

Assessment of persistence of *Besnoitia besnoiti* (Henry, 1913) bradyzoites in *Stomoxys calcitrans* (Diptera: Muscidae)

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SUMMARY

The Stable fly, *Stomoxys calcitrans*, is a common and worldwide distributed blood-feeding fly. It is the vector of numerous pathogens including *Besnoitia besnoiti*, a cyst-forming coccidian parasite of cattle. Infection of stable flies occurs either following the dissection of cutaneous *B. besnoiti* bradyzoites cysts or consumption of tachyzoite-infected blood. Determinants governing the vector competence of *S. calcitrans* for *B. besnoiti* remain poorly known. We addressed here the persistence of *B. besnoiti* bradyzoites on mouthparts and in digestive tract of *S. calcitrans*. Twelve batches of 200 laboratory reared *S. calcitrans* were fed on *B. besnoiti* chronically infected cows either 5 min (three batches, interrupted blood meal) or 15 min (nine batches, complete blood meal). After this infective exposure, flies completed their meal with *B. besnoiti*-free blood during one hour, either immediately (short exposure) or 48, 96, 144h later. Quantitative PCR was used for *B. besnoiti* DNA detection in the recipient blood, the mouthparts and the abdominal content. Recipient blood was microscopically examined to find entire parasites. Very few motile parasites were observed until 48 h in the recipient blood. Beyond this point, only parasite DNA traces were recovered in some abdominal content of stable flies. The fast destruction of bradyzoites in 48 h suggests that mechanical transmission could occur only in brief time periods between feeds. New experimental infections are required to assess if these observed parasites could induce seroconversion in susceptible hosts or are infective for cell cultures.

Keywords: Cattle besnoitiosis, bradyzoites, stable fly, mechanical vector

RÉSUMÉ

Évaluation de la persistance des bradyzoïtes de *Besnoitia besnoiti* (Henry, 1913) chez *Stomoxys calcitrans* (Diptera : Muscidae)

Le Stomoxe, *Stomoxys calcitrans*, est une mouche piqueuse banale et cosmopolite vectrice de nombreux agents pathogènes, dont *Besnoitia besnoiti*, protozoaire parasite des bovins. Les mouches se contaminent soit en perçant des kystes à bradyzoïtes cutanés soit en ingérant des tachyzoïtes sanguins. La compétence vectorielle de *S. calcitrans* pour *B. besnoiti* demeurent mal connus. Nous avons évalué la persistance des bradyzoïtes de *B. besnoiti* chez *S. calcitrans* (proboscis et tube digestif). Douze lots de 200 *S. calcitrans* élevés en laboratoire ont été nourris sur des vaches chroniquement infectées par *B. besnoiti* pendant 5 min (trois lots, repas interrompu) ou pendant 15 min (neuf lots, repas complet). Les mouches ont ensuite été exposées pendant une heure à du sang indemne de *B. besnoiti* soit immédiatement (exposition courte) soit 48, 96, 144h plus tard. La PCR quantitative a été utilisée pour la détection d'ADN de *B. besnoiti* dans le sang receveur, les parties buccales et le contenu abdominal. Le sang receveur a été examiné au microscope pour rechercher des parasites entiers. Quelques parasites mobiles ont été observés jusqu'à 48 h dans le sang receveur. Au-delà de ce point, seules des traces d'ADN parasitaire ont été trouvées dans le contenu abdominal des mouches. La destruction rapide des bradyzoïtes en 48 h suggère que la transmission mécanique ne peut se produire que peu de temps après le repas infectant. D'autres infections expérimentales sont nécessaires pour évaluer si ces parasites peuvent induire une séroconversion des hôtes sensibles ou demeurent infectieux pour des cultures cellulaires.

Mots-clés : Besnoitiose bovine, bradyzoïtes, mouche charbonneuse, vecteur mécanique

Introduction

The cosmopolitan Stable fly *Stomoxys calcitrans* (L.) (Diptera: Muscidae) is one of the major arthropod pests of livestock and occasionally a biting nuisance of humans [14, 28]. Both sexes of adult imagoes are obligate blood feeders. Their economic impact may exceed annually US\$2 billion in the United States alone for the cattle industry [29] inducing milk production reduction and weight losses [5, 7, 22]. They are persistent feeders, inflicting painful bites resulting in interruption of blood meal by host defensive behaviour [26]. The blood meal completion is then performed on the same host or on nearby animals [11]. Besides the animal harassment and blood losses, they are vectors of more than 20 arthropod-borne infectious agents including viruses, bacteria, rickettsia, protozoa, and helminths of veterinary

and zoonotic importance [3]. The frequent interrupted blood feeding is known to dramatically increase the risk of mechanical pathogen transmission by regurgitation of part of a previous blood meal or passive transfer of contaminated blood on mouthparts [8]. This jeopardy is moreover enhanced by the survival ability of the implied pathogens inside or on the vector. For instance, *Trypanosoma evansi* remains infective up to 480 min in *Stomoxys niger niger* in the digestive tract but less than 7 minutes in or on the proboscis [27].

Among this wide panel of pathogens, the protozoan *Besnoitia besnoiti* (Henry, 1913), a cyst-forming Apicomplexa, has received more attention since 2010 when it has been considered as a re-emerging parasite by the European Food Safety Authority [12]. It is the causative agent of cattle besnoitiosis which has spread for about 20

years from its European historic enzootic areas (Portugal, Spain, and southern France) [16] towards various European countries: Italy [1], Germany [18], Switzerland [4], Hungary [15], Croatia [9], Greece [20], Belgium [30] and Ireland [23]. Intensification of international cattle trading could probably contribute largely to this parasite importation in new countries [19]. In recently infected cattle herds, the seroprevalence increases quickly and up to 20% of newly contaminated cattle could exhibit clinical signs with fever and oedemas, sometimes mortality and abortion, male transient or definitive sterility and, later, for the chronic phase of the disease, numerous scleroderma cysts containing thousands of bradyzoites [16, 21]). Blood-sucking flies including *S. calcitrans*, could participate to the rapid and local spread of the disease within herds by piercing skin bradyzoite cysts or ingesting tachyzoites in blood [6, 17]. Among the various factors affecting the vector transmission of cattle besnoitiosis, the survival of *B. besnoiti* in *S. calcitrans* between two successive blood meals remains poorly studied [6]. However, this important feature could be implied in dissemination of the parasites between herds, taking account that stable flies could feed at least once a day [24] and are able to fly up to 7.1 km per day when searching for a host [2]. Estimation of persistence has been based mainly on the inoculation to susceptible rabbits of triturated abdominal contents of infected *S. calcitrans* at different intervals of time after an infective blood meal [6]. No clinical signs have been observed on rabbits which were inoculated with triturated abdominal contents of *S. calcitrans* 3 hours after the infective blood meal [6]. The same study performed on tabanids has showed that parasite could remain viable and infective to rabbits up to 24 h post feeding [6]. Nevertheless, inoculation of triturated abdominal contents is insufficient to assess the suspected natural route of contamination which involves parasite passage via the mouthparts. Moreover, presence of *B. besnoiti* DNA in or on stable fly proboscis and in recipient blood (where flies were allowed to achieve their blood meal) has been recorded until 24 h after the exposure to skin of cow in scleroderma stage of the disease [17]. Founded on these previous results, the aim of this study was to investigate the persistence of *B. besnoiti* within and on the mouthparts of stable flies and within their digestive tract beyond 24 h following an initial blood meal on *B. besnoiti* chronically infected cows.

Material and methods

SOURCE AND MAINTENANCE OF INSECTS

Stable flies were obtained from a colony established and maintained in the laboratory of the École Nationale Vétérinaire de Toulouse (ENVT, France) since 2009 as previously described [24].

A total of three thousand newly emerged stable fly adults aged 4 ± 2 days old was used with a 1: 1 sex ratio for this study.

SOURCE OF *BESNOITIA BESNOITI* BRADYZOITES

Two cows (Limousine breed, number 1330, Blonde d'Aquitaine breed, number 1575) suffering from severe chronic besnoitiosis were used as a source of *B. besnoiti* bradyzoites. They came from commercial herds in Tarn and Haute-Garonne French departments respectively. They were 16 months old (1330) and 6 years old (1575). They were referred to the large animal hospital of the ENVT for necropsy examination. Serological confirmation of cattle besnoitiosis was performed by in-house Western blot analysis (see below). For the following experiments (experiments 2 and 3, see below), three selected skin areas (right shoulder, back line and right hip of 90 cm²) were shaved the day before the trials. The skin parasite burden per 500 mg of skin sample was assessed with quantitative PCR (see below) from biopsies of three skin locations described above (Biopsy Punch 8 mm[®], Kruuse, Langeskov, Denmark).

SOURCE OF *BESNOITIA BESNOITI* FREE BLOOD

A seven year-old cow maintained at the ENVT provided uninfected *Besnoitia besnoiti* blood for experimental transmission trials. This cow had not received any insecticide treatment for seven years. It was serologically negative for *B. besnoiti* during this period as regularly confirmed by in-house Western blot analysis. The blood was collected for all experiments the day before the experiment in 4-ml tubes containing 6.3 mg EDTA-K3 (Terumo Europe N.V. [®], Leuven, Belgium) and stored at 4°C for 24 hours.

EXPERIMENTAL DESIGN

Batches of two hundred stable fly imagoes per cage were starved 48 hours for blood and 24 hours for bee honey before the trials. Tap water was available *ad libitum*.

EXPERIMENT 1 – CONTROL GROUP

To assess the absence of a *B. besnoiti* contamination of the laboratory-bred stable flies, a batch of 200 *S. calcitrans* was fed on 8 ml uninfected blood from the *B. besnoiti*-free cow contained in a double-chambered glass feeder placed in contact with the upper side of the cage during 1 h. The flies were maintained in mesh cages (30 × 30 × 30 cm). The glass feeder containing the uninfected blood was warmed up to 38.4°C and was closed with two Parafilm membranes (Parafilm 3 M[®], Pechiney Plastic Packaging, Chigago, IL, USA) for fly bites. The stable flies were maintained in a room at $22 \pm 2^\circ\text{C}$, with 12:12 hours light:dark photoperiod and $40 \pm 10\%$ relative humidity (RH). After the exposure, two ml of the recipient blood were collected, one ml used for qPCR analysis and one ml diluted in 5 ml of sterile PBS for extemporaneous microscopic examination of *B. besnoiti* presence (Zeiss Telaval 31[®], Carl Zeiss, Le Pecq, France). All the flies were killed at -20°C for 1 h and dissected [17]. The proportions of engorged flies were evaluated in each

assay. Mouthparts were manually removed and pooled into a grinding tube containing 1.4 ml of PBS (Bio-Rad®, Marnes-la-Coquette, France). Abdominal contents were collected and pooled into the same sterile 4-ml tube containing 60 USP U Lithium Heparin (Terumo Europe N.V.®, Leuven, Belgium) and 2 ml of PBS. Presence of *B. besnoiti* DNA was assessed by quantitative PCR (see below) in mouthpart, abdominal content and recipient blood samples. This trial was then repeated twice again.

EXPERIMENT 2 – IMMEDIATE TRANSFER OF *B. BESNOITI* BRADYZOITES BY *S. CALCITRANS* AFTER THE INFECTED BLOOD MEAL

The aim of this trial was to assess the undelayed transfer of bradyzoites by stable flies following an interrupted blood meal on a chronically infected cow to a *B. besnoiti*-free bovine blood contained in an artificial glass feeder.

A cage of 15 × 15 × 15 cm enclosed by thin wire mesh containing 200 *S. calcitrans* was put on each of the three skin locations. Stable flies were allowed to ingest blood for 5 minutes [10] on cow number 1330 (blood meal 1). Immediately after, *B. besnoiti*-free blood (*ie* recipient blood) was given to these flies to achieve their blood meal during 1 h in the same laboratory conditions as for experiment 1 (blood meal 2). After one hour, all stable flies were killed and dissected as previously described for the control group for qPCR analyses. Two milliliters of the recipient blood were collected and processed by qPCR and microscopic examination

EXPERIMENT 3 – DELAYED TRANSFER AND PERSISTENCE OF *B. BESNOITI* BRADYZOITES WITHIN *S. CALCITRANS*

The aim of this trial was to assess the persistence of *B. besnoiti* infection within stable flies after a complete blood meal on chronically infected cows. The skin locations described previously in experiment 2 were used. Different batches of 300 stable flies maintained in a cage, as previously described, performed a complete blood meal during 15 min on one of the two cows and one of the three skin areas (blood meal 1). For each skin location (and for the corresponding batch of flies), the second blood meal, consisting of uninfected blood (blood meal 2), was then presented either 48 h (cow 1330), 96 h or 144 h (cow 1575) later. Flies were provided with water and honey bee immediately at the end of the cow skin exposure until the second blood meal. The batches of stable flies and the remaining blood in the glass feeder were analyzed as defined in experiment 2 after the second blood meal.

LABORATORY PROCEDURES

Serological analysis

The sera from the uninfected and the two chronically infected cows were analyzed by an in-house Western-Blot as previously described [17].

Detection of *B. besnoiti* DNA

Quantitative PCR was used to detect *B. besnoiti* DNA from infected bovine skin samples (after their necropsies) in the three skin areas used for infection of stable flies, from mouthparts and abdominal contents of *S. calcitrans* and from blood of glass feeders (before and after transmission trials). Mouthparts of stable flies were ground with the TeSeE™ Purification Kit (Bio-Rad, Marnes-la-Coquette, France) according to the manufacturer's recommendation. DNA extraction was achieved with the commercial kit QIAmp® DNA Mini Kit (Qiagen®, Courtabœuf, France). *B. besnoiti* ITS-1 amplification was performed with the commercial PCR kit AdiaVet™ Besnoitia (BioX-Diagnostics®, Jemelle, Belgium) and with the Stratagene MX3005P thermal cycler (Agilent Technologies®, La Jolla, CA). Positive and negative controls were provided by the manufacturer. Results were analyzed using the MxPro QPCR version 4.10 software (Agilent Technologies®, La Jolla, CA). A threshold cycle (Ct) value, equal or superior to 40 corresponded to any parasite DNA detection.

Standard curve and reproducibility of the detection of *B. besnoiti* ITS-1

Purified *B. besnoiti* tachyzoites were isolated from the skin of chronically infected heifer coming from the French Dordogne department. They have been maintained on Vero Cells at the laboratory since 2015. They were at the weekly passage 124. They were used to generate a standard curve assessing the detection limit of the qPCR of the commercial PCR kit AdiaVet™ Besnoitia. DNA extraction of a suspension of 10⁵ *B. besnoiti* culture tachyzoites was performed as described above and serially diluted 10-fold in PBS ranging from 10⁵ to 1 parasite equivalents per milliliter of PBS (par. eq./mL). The standard curve was generated with Ct values (*y*-axis) plotted against the log of the number of par. eq. (*x* axis). The amplification efficiency (E) of the qPCR was calculated with the formula $E = (10^{-(1/S)}) - 1$ where S was the slope of the standard curve. To determine the inter-assay reproducibility, three replicates of 10-fold serial dilutions of *B. besnoiti* DNA (between 1 and 10⁵ tachyzoites) were performed. The intra-assay reproducibility was assessed on three replicates using one range of the previous DNA dilutions used in the inter-assay reproducibility.

DATA ANALYSIS

The obtained CT values were transformed into the number of parasites according to the equation of the linear

regression of the standard curve estimated with Microsoft™ Excel 2013 software (Microsoft Corporation™, Redmond, Washington, USA). The arithmetic means, standard deviations, and coefficients of variation of the dilution ranges were computed with Microsoft™ Excel 2013 software. Parasite burden between the two cows considering all skin locations together were compared with the permutation test (exact procedure) implemented in the software package StatXact® release 10.0 (Cytel Software Corporation, Cambridge, Massachusetts, USA) with a significant threshold of 0.05.

Results

The efficiency (E) of the qPCR by combination of the intra- and inter-assays was 92%, with a strong linear correlation ($R^2 = 0.9996$), a y-intercept value of 40.405, and a slope of -3.5318 (Figure 1). The detection limit was found to be 10 parasite equivalents, corresponding to a mean Ct value of 36.82 ± 0.87 . Amplifications of lower numbers of parasite equivalents (< 10) provided inconstant and non-reproducible Ct values even when after increase of the number of amplification cycles (data not shown). The coefficients of variation were low, ranged from 0.34% for 10^4 par. eq./ml to 2.36% for 10 par. eq./ml showing strong reproducibility.

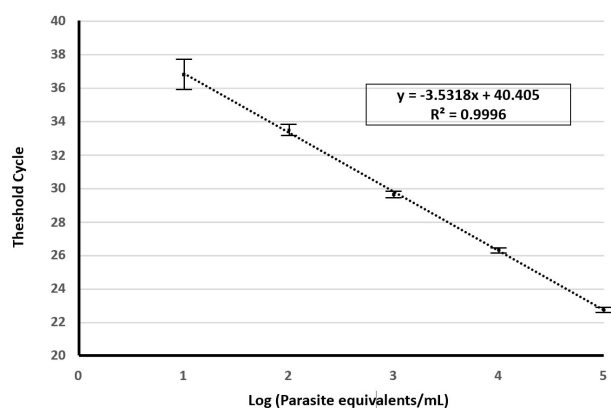


FIGURE 1: Reportable range (\pm standard deviation) for *B. besnoiti* detection and quantification by qPCR. qPCR was carried out with serial diluted DNA extracted from parasite cultivated samples, ranging from 10^5 to 10 par. eq./mL, tested in six replicates each (combination of 3 intra- and 3 inter-assays). The equation obtained by linear regression is indicated in the chart.

Neither batches of *S. calcitrans* nor the cattle recipient blood were found positive for *B. besnoiti*-specific qPCR among the three replicates of experiment 1.

The skin parasite loads, determined by qPCR, varied from 5,866,678 to 13,253,057 parasite equivalents per 500 mg of skin sample, whatever the skin location and the cow (Table I). The mean numbers of parasite equivalents, considering all the three skin locations together, were $11,745,994 \pm 2,610,311$ for the cow 1330 and $10,790,931 \pm 4,264,528$ for the cow 1575 and were not significantly different ($p > 0.05$).

Mortality rates of stable flies maintained for 48, 96 and 144 hours after the infectious blood meal intervals ranged from 5

to 10 %. The proportions of engorged flies, assessed visually by the same operator during the whole study, reached 65 – 70% for experiment 2 (5 min of exposure) and varied from 85 to 90% for experiments 1 and 3 (15 min of exposure).

For the experiment 2 (immediate transfer), the number of parasite equivalents was low (less than 100 parasite DNA equivalents) in mouthparts and recipient blood compartments and highest in the abdominal contents, more than 150,000 parasite equivalents (Tables I and II). One or two *B. besnoiti* bradyzoites were observed motile in the recipient blood (Table III).

Parasite DNA continued to be detectable 48h after feeding (experiment 3) but the number of parasite equivalents has dramatically decreased in *S. calcitrans* (less than 20, Table I). No parasite DNA was found in one out three mouthpart samples and two out three abdominal contents (Table I). Some rare motile parasites (one or two) were observed in the recipient blood (Table III). In parallel, no parasite DNA could be detected in the recipient blood because this number was below the detection limit of the qPCR (Figure 1, Table II). Beyond 48h interval between blood meals, no parasite DNA was recovered on *S. calcitrans* mouthparts whereas parasite DNA traces were still found in abdominal contents corresponding to less than 10 parasite equivalents (Table I). At 144h, no evidence of parasite DNA was recovered. In the recipient blood, neither motile parasites nor parasite DNA were detected at 96 and 144h intervals (Tables II and III).

Discussion

This study investigated the persistence of *B. besnoiti* in *S. calcitrans*, using a quantitative molecular approach after exposure to infected cattle during 5 or 15 min. The parasite burden in cows and stable flies was assessed with the standard curve which showed good reproducibility and sensitivity. These parasite equivalent data were not provided in previous studies [17, 25].

Following the results of the experiment 1, the laboratory colony of stable flies established for eight years was thus considered *B. besnoiti* free. It was not possible to use the same cow for all persistence trials because infected cattle should be quickly culled for ethical reasons. Both cows were considered suitable and equivalent sources of *B. besnoiti* bradyzoites for stable flies, no significant difference of parasite burden was found between them. However, parasite cyst distribution was subjected to parasite burden variations as reported before [6, 13, 25].

The conditions of experiment 2 were close to field conditions, where interrupted blood meals are the rule [26]. As previously demonstrated [6, 17], stable flies were able to contaminate their mouthparts and to ingest *B. besnoiti* bradyzoites by piercing cysts in cow skin. This verification was crucial for the exploration of the long-term parasite persistence (experiment 3).

		Time interval between blood meals 1 and 2 (h)			
		0 ^a	48 ^b	96 ^b	144 ^b
Mouthparts	Shoulder	65	18	ND	ND
	Back line	65	ND	ND	ND
	Hip	< 10	< 10	ND	ND
Abdominal content	Shoulder	162,603	< 10	ND	ND
	Back line	598,971	ND	< 10	ND
	Hip	162,603	ND	< 10	ND

^a interrupted blood feeding on cow number 1330 with skin Ct values: 15 (shoulder and back line), 16 (hip) corresponding to 13,253,057 and 8,731,866 *B. besnoiti* bradyzoite equivalents respectively in 500 mg of skin sample

^b complete blood feeding on cow number 1575 with skin Ct values: 17 (shoulder), 15 (back line and hip) corresponding to 5,866,678 and 13,253,057 *B. besnoiti* bradyzoite equivalents respectively in 500 mg of skin sample

ND: *B. besnoiti* DNA not detected

TABLE I: Number of *B. besnoiti* bradyzoite equivalents in *S. calcitrans* mouthparts and abdominal contents at the end of the blood meal 2

		Time interval between blood meals 1 and 2 (h)			
		0 ^a	48 ^b	96 ^b	144 ^b
Cow skin location for blood meal 1	Shoulder	240	ND	ND	ND
	Back line	34	ND	ND	ND
	Hip	240	ND	ND	ND

^a interrupted blood feeding on cow number 1330 with skin Ct values: 15 (shoulder and back line), 16 (hip) corresponding to 13,253,057 and 8,731,866 *B. besnoiti* bradyzoite equivalents respectively in 500 mg of skin sample

^b complete blood feeding on cow number 1575 with skin Ct values: 17 (shoulder), 15 (back line and hip) corresponding to 5,866,678 and 13,253,057 *B. besnoiti* bradyzoite equivalents respectively in 500 mg of skin sample

ND: *B. besnoiti* DNA not detected

TABLE II: Number of *B. besnoiti* bradyzoite equivalents in the recipient blood at the end of the blood meal 2

		Time interval between blood meals 1 and 2 (h)			
		0 ^a	48 ^b	96 ^b	144 ^b
Cow skin location for blood meal 1	Shoulder	+	+	-	-
	Back line	+	+	-	-
	Hip	+	+	-	-

^a interrupted blood feeding on cow number 1330 with skin Ct values: 15 (shoulder and back line), 16 (hip) corresponding to 13,253,057 and 8,731,866 *B. besnoiti* bradyzoite equivalents respectively in 500 mg of skin sample

^b complete blood feeding on cow number 1575 with skin Ct values: 17 (shoulder), 15 (back line and hip) corresponding to 5,866,678 and 13,253,057 *B. besnoiti* bradyzoite equivalents respectively in 500 mg of skin sample

+: one or two parasites seen

-: no detected parasite

TABLE III: Visual examination of parasites in recipient blood at the end of the blood meal 2

No difference concerning mortality rates was found between batches of *S. calcitrans* exposed to bradyzoites and control batches suggesting that ingested bradyzoites do not interfere with the longevity of stable flies at least during the interval of time considered in this study. As expected, the longest exposure time has led to highest engorgement rates and probably (but not assessed here) an increase of parasite burden within stable flies.

A complete destruction and evacuation of bradyzoites has occurred 144h after the blood meal 1. The parasites can be

retained viable in the mouthparts but probably for a very short time between feeds because they did not provide conducive conditions for *B. besnoiti* persistence. Indeed, no transmission of parasites to rabbits has been observed one hour after an infective blood meal [6]. Moreover, all along these trials, the stable flies had the opportunity to clean their mouthparts through different ways: grooming behavior, washing in water and bee honey, but also by transfer to the newly presented blood. Feeding regurgitation of viable parasites from the digestive tract is an alternative to mouthparts contamination [8]. However, the hostile environmental conditions of the fly

gut did not probably allow the parasite survival due to the presence of numerous proteases and lysins which can destroy quickly pathogens, such as shown for *Trypanosoma* sp. [27].

To conclude, the observation of some motile *B. besnoiti* bradyzoites 48h after an infective blood meal in the recipient blood could be considered as anecdotic, and the motility does not mean that these parasites are viable and virulent. The protocol used here was not addressed to assess these both pivotal features for transmission. As noticed by Bigalke (1968), if mechanical *B. besnoiti* transmission by *S. calcitrans* is of significance in the epidemiology of cattle besnoitiosis, the conditions under it may occur are very restrictive and probably limited between cattle belonging to the same herd. No definitive conclusion can be drawn concerning the maintenance of virulence of these parasites at 48h and this point required further investigations by inoculation to susceptible hosts and cell cultures. Aged parasites may have probably loss their infectious potential. Moreover, it remains unknown if these extremely low parasite burden could induce a host immunological response. Indeed, the minimal dose of parasite required to induce host seroconversion has never been clearly elucidated to the authors' knowledge and should be determined.

Conflict of interest

The authors declare no conflicts of interest.

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