège (N = 105), Hautes-Alpes (N = 33) and Alpes de Haute-Provence (N = 22). These animals did not show any clinical sign of bovine besnoitiosis and their status (infected or not) was unknown at the time of sampling. Skin biopsies for PCR were collected from the ear, neck and tail base, transported individually in an identified dry tube (Corning MCT-150-G) and stored at 4 °C until PCR analysis. Blood samples for western blot (WB) analyses were drawn from the jugular vein during bleeding and collected in BD Vacutainer® blood collection tubes without anticoagulants.

Asymptomatic cattle from herds showing high seroprevalence

Eight farms located in different regions of France (two in the center, six in the south) were included in the study (Table I). Both dairy (N = 4) and beef cattle herds (N = 4) were selected. Farms A, B and C were recruited in the spring of 2017, farms D, E and F in the spring of 2018 and farms G and H in the spring of 2019. These farms were chosen because i) all had experienced at least one clinical case of bovine besnoitiosis before the beginning of the survey, ii) the ELISA-seroprevalence established just before the beginning of the survey was high (over 40%), and iii) the cattle producer volunteered to participate in the study. Overall, 518 seropositive individuals were tested by real-time PCR on skin biopsies. Detailed information is provided in Table I.
The skin sample for PCR was taken without anesthesia at the tail base using biopsy punches (8 mm diameter, Kruuse 273693). This area was previously cleaned with Betadine (Viatris 9451.8). The skin fragment was placed in a dry tube (Corning MCT-150-G), identified with the bovine individual ID and stored at + 4 °C before analysis. Aluminum spray (Aluspray® Vetoquinol) was sprayed on the biopsied area.

DNA Extraction And Quantitative Real-time PCR

In the ENVT laboratory, DNA was extracted from tissue biopsies using a commercial kit (QIAamp® DNA Mini Kit, Qiagen, Courtaboeuf, France). Following the manufacturer's recommendations, 50 mg aliquots of tissue biopsies were processed after an over-night incubation with proteinase K. *Besnoitia spp.* internal transcribed spacer 1 (ITS-1) amplification was performed with the commercial PCR kit AdiaVetTM Besnoitia (AES Chemunex, Bruz, France). The quantitative PCR was performed with the Stratagene MX3005P thermal cycler (Agilent Technologies, La Jolla, CA, USA). Positive and negative controls were provided by the manufacturer. Results were analyzed using the MxPro QPCR version 4.10 software (Agilent Technologies). When the Ct value was inferior or equal to 36, the parasite DNA was considered detected and the animal was deemed to be a super-spreader, when Ct values ranged between 36 and 40, parasite DNA was at the limit of detection, and a Ct superior to 40 was considered as a negative real-time PCR result.

Serology

Sera were separated by centrifugation and tested for *B. besnoiti* antibodies using a commercial ELISA kit (ID Screen® Besnoitia Indirect 2.0, IDVET). Serological analyses were performed by the Departmental Veterinary Laboratory of Ariège (LVD 09) for farms A, B, C and D, LVD 26 (Drôme) for farms G and H, LVD 18 (Cher) for farm E, and LVD 36 (Indre) for farm F.

WB analyses were performed on animals necropsied in the ENVT facilities, on animals collected in the slaughterhouses, and when doubtful ELISA results were recorded, on farms A to H. For tachyzoite-based WB analysis, the coated membranes and the immunoblots were performed as previously described [9]. Three main antigenic reactivity areas are described [19]: area I, 12–20 kDa; area II, 23–38 kDa and area III, 60–90 kDa. The minimal criterion for serological positivity was the recognition of at least four bands in at least two domains. This test is considered to be highly specific [20].

Farm monitoring

Real-time PCR analyses were performed within a week after the skin biopsy samples were collected. The results were immediately communicated to farmers and veterinarian practitioners in charge of the farm so that animals deemed to be super-spreaders (Ct ≤ 36) could be culled as soon as possible. In this way,
super-spreaders were discarded from the herd before the high activity period of hematophagous flies began. However, this culling strategy was only applied on farms A, D and H. To assess whether the status of individuals persisted over time, PCR samples were collected again ten months later on six animals considered to be super-spreaders on farm C, and seven animals with doubtful or negative PCR results were sampled three years later on farm A.

To assess the ability of this strategy to reduce the number of newly acquired *B. besnoiti* infections, serological analyses were performed on previously seronegative animals and young ones (over six months old) that were not present on the farm at the previous sampling date. The time intervals between the initial and subsequent analyses differed per farm as follows: 12, 24 and 36 months for farm A, 12 and 24 months for farm D, 12 months only for farms B, C and H.

**Statistical Analyses**

In chronically infected cattle necropsied in the ENVT facilities, comparisons between mean Ct values from skin samples and mean Ct values from internal organs, and then between mean Ct values from skin of the upper side of the body and from skin of the lower side of the body, were performed using Mann-Whitney-Wilcoxon (MWW) rank tests. For cattle sampled at slaughterhouses, Ct values recorded in the neck, ear and tail base of the same individual were compared using the Friedman rank test. Individual variability of Ct values obtained in asymptomatic infected animals from the eight farms studied were presented according to classes of Ct (in steps of three Ct units between Ct ≤ 15 to NoCt). Distribution of individuals within these Ct classes was compared between farms using the Fisher exact chi-square test. The relationship between Ct values and ELISA sample to positive ratio (S/P value) was assessed on a subset of 147 animals (farms A, B, C and D) for which all serological analyses were performed in the same laboratory (LVD 09). The Spearman correlation was calculated, with individuals showing no DNA detection in their skin biopsies scored with a Ct value of 45. A binary logistic regression model was fitted to investigate factors related to the Ct value obtained in real-time PCR on the skin biopsies (positive for Ct value < 40 versus negative for Ct ≥ or equal to 40). These factors included farm (A, B, C or D), age (below or above 24 months old) and ELISA S/P value (below and above 110%). Again, only the 147 individuals from farms A, B, C and D were used in this analysis. All statistical analyses were done using R software (version 3.5.2, R foundation).

**Results**

**Cattle in scleroderma phase**

All tissues from the 14 animals in the chronic phase of the disease yielded real-time PCR positive results (Figs. 1A and 1B). A significant higher DNA parasite load (p < 0.05) was found in skin samples (mean Ct value: 17.0 [95% range: 12.6–34.2]) compared to internal organs (mean Ct value: 28 [95% range: 12.3–
The parasite DNA load was higher \((p < 0.05)\) in the upper respiratory tract than in the lower respiratory tract (lung).

Mean \(Ct\) values in skin samples ranged from 15.0 (tail base) to 20.0 (udder) but no significant difference was observed between the lower body parts (right anterior and posterior barrels, udder, interior of the right thigh, dewlap, umbilical zone) and the upper body parts (back line, right flank, right shoulder).

### Asymptomatic Infected Animals

#### From slaughterhouses

Out of 160 animals sampled at three slaughterhouses, 113 (70.6%) yielded negative WB results (Table II). Among them, no parasite DNA was found in 97 individuals (85.8%) whatever the sampled area (tail base, ear or neck). Nevertheless, parasite DNA was detected in low amounts in 14 animals (12.3%) in at least one location and in large amounts in only two animals (1.7%). Forty-seven animals (29.4%) exhibited a positive WB analysis. No parasite DNA was detected in half of them (24 animals) whatever the location of the skin sample. Parasite DNA was detected in the 23 remaining individuals, eight with low parasite loads only and 15 with at least one high parasite DNA load.

Among the 23 animals showing positive parasite DNA detection, seven had only one positive location, 12 had two positive sampling sites and only four had three positive locations. Regarding the WB-positive individuals showing at least a CT value below or equal to 36 in PCR \((N = 17)\), the tail base was positive in 12 cases (70%), and neck and ear samples were positive in only seven animals (41%).

#### From farms

Out of the total number of cattle examined \((N = 518)\), 75 (14.5%) yielded a highly positive real-time PCR result \((Ct \leq 36)\) on skin samples taken from the tail base, 92 (17.7%) were “doubtful” with a parasite DNA amount close to the limit of detection \((36 < Ct \leq 40)\), and 351 individuals (67.8% of the total) were “negative”, i.e., No Ct (Fig. 2).

This general trend of the distribution of individuals into three main categories of \(Ct\) values was similar in the eight farms studied, with the category “No \(Ct\)” predominating, i.e., no parasite DNA detected, regardless of the animals’ geographic origin, size, type of production (dairy versus beef cattle farms) or history of the disease. However, some variations in the balance between the three categories were noted according to the farm \((p\text{-value} = 2.8.10^{-6})\) (Fig. 3). The proportion of individuals with a \(Ct\) value below or equal to 36 varies from 8.8–28.0% depending on the farm (Fig. 3). In six out of eight farms, the percentage of cattle with “negative” real-time PCR results was greater than 60.0% (Farm B, C, D, E, F and H) (Fig. 3). Farms A and G had lower proportions of individuals with a “negative” result (46.9% and 36.0% respectively) and higher proportions of individuals with a “doubtful” result (28.1% and 36.0% respectively).
A weak but significant negative correlation between S/P values in ELISA and Ct values in real-time PCR (r = -0.26; p-value = 0.0015) was evidenced (Fig. 4) in a subset of 147 individuals, suggesting that the higher the quantity of parasite DNA load in a skin sample, the higher the level of specific antibodies. Interestingly, all individuals with Ct values below 30 showed antibody titers over 110%. However, a considerable range of antibody titers was observed in negative real-time PCR cases.

The potential influence of some factors (farm, age and optical densities in ELISA) on the PCR status of an individual (positive when Ct value < 40 versus negative for Ct > or equal to 40) were analyzed by fitting multivariable logistic regression models (Table III). All investigated factors had a significant effect on the binary positive/negative PCR response. In this study, animals below 24 months old and animals with high ELISA optical densities (over 110%) had significantly higher odds of having a positive real-time PCR response.

**Farm Monitoring**

After receiving the results, two types of strategies were followed. Farmers A, D and H decided to cull or to separate in a confined place the animals deemed to be super-spreaders (i.e., with Ct values below or equal to 36) in the week following the reception of the results. In contrast, farmers B and C decided to keep the super-spreaders in their herds, either because these animals were of high genetic merit (farm B) or because they were young replacement heifers (farm C). The subsequent course of besnoitiosis infection strongly differed between these two groups of farms (Table IV): 12 months after the initial detection, only a few animals showed a seroconversion on farms A (1/30), D (0/48) and H (1/23), while the seroconversion rates were higher on farms B (15/30 within 6 months) and C (12/14 within 12 months). Interestingly, on farm A, no seroconversion was noted 24 and 36 months after the detection and culling of super-spreaders although seropositive cows were still present and in contact with seronegative animals in the herd. In contrast, farm D exhibited more seroconversions (6/14) 24 months after the initial detection of super-spreaders.

Seven cattle from farm A showing initial PCR results that were negative or questionable (36 < Ct ≤ 40) were tested again 36 months later and similar results were obtained. This suggests that the status of non “super-spreaders” could be stable over time (Table V). Similarly, six animals considered to be super-spreaders from farm C were tested again 10 months later; the status of these animals had not changed.

**Discussion**

When the serological prevalence is below 10% in a cattle herd, the rapid detection and subsequent culling or separation of all seropositive individuals is currently the most effective way to prevent the within-herd spread of besnoitiosis [15]. For seroprevalence above 10% or due to specific conditions on a farm, this sanitation strategy is not practically or economically feasible.
In this context, this study was carried out to propose a selective culling strategy to cattle breeders who have herds with a high seroprevalence and are unable to manage two separate batches of animals. Current observations agree that all seropositive cattle on a farm do not contribute equally to the transmission of *B. besnoiti*. Individuals showing a clinical phase have an enriched-cysts dermis [21], and their culling should reduce the transmission pressure within the herd. However, this measure cannot totally control besnoitiosis in a herd because a subset of seropositive animals may also present a risk of parasite transmission to seronegative animals on the farm [22]. Indeed, among seropositive cattle that have never shown any obvious clinical sign of the disease (febrile phase and/or scleroderma), it is reasonable to consider that some have no or few cysts in the dermis while others may carry a significant number of cysts in their tegument [23]. This category of animals could serve as a reservoir of parasites and could play a major role in the transmission of the parasite via hematophagous flies. According to this assumption, the identification and selective culling of cattle that have high concentrations of bradyzoïte cysts in their skin, even if they do not exhibit clinical signs, could be a major tool to control the disease in endemic areas. Previous studies carried out on a small number of cattle have shown that the PCR method allowed *B. besnoiti* DNA to be detected in skin samples collected from seropositive cattle that had no clinical signs [17].

In the present study, the use of a real-time PCR analysis on a skin sample made it possible to distinguish between individuals with a high parasite DNA load and those with a low or no parasite DNA load. In the severe chronic phase with elephant skin, bradyzoïte cysts are easily detected in the dermis, the scleral conjunctiva and mucous membranes of the upper respiratory tract by histology [24] and real-time PCR [17, 25]. Experimental infections of rabbits with *B. besnoiti* have also shown that the nasal mucosa is highly infiltrated by *B. besnoiti* cysts after experimental infection with bradyzoïtes [26]. This tropism of cysts for the upper respiratory tract is probably responsible for the mucopurulent nasal discharge sometimes observed in animals during the clinical phase of the disease, but remains unexplained to date. This feature was also observed in samples taken from the skin and organs of the 14 chronically infected cattle of the present study: the skin samples showed significantly higher parasite DNA loads than the internal organs, except for the nasal mucosa whose mean Ct value was 16.1 +/- 3.0. The skin is therefore the site of predilection for cysts, and considering the results obtained in cattle showing scleroderma, all skin biopsies, regardless of the site sampled, yielded Ct values below 34.2, i.e., a response considered to be strongly positive. Schares et al. [18] collected samples from 77 different skin locations in four chronically infected cattle and observed a significant difference in the distribution and detectability of parasite DNA between skin regions. *B. besnoiti* therefore is not equally distributed in the skin of infected cattle. The tail base with an average Ct value of 15.5 +/- 0.5 was particularly rich in parasite DNA and provided an easily accessible site for a biopsy to be carried out on a living animal on a farm. This further reinforces the assumption that the transmission of the parasite is due to hematophagous flies and is facilitated by the cutaneous tropism of the cysts, present in large numbers in the dermis and in the connective tissue [18]. In contrast, PCR analyses revealed more heterogeneous results in asymptomatic infected animals [23, 27]. In 24 out of 47 seropositive cattle in the slaughterhouse study, molecular amplification did not yield any positive result regardless of the site from which the skin sample was
taken, while these individuals were confirmed to be infected by WB serology. These negative results cannot be attributed clearly in the PCR sample process because i) the quality and the efficiency of the DNA extraction from skin tissue was verified by the amplification of bovine GAPDH, an internal control of extraction and amplification steps, and ii) the limit of detection of the commercial PCR tool was equivalent to the DNA of 2.5 parasites per reaction [6]. Moreover, even though B. besnoiti may cross with Neospora caninum [28], the high specificity of WB analysis in the diagnosis of besnoitiosis excludes a false positive result for such a large number of animals [20]. However, during the course of infection, clinically affected animals may experience an apparent recovery even though they remain infected life-long, making parasite DNA detection more difficult [29]. A strong humoral and cell-mediated immune responses [30] could significantly reduce the dermal cysts load, which could explain the negative PCR results of some skin regions [21, 16]. Samples taken from three locations (tail base, neck and ear) on the same individual did not give similar qPCR results. From the 39 cattle that tested PCR positive in at least one of the three areas sampled, it was not possible to determine one location that gave a consistent positive qPCR result. Therefore, contrary to animals showing severe scleroderma, Besnoitia cysts appear to be heterogeneously distributed in the skin of asymptomatic cattle. As it is not feasible in field conditions to take several skin biopsies at the same time from one asymptomatic animal, it is essential to sample the skin site with the highest probability of having a cyst. The tail base yielded a positive result in 70% of seropositive animals with at least one CT value below or equal to 36, while the positive rate for neck and ear samples was below 50%. An optimal compromise between the requirement for maximum sensitivity and the feasibility of sampling in field conditions therefore would be to designate the tail base as the location to sample to assess the status of one animal. However, this does not mean that a negative PCR result on a skin fragment taken from the tail base can be used to "qualify" this animal as "not dangerous for transmission".

We therefore chose this easily accessible area when the animals were in a corridor or headlocks to collect skin biopsies in our farm study. The distribution of PCR Ct values were similar in the eight farms, with a minority of individuals having a high parasite DNA load, regardless of geographical origin, production system, breed or herd size. Among asymptomatic seropositive cattle, a small proportion carried a much larger quantity of parasite DNA than others, and these animals are likely to be a source of contamination for their naive congeners in the presence of the vectors. These results suggest high individual heterogeneity in the transmission capacity of besnoitiosis, however, this must be confirmed in further studies. With such a distribution, a small number of individuals could be called “super-spreaders”. According to Vanderwaal et al. [31], many parasitic infections, including helminths, arthropods and microparasites, are commonly aggregated in host populations. In other words, a single individual with a very high parasite load represents a perennial source of parasites with a considerably high potential for transmission. This heterogeneity in transmission is a challenge for infectious disease dynamics and control. An 80 − 20 "Pareto" rule has been proposed to describe this heterogeneity, whereby 80% of transmission is accounted for by 20% of individuals, called super-spreaders [32, 33, 34]. Vanderwaal et al. [31] notes that criterion V (or R0) can be defined as the number of new infections induced by one infected individual [35]. The value of V depends on three components: the infectiousness of infected hosts, the
contact rate between infected and healthy subjects, and the duration of infection. This approach has been transposed and adapted to the bovine – *B. besnoiti*-stable fly system [36]. In addition to these three components, two other parameters contribute to the efficiency of the transmission of *B. besnoiti*: i) the interactions between the parasite and its vector, and ii) the level of activity of the latter, i.e., the density of adult flies. The value of *V* then is the result of the following: 

\[ V = \text{(infectiousness of infected hosts)} \times \text{(rate of contact between infected and healthy cattle)} \times \text{(persistence over time of infection in the host)} \times \text{(interaction between *Besnoitia besnoiti*-vector)} \times \text{(vector density)}. \]

In order to counter the spread of besnoitiosis, researchers, veterinarians and cattle producers must act on each of these *V* components in accordance with current knowledge. As noted by Bigalke [7], if mechanical *B. besnoiti* transmission by *Stomoxys calcitrans* is of significance in the epidemiology of cattle besnoitiosis, the lack of parasite persistence on the mouthparts of stable flies suggests that mechanical transmission may only occur during brief periods of time [37]. This is in contrast with many bloodsucking vectors which are able to transmit pathogens for the rest of their life, such as Culicoides vectors of Bluetongue virus [38] or phlebotomine sand fly vectors of leishmaniasis [39]. Therefore, the long persistence of infectivity in a minority of subclinical carriers could contribute to the insidious spread of the disease within the herd.

In the present study, PCR analysis of skin biopsies performed on 518 cattle highlights the heterogeneity of this infectivity, revealing a limited number of potential super-spreaders on each of the eight farms studied. This long persistence of super-spreader status was confirmed on only one farm by a second PCR analysis one year after the initial evaluation. This heterogeneity is largely shaping the epidemiology and transmission of infectious diseases. It opens herein the prospect of a new means to control besnoitiosis by identifying and selectively culling these super-spreaders. A significant positive correlation between the antibody titers and the parasite DNA load evaluated by real-time PCR on a skin fragment was evidenced in 147 asymptomatic individuals which were analyzed in the same laboratory with the same ELISA commercial kit. A similar observation was previously performed by Schares et al. [25] who noted a positive correlation between antibody titer and the amount of parasite DNA detected by PCR in 43 symptomatic cattle from the same cattle herd. Similarly, Frey et al. [29] found the highest parasite DNA loads in cattle with the highest antibody levels. Unfortunately, this correlation was not strong enough to use antibody titers as a proxy of real-time PCR analysis to detect super-spreaders, and many animals showed high antibody titers while yielding negative PCR results. The influence of some factors such as farm, age and antibody titer on the parasite load in the skin of cattle were confirmed. However, the multivariate analysis did not allow the determination of a typical profile of a super-spreader individual.

We proposed to farmers that they cull very quickly or, at the minimum, isolate highly contaminated animals from the rest of the herd to reduce the transmission pressure. The ultimate goal of such a control strategy is to protect the healthy herd from new infections and to progress step by step towards a disease-free herd. However, many difficulties were encountered. The motivation of farmers to apply the recommendations differed from farm to farm. When cattle considered at risk of transmission were eliminated or segregated as soon as possible (farms A and D), the serological incidence was relatively low in the following months and years. This result was very encouraging and showed the potential efficacy of this strategy in reducing the parasite transmission within a herd. In contrast, these
recommendations were not followed on farms B and C, and serological incidences in the following months and years were very high. On farm C, we accurately monitored heifers with highly positive serology and PCR results at two sampling periods (May 2017 and March 2018). Although the number of animals examined was low, it seemed that the super-spreader status persisted over time. These findings reinforce the desirability of eliminating these individuals as quickly as possible. Nevertheless, given the small number of farms that were included in this study, these assumptions need to be confirmed in further studies.

Conclusion

This survey demonstrated that parasite DNA load is massive in the skin of animals suffering from severe scleroderma of besnoitiosis. In contrast, this parasite DNA load seems to be more heterogeneous in asymptomatically infected individuals. A real time PCR analysis on a skin sample taken at the tail base can provide more accurate information on the infectiousness status of the bovine compared to other locations and allows for the detection of super-spreaders in a herd. All seropositive animals from eight farms were tested and the proportion of super-spreaders was low on all of the farms studied. A new strategy of bovine besnoitiosis control using the rapid culling of these super-spreaders was tested and gave encouraging results: the number of new infections was reduced compared to herds where super-spreaders were kept in the herd. However, due to the small number of farms included in the present study, these preliminary results need to be confirmed at a large scale.

Abbreviations

EFSA: European Food and Safety Authority; LVD: Departmental Veterinary Laboratory; Real-time PCR: Real-time polymerase chain reaction; IFAT: Indirect fluorescent antibody test; ELISA: Enzyme-linked immunosorbent assay; WB: Western blot; ITS rDNA: Internal transcribed spacer ribosomal DNA; SD: standard deviation; Ct: threshold cycle.

Declarations

Ethics approval and consent to participate

The biopsies performed at the envt and at the slaughterhouse, were collected from already dead animals: ethics not applicable. The biopsies performed in the farms were supervised and operated by a practicing veterinarian with the consent of the breeders.

Consent for publication

All authors read and approved to publication the final version of the manuscript.

Competing interests
The authors declare that they have no competing interests.

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**Author’s contributions**

CG collected the samples, performed the laboratory work, performed data analysis and wrote the manuscript. LB collected the samples, performed data analysis and wrote the manuscript. FP collected the samples, performed the laboratory work. JPA, MR, XD participated in serological screening. JP, EL, JLM, KV collected the samples. CB, CL provided project’s coordination. FC supervised the statistical analysis. PJ planned and supervised the study, collected the samples and wrote the manuscript.

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**References**

1. Besnoit C, Robin V. Sarcosporidiose cutanée chez une vache. Rev Méd Vet. 1912;37:649–63.
2. EFSA Bovine besnoitiosis. : an emerging disease in Europe. EFSA J. 2010;8;1499–514.
3. Cortes HCE, Leitão A, Gottstein B, Hemphill A. A review on bovine besnoitiosis: a disease with economic impact in herd health management, caused by *Besnoitia besnoiti* (Franco and Borges). Parasitol. 2014;141:1406–17.

4. Jacquiet P, Liénard E, Franc M. Bovine besnoitiosis: epidemiological and clinical aspects. Vet Parasitol. 2010;174:30–6.

5. Basso W, Schaeres G, Gollnick NS, Rütten M, Deplazes P. Exploring the life cycle of *Besnoitia besnoiti* - experimental infection of putative definitive and intermediate host species. Vet Parasitol. 2011;178:223–34.

6. Esteban-Gil A, Grisez C, Prevot F, Florentin S, Decaudin A, Picard-Hagen N, Berthelot X, Ronson P, Alzieu JP, Marois M, Corboz N, Peglion M, Vilardell C, Liénard E, Bouhsira E, Castillo JA, Franc M, Jacquiet P. No detection of *Besnoitia besnoiti* DNA in the semen of chronically infected bulls. Parasitol Res. 2014;113:2355–62.

7. Bigalke RD. New concepts on the epidemiological features of bovine besnoitiosis as determined by laboratory and field investigations. Onderstepoort J Vet Res. 1968;35:3–137.

8. Sharif S, Jacquiet P, Bouhsira E, Prevot F, Grisez C, Raymond-Leutron I, Semin M, Geoffré A, Trumel C, Franc M, Liénard E. *Stomoxys calcitrans*, mechanical vector of virulent *Besnoitia besnoiti* from chronically infected cattle to susceptible rabbit. Med Vet Entomol. 2019;33:247–55.

9. Liénard E, Salem A, Grisez C, Prévot F, Bergeaud JP, Franc M, Gottstein B, Alzieu JP, Lagalisse Y, Jacquiet P. A longitudinal study of *Besnoitia besnoiti* infections and seasonal abundance of *Stomoxys calcitrans* in a dairy cattle farm of southwest France. Vet Parasitol. 2011;177:20–7.

10. Olias P, Schade B, Mehlhorn H. Molecular pathology, taxonomy and epidemiology of *Besnoitia species* (Protozoa: Sarcocystidae). Infect Genet Evol. 2011;11:1564–76.

11. Bigalke RD, Schoeman JH, McCully RM. Immunization against bovine besnoitiosis with a live vaccine prepared from a blue wildebeest strain of *Besnoitia besnoiti* grown in cell cultures. 1. Studies on rabbits. Onderstepoort J Vet Res. 1974;41(1):1–5.

12. Tainchum K, Shukri S, Duvallet G, Etienne L, Jacquiet P. Phenotypic susceptibility to pyrethroids and organophosphate of wild *Stomoxys calcitrans* (Diptera: Muscidae) populations in southwestern France. Parasitol Res. 2018;117:4027–32.

13. Reissert-Oppermann S, Bauer B, Steuber S, Clausen PH. Insecticide resistance in stable flies (*Stomoxys calcitrans*) on dairy farms in Germany. Parasitol Res. 2019;118:2499–507.

14. Cortes HC, Reis Y, Waap H, Marcelino E, Vaz Y, Nunes T, Fanzendeiro I, Caeiro V, Leitao A,. Longitudinal study of Besnoitia *besnoiti* infection prevalence rates in a beef cattle herd in Alentejo, Portugal. In: Proceedings of the COST 854 Final Conference, Liege, Belgium. 2006b;20.

15. Alzieu JP, Jacquiet P, Boulon C, Mejean F, Lemaire-Meyer M, Desclaux X, Rameil M, Perez L, Prevot F, Grisez C. L’expérience de dépistage et d’éradication de la besnoitiose bovine sur le plateau ardéchois du Coiron entre 2010 et 2019. Bull GTV. 2019;96:57–62.

16. Langenmayer MC, Gollnick NS, Majzoub-Altwec M, Schar JC, Schaeres G, Hermanns W. Naturally acquired bovine besnoitiosis: histological and immunohistochemical findings in acute, subacute, and
chronic disease. Vet Pathol. 2015;52:476–88.

17. Cortes HCE, Reis Y, Gottstein B, Hemphill A, Leitao A, Muller N. Application of conventional and real-time fluorescent ITS1 rDNA for detection of Besnoitia besnoiti infections in bovine skin samples. Vet Parasitol. 2007;146:352–6.

18. Schares G, Langenmayer MC, Majzoub-Altweck M, Scharr JC, Gentile A, Maksimov A, Schares S, Conraths FJ, Gollnick NS. Naturally acquired bovine besnoitiosis: Differential distribution of parasites in the skin of chronically infected cattle. Vet Parasitol. 2016;216:101–7.

19. Cortes HCE, Nunes S, Reis Y, Staubli D, Vidal R, Sager H, Leitao A, Gottstein B. Immunodiagnosis of Besnoitia besnoiti infection by ELISA and Western Blot. Vet Parasitol. 2006;141:216–25.

20. García-Lunar P, Ortega-Mora LM, Schares G, Gollnick NS, Jacquiet P, Grisez C, Prevot F, Frey CF, Gottstein B. et Alvarez-García G. An Inter-Laboratory Comparative study of serological tools employed in the diagnosis of Besnoitia besnoiti infection in bovines. Transboundary Emerg Dis. 2013;60:59–68.

21. Gollnick NS, Scharr JC, Schares G, Langenmayer MC. Natural Besnoitia besnoiti infections in cattle: chronology of disease progression. BMC Vet Res. 2015;11–32.

22. Villa L, Gazzonis AL, Zanzani SA, Perlotti C, Sironi G, Manfredi MT. Bovine besnoitiosis in an endemically infected dairy cattle herd in Italy: serological and clinical observations, risk factors, and effects on reproductive and productive performances. Parasitol Res. 2019;118:3459–68.

23. Liénard E, Grisez C, Alzieu JP, Prévot F, Bardoux P, Blanchard B, Salem A, Franc M, Jacquiet P. Blurred epidemiology of bovine besnoitiosis: parasite detection in skin among seropositive cattle. ApiCOWplexa. Apicomplexa in farm animals. Int Meet Lisbon. 2012;p. 57:122.

24. Basson PA, McCully RM, Bigalke RD. Observations on the pathogenesis of bovine and antelope strains of Besnoitia besnoiti (Marotel, 1912) infection in cattle and rabbits. Onderstepoort J Vet Res. 1970;37:105–26.

25. Schares G, Maksimov A, Basso W, More G, Dubey JP, Rosenthal B, Majzoub M, Rostaher A, Selmaire J, Langenmayer MC, Scharr JC, Conraths FJ, Gollnick NS. Quantitative real time polymerase chain reaction assays for the sensitive detection of Besnoitia besnoiti infection in cattle. Vet Parasitol. 2011;178:208–16.

26. Liénard E, Pop L, Prevot F, Grisez C, Mallet V, Raymond-Letron I, Bouhsira É, Franc M, Jacquiet P. Experimental infections of rabbits with proliferative and latent stages of Besnoitia besnoiti. Parasitol Res. 2015;114:3815–26.

27. Alzieu JP, Jacquiet P, Liénard E, Grisez C, Prévot F, Malavieille R, Desclaux X, Nussbaum S, Bergeaud JP, Franc M, Schelcher F, Corboz N, Bastien F, Guerrier-Chatelet MC, Gavet L, Schneider V, Boulon C. Réémergence de la besnoitiose bovine: démarche diagnostique et possibilités de contrôle. Bull GTV. 2011;58:71–85.

28. Gondim LFP, Mineo JR, Schares G. Importance of serological cross-reactivity among Toxoplasma gondii, Hammondia spp., Neospora spp., Sarcocystis spp. and Besnoitia besnoiti. Parasitology. 2017, 144:851–868.
29. Frey CF, Gutiérrez-Expósito D, Ortega-Mora LM, Benavides J, Marcen JM, Castillo JA, Casasus I, Sanz A, García-Lunar P, Esteban-Gil. A, Álvarez- García, G. Chronic bovine besnoitiosis: Intra-organ parasite distribution, parasite loads and parasite-associated lesions in subclinical cases. Vet Parasitol. 2013;197:95–103.

30. Alvarez-García G, García-Lunar P, Gutiérrez-Expósito D, Shkap V, Ortega-Mora LM. Dynamics of Besnoitia besnoiti infection in cattle. Parasitol. 2014;14:1419–35.

31. VanderWaal KL, Ezenwa VO. Heterogeneity in pathogen transmission: mechanisms and methodology. Funct Ecol. 2016;30:1606–22.

32. Cooper L, Kang SY, Bisanzio D, Maxwell K, Rodriguez-Barraquer I, Greenhouse B, et al. Pareto rules for malaria super-spreaders and super-spreading. Nat Commun. 2019;10:3939.

33. Wilson K, Bjørnstad ON, Dobson AP, Merler S, Pog-layen G, Randorlf SE, Read AF, Skorping A. Heterogeneities in macroparasite infections: patterns and processes. In: Hudson PJ, Rizzoli A, Grenfell BT, Heesterbeek H, Dobson AP, editors. The Ecology of Wildlife Diseases, Oxford University Press, Oxford. 2002:6–44.

34. Woolhouse ME, Dye C, Etard JF, Smith T, Charlwood JD, Garnett GP, Hagan P, Hii JL, Ndlovu PD, Quinnell RJ, Watts CH, Chandiwana SK, Anderson RM. Heterogeneities in the transmission of infectious agents: implications for the design of control programs. Proc Natl Acad Sci U S A. 1997;94:338–42.

35. Lloyd-Smith JO, Schreiber SJ, Kopp PE, Getz WM. Superspreading and the effect of individual variation on disease emergence. Nature. 2005;438:355–9.

36. Sharif S. Stomoxys calcitrans: évaluation du rôle dans la transmission de Besnoitia besnoiti et nouveaux moyens de contrôle. 2018, PhD, Pathologie, Toxicologie, Génétique et Nutrition, Institut National Polytechnique de Toulouse.

37. Sharif S, Jacquiet P, Prevot F, Grisiez C, Bouhsira E, Franc M, Liénard E. Assessment of persistence of Besnoitia besnoiti (Henry, 1913) bradyzoites in Stomoxys calcitrans (Diptera: Muscidae). Revue Méd Vét. 2017;168:197–203.

38. Paslaru AI, Mathis A, Torgerson P, Veronesi E. Vector competence of pre-alpine Culicoides (Diptera: Ceratopogonidae) for bluetongue virus serotypes 1, 4 and 8. Parasites Vectors. 2018;11:466.

39. Bates PA. Transmission of Leishmania metacyclic promastigotes by phlebotomine sand flies. Int J Parasitol. 2007;37:1097–106.

**Tables**

Table I: Description of the eight farms studied.
| Farm          | Husbandry | Breed                | Number of individuals examined | Initial* seroprevalence (%) | Number of clinical cases (period) |
|---------------|-----------|----------------------|-------------------------------|----------------------------|---------------------------------|
| A (Aveyron)   | Dairy cattle | Prim'Holstein       | 32                            | 42                         | 2 (2016)                        |
| B (Tarn)      | Beef cattle | Blonde d'Aquitaine  | 49                            | 67 (> 2 years) 50 (< 2 years) | 6 (2014–2016)                  |
| C (Ariège)    | Beef cattle | Limousine           | 32                            | 92                         | 3 (2015–2017)                  |
| D (Ardèche)   | Beef cattle | Charolaise          | 34                            | 51                         | ND                              |
| E (Cher)      | Dairy cattle | Prim'Holstein       | 161                           | 74                         | ND                              |
| F (Indre)     | Beef cattle | Charolaise          | 163                           | 80                         | 6 (2016–2018)                  |
| G (Ardèche)   | Dairy cattle | Montbéliarde        | 25                            | 52                         | ND                              |
| H (Ardèche)   | Dairy cattle | Montbéliarde        | 22                            | 88                         | 4 (2018)                       |

ND: not documented

*The month before PCR-skin sample analysis.

Table II: distribution of 160 necropsied cattle according to PCR Ct values and western blot (WB) results (slaughterhouses in southern France, endemic areas of besnoitiosis).

| WB         | PCR results | Total |
|------------|-------------|-------|
|            | Ct ≤ 36     | 36 < Ct ≤ 40 | No Ct |
| Positive   | 15          | 8     | 24   |
| Negative   | 2           | 14    | 97   |
| Total      | 17          | 22    | 121  |

Table III: results from the multivariable logistic regression model fitted to the binary positive/negative real-time PCR response for 147 cattle.
| Variable                                      | OR (Odds Ratios) | 95% CI *  | p-value   |
|----------------------------------------------|------------------|-----------|-----------|
| Farm A                                       | Reference        |           |           |
| Farm B                                       | 0.36             | 0.13–0.94 | 392.10⁻²  |
| Farm C                                       | 0.12             | 0.03–0.43 | 1.86.10⁻³ |
| Farm D                                       | 0.01             | 0.00-0.07 | 5.21.10⁻⁶ |
| Age : heifer ≤ 24 months                     | Reference        |           |           |
| Age : cow > 24 months                        | 0.09             | 0.02–0.38 | 1.85.10⁻³ |
| OD** < 110%                                  | Reference        |           |           |
| OD** ≥ 110%                                  | 2.60             | 1.18–5.93 | 1.98.10⁻² |

* 95% CI: 95% confidence interval

** OD: optical densities in ELISA

Table IV: Number of animals showing a seroconversion and total number of animals examined, 12 months after the initial detection of super-spreaders, according to the strategy used by farmers

| Strategy                                      | Farm | 12 months* |
|-----------------------------------------------|------|------------|
| Rapid culling of super-spreaders              | A    | 1/30       |
|                                               | D    | 0/48       |
|                                               | H    | 1/23       |
| Conservation of super-spreaders on the farm   | B    | 5/10       |
|                                               | C    | 12/14      |

*Time after the initial detection of super-spreaders

Table V: Real-time PCR results obtained on skin biopsies 12 months (farm C) or 36 months (farm A) after the initial evaluation.
| Farm | Animal | First real-time PCR result (Ct value) | Second real-time PCR results (Ct value) |
|------|--------|--------------------------------------|----------------------------------------|
|      |        |                                      | 10 months later | 36 months later |
| A    | 1      | 38                                   | NoCt                                   |
|      | 2      | 38                                   | 38                                     |
|      | 3      | 39                                   | NoCt                                   |
|      | 4      | NoCt                                 | NoCt                                   |
|      | 5      | NoCt                                 | NoCt                                   |
|      | 6      | 37                                   | NoCt                                   |
|      | 7      | NoCt                                 | NoCt                                   |
| C    | 1      | 34                                   | 35                                     |
|      | 2      | 26                                   | 26                                     |
|      | 3      | 21                                   | 24                                     |
|      | 4      | 25                                   | 16                                     |
|      | 5      | 29                                   | 26                                     |
|      | 6      | 29                                   | 36                                     |

**Figures**
Figure 1

Relationship between Ct values in real-time PCR and optical densities in ELISA in a subset of 147 individuals. The solid black line shows the estimated linear regression line. The dashed horizontal line shows the ELISA DO 110% threshold. The vertical dashed line shows the PCR Ct value 30 threshold. Cattle with negative PCR result were scored a Ct value of 45.
Figure 2

Distribution of B. besnoiti seropositive individuals according to the class of Ct in real-time PCR on the eight farms (detailed results)
Figure 3

Distribution of 518 B. besnoiti seropositive individuals according to the class of Ct in real-time PCR (pooled results from the eight farms)
Real-time PCR results (expressed as mean Ct values and SD) of 14 B. besnoiti infected cattle showing severe scleroderma, slaughtered in the facilities of the National Veterinary School of Toulouse. 1A: skin samples; 1B: internal organs