Dynamics of *Besnoitia besnoiti* infection in cattle

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SUMMARY

Bovine besnoitiosis is caused by the cyst-forming apicomplexan parasite *Besnoitia besnoiti*. This disease progresses in two sequential phases: a febrile acute phase with oedemas and respiratory disorders, and a chronic phase characterized by the presence of subcutaneous tissue cysts and skin lesions. Serious consequences of the infection are poor body condition, sterility in bulls and eventual death. The role of host/parasite-dependent factors, which play a major role in the pathogenesis of the disease, is not yet fully elucidated. Isolate/strain virulence, parasite stage, dose and the route of parasite inoculation were studied under different experimental conditions, which make it difficult to compare the results. Data on host-dependent factors obtained from naturally infected cattle showed that (i) the seroprevalence of infection is similar in both sexes; (ii) seropositivity increases with age; (iii) both beef and dairy cattle are susceptible to the infection; and (iv) the cell-mediated immune response is likely to play a major role because a T cell response has been observed around several tissue cysts. Whether colostral antibodies are protective and to what extent the humoral immune response might reflect the disease/protection status require further research. Thus, a well-established experimental bovine model could help to clarify these important questions. The dynamics of *B. besnoiti* infection in cattle and available knowledge on relevant factors in the pathogenesis of the infection are reviewed in the present work.

Key words: *Besnoitia besnoiti*, cattle, pathogenesis, anasarca, scleroderma, parasite-host-dependent factors.

INTRODUCTION

Bovine besnoitiosis is a cattle disease caused by the cyst-forming apicomplexan parasite *Besnoitia besnoiti*. Although low mortality rates are reported (up to 10%) (Bigalke, 1968), it is a chronic and a debilitating disease that causes local and systemic clinical signs. Thus, severe economic losses are incurred, mainly due to poor body condition, decreased milk production, and transient or definitive sterility in bulls. Additionally, the hides of affected animals are of low value for leather production (reviewed by Jacquiet *et al*. 2010 and Álvarez-García *et al*. 2013).

In Europe, during the last century, the disease was restricted to the Pyrenees and Alentejo region in Portugal. However, recent outbreaks of bovine besnoitiosis have been reported in other European countries, and it is considered to be re-emergent due to the increased number of cases and the geographic dissemination over the last two decades (EFSA Journal, 2010; reviewed by Álvarez-García *et al*. 2013). The disease has been described in France, Portugal, Spain, Germany, Switzerland, Italy and Hungary (Besnoit and Robin, 1912; Cortes *et al*. 2006*b*; Alzieu, 2007; Fernández-García *et al*. 2009;

Rostaher *et al*. 2010; Gentile *et al*. 2012; Basso *et al*. 2013; Hornok *et al*. 2014).

Bovine besnoitiosis is also present in Africa, where it is widely distributed in sub-Saharan countries. In particular, the disease seems to be spreading in smallholder farming areas, limiting livestock production (as reported by farmers in Zimbabwe) (Chatikobo *et al*. 2013), and it has been reported on several occasions in South Africa from the 1950s until the present day (Bigalke, 1981; Dubey *et al*. 2013). The disease is also present in Israel (Neuman, 1972; Frank *et al*. 1977; Goldman and Pipano, 1983), and there have also been reports of it in Russia, China, Kazakhstan, Korea, Uzbekistan and Venezuela (Olias *et al*. 2011), although these have not been confirmed. The existence of *B. besnoiti* infection in dairy, beef and mixed cattle from South Australia (Nasir *et al*. 2012) and Brazil (Uzêda *et al*. 2014) could not be confirmed using complementary serological tools (enzyme-linked immunosorbent assay (ELISA), immunofluorescence antibody test (IFAT) and western blot). Confirmation of 17 and 9% seroprevalence rates reported in Egypt for cattle and water buffalo, respectively, is also necessary (Ashmawy and Abu-Akkada, 2014).

The current spread of besnoitiosis in Europe has emphasized the need for prevalence studies to identify areas where the disease may be endemic and to determine the extent of the outbreaks (EFSA

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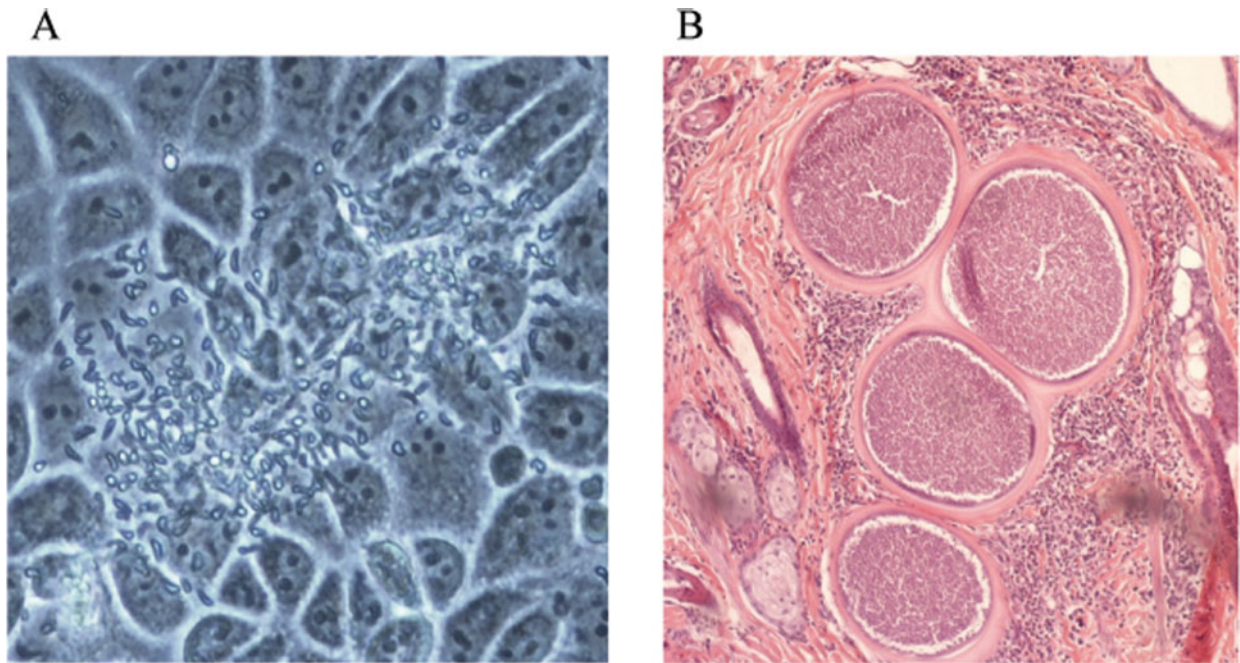
Fig. 1. Asexual stages of *Besnoitia besnoiti*. (A) Tachyzoites proliferating in a monolayer cell culture; (B) Tissue cysts in a haematoxylin-eosin-stained skin section.

Journal, 2010). Recent seroprevalence studies carried out in Spain have confirmed that (i) the disease is found in beef cattle kept under extensive husbandry conditions and (ii) it is endemic and extremely widespread in northern mountainous regions (87·3% herd prevalence and 50% animal prevalence) (Urbasa-Andía Mountains and the Pyrenees) (Álvarez-García *et al*. 2014; Gutiérrez-Expósito *et al*. in press). Rinaldi *et al*. (2013) applied an ELISA to test sera from 88 cattle farms in southern Italy, where the disease had not been previously reported. Serological findings of 83 and 44·1% herd and animal prevalence, respectively, were reported. However further confirmation is required as no additional independent assays were used. In contrast, in non-endemic areas in central Spain, the number of seropositive animals increased up to 90·5%, and 43·2% exhibited clinical signs (Fernández-García *et al*. 2010). The incidence of clinical cases reported also varied depending on whether the disease was emerging (15–40% per year) or endemic (1–10% per year) (Cortes *et al*. 2006*a*; Fernández-García *et al*. 2010; reviewed by Jacquiet *et al*. 2010; Schares *et al*. 2010; Liénard *et al*. 2011). These studies support the hypothesis that infection spreads slowly within affected herds and could last at least several months or years.

Given the importance of the disease, the dynamics of *B. besnoiti* infection in cattle is reviewed in depth, and available knowledge on relevant parasite/host-dependent factors in the pathogenesis of the infection is summarized. Moreover, important questions requiring further clarification are also raised, taking into account the limitations of the few experimental infections carried out in cattle in the past.

BESNOITIA BESNOITI LIFE CYCLE AND TRANSMISSION

Besnoitia besnoiti is taxonomically closely related to *Toxoplasma gondii* and *Neospora caninum* (Ellis *et al*. 2000). Parasites of the genus *Besnoitia* are classified in the phylum Apicomplexa, family Sarcocystidae, sub-family Toxoplasmatinae, and this genus comprises 10 species (*B. besnoiti*, *B. bennetti*, *B. jellisoni*, *B. wallacei*, *B. tarandi*, *B. darlingi*, *B. caprae*, *B. akadoni*, *B. neotomofelis* and *B. oryctofelisi*) (Nganga *et al*. 1994; Dubey and Lindsay, 2003; Dubey *et al*. 2003*a*,*b*, 2004, 2005; Oryan and Azizi, 2008; Dubey and Yabsley, 2010). Among these species, *B. besnoiti*, *B. tarandi*, *B. caprae* and *B. bennetti* cause a similar disease in different species of ungulates (bovids, wild ruminants, goats and equids, respectively).

The definitive host of *B. besnoiti* is unknown; thus, its life cycle remains to be elucidated. The role of a carnivorous species was suspected, although such a species has not been identified yet (Diesing *et al*. 1988; Basso *et al*. 2011). Cattle and wild bovids act as intermediate hosts of this parasite (Pols, 1960; Basson *et al*. 1965). Both the fast multiplying tachyzoites and slowly multiplying bradyzoites in tissue cysts (Fig. 1) develop in these animals, leading to the acute and the chronic stage, respectively (reviewed by Jacquiet *et al*. 2010; Olias *et al*. 2011; reviewed by Álvarez-García *et al*. 2013).

Both tachyzoites and bradyzoites are crescent-shaped and contain common and typical apicomplexan organelles that have been previously detailed in bradyzoites. Bradyzoites are located inside tissue

cysts composed of three layers: the outer layer, with connective-like tissue, the middle layer, which includes host nuclei, and the parasitophorous vacuole membrane (PVM) containing the bradyzoites. The host cell nucleus is enclosed in the cyst layer above the PVM, which is a feature that is distinct from other tissue cyst-forming coccidian parasites (Dubey *et al*. 2003*a*, 2013; Fernández-García *et al*. 2009).

Mechanical transmission of *B. besnoiti* was achieved experimentally either with a syringe or with blood-sucking insects, such as tabanids, tsetse flies and *Stomoxys calcitrans* (Pols, 1960; Bigalke, 1968). The role of arthropods in parasite transmission is also supported by the seasonal incidence of the disease, with a restricted range of spread. Thus far, no cyclic transmission has been suggested because rather close contact is required for successful transmission (Schulz, 1960; Bigalke, 1968; Liénard *et al*. 2013). Both acutely and chronically infected cattle may serve as reservoirs of the parasite for blood-sucking vectors. First, tachyzoites are thought to be present in blood only during the febrile reaction, and second, bradyzoites released from cysts may be successfully transmitted from long-lived chronically infected cattle that may act as reservoirs for naïve animals (Pols, 1960; McCully *et al*. 1966; Bigalke *et al*. 1967). Studies supporting the triad including cattle, wild ruminants and blood-sucking arthropods playing an important role in natural transmission of bovine besnoitiosis have not been conclusive. In recent studies performed in the Pyrenees, where a high density of wild ruminant species, cattle and tabanids is found, specific anti-*Besnoitia* spp. antibodies were found only in two specimens, a roe and a red deer (Gutiérrez-Expósito *et al*. 2013); and, despite the opportunistic feeding behaviour of tabanids, they fed mainly on red deer and cattle, with a preference for red deer (Baldacchino *et al*. 2014). Accordingly it was suggested that two wild ruminant species might serve as an intermediate host, and tabanids might be involved in parasite transmission. However, in both cases parasite detection is not yet confirmed.

In addition, there is epidemiological evidence that supports the hypothesis that horizontal transmission (e.g. direct contact throughout natural mating, wounds and lacerations) could be an important means of parasite dissemination in a herd since an association between the disease prevalence and the animals' age has been reported. Moreover, Bigalke (1968) suggested that the bradyzoite stage might be able to cross mucous membranes. However, this transmission route has not been conclusively proven (reviewed by EFSA Journal, 2010 and Álvarez-García *et al*. 2013). Recent reports of the disease in Spain, Italy, Germany and Switzerland have indicated the potential of introducing *B. besnoiti* into a non-endemic area through the introduction of infected animals into a naïve herd (reviewed by Álvarez-García *et al*. 2013). In fact, several studies

support the hypothesis that sub-clinical cases may act as reservoirs for infection and spread of the disease in a herd (Basso *et al*. 2013; Frey *et al*. 2013).

DYNAMICS OF *B. BESNOITI* INFECTION IN CATTLE

Pathogenesis, clinical signs and pathological findings

Transmission experiments have shown that once bovines are infected with *B. besnoiti*, the incubation period may vary from 1 to 13 days, depending on the method of the infection with an average of 13 days in natural infections (Bigalke, 1968; Basson *et al*. 1970; Bigalke and Prozesky, 2004). Bovine besnoitiosis progresses in two sequential phases (reviewed by Jacquiet *et al*. 2010 and Álvarez-García *et al*. 2013): the febrile acute phase, also known as anasarca phase, caused by the fast-replicating tachyzoite stage in endothelial cells, tunica media, adventitia and mononuclear cells, and characterized initially by fever and later by oedema that may last 4–5 weeks post-infection (wpi), followed by the chronic or scleroderma phase, when pathognomonic tissue cysts develop and cause skin lesions (Basson *et al*. 1970). Subsequently, affected animals remain persistently infected. Oedemas and tissue cysts may be present simultaneously from 11 days post-infection (dpi) until 4–5 wpi; this time interval is also known as the sub-acute phase (Basson *et al*. 1970).

Febrile acute stage. Initially, the infection is characterized by hyperthermia (40·8 to 41·6 °C), which may cause abortions (Pols, 1960; Bigalke, 1968; Givens and Marley, 2008), and non-specific clinical signs that may go unnoticed, such as depression, swelling of the superficial lymph nodes, weight loss, photophobia, ocular and nasal discharge, and increased heart and respiratory rates (Pols, 1960; Schulz, 1960). During this febrile acute stage, which may last for 3 to 10 days, tachyzoites, as obligate intracellular parasites, start invading the endothelial cells of blood vessels, causing degenerative and fibrinoid necrotic vascular lesions, vasculitis, and thrombosis, which subsequently cause congestion, haemorrhages and infarcts (Pols, 1960; Basson *et al*. 1970). It has also been suggested that a possible toxic effect of the parasite might be its ability to cause increased vascular permeability (Bigalke and Prozesky, 2004). These lesions are mainly located in medium-sized and smaller veins as well as some arteries in the skin and testes (McCully *et al*. 1966; Basson *et al*. 1970). Thus, vascular lesions play a key role in the pathogenesis of bovine besnoitiosis. Rapidly multiplying parasites have been observed up to 10–12 days after infection; at this point, most vascular lesions have already been observed (Basson *et al*. 1970). Released tachyzoites from endothelial cells and parasitized mononuclear cells are disseminated by the blood circulation (Pols, 1960). Free parasites enter new cells to repeat the

Fig. 2. Clinical signs of chronic bovine besnoitiosis. (A) Tissue cysts in the sclera and palpebral conjunctiva; (B) Elephant skin; (C) Nodules in the udder.

parasite lytic cycle. Proliferative organisms may be detected in blood from the 3rd up to 12th day of the beginning of the febrile reaction (Bigalke, 1968; Basson *et al*. 1970). The rapid multiplication of the parasite in these locations causes increased vascular permeability, and subsequently it produces oedema that initially appears in the head and neck areas, although it may progress to the limbs and ventral parts of the body, such as the breast and scrotum, where it becomes more visible (Pols, 1960; Basson *et al*. 1970). Parasites may cause acute ophthalmitis, likely accompanied by oedema, increased lacrimation and photophobia (McCully *et al*. 1966). Haemorrhages, necrosis, pseudo-membranes, oedemas and erosions may be found in the upper respiratory tract. In severe cases, oedemas in alveolar and interstitial tissues in the lungs, accompanied by pneumonitis and emphysema, may also cause severe respiratory disorders. Oedemas in joints cause painful movements that may lead to permanent posterior lameness.

Tachyzoites, together with a few haemorrhagic and necrotic foci, may also be present in different locations such as the iris, muscles, lymph nodes and lungs with eosinophils and mononuclear infiltration. Interestingly, Dubey *et al*. (2013) reported for the first time that acutely infected animals may die of nephrotic syndrome that leads to severe hypoalbuminaemia, hyperproteinuria and mild leucocytosis.

Scleroderma stage. Chronic infection is produced by the slow replication of bradyzoites inside tissue cysts with tropism for connective tissues mainly on the mucous membranes, superficial skin layers and male genital tract (Pols, 1960; Bigalke, 1981; Kumi-Diaka *et al*. 1981). There is no evidence of parasitaemia in chronically infected cattle, although Besnoit and Robin (1912) suggested the occurrence of periodic parasite release into the blood.

Cyst formation occurs immediately after extra-cystic proliferation terminates at approximately 11 dpi in blood vessel walls and also extravascularly,

and the appearance of cysts is near synchronous (Bigalke, 1968). Young cysts may contain 1–4 bradyzoites (Dubey *et al*. 2013). The hypertrophic cells become multinucleated and rapidly enlarge until 4 wpi, when their growth gradually diminishes (Basson *et al*. 1970). At 4–5 wpi, tissue cysts measuring more than $100 \, \mu$m are already developed, and cyst development apparently reaches completion at 10 wpi (Basson *et al*. 1970). In a recent study carried out with different antibody markers, host cyst cells showed features of myofibroblasts (Dubey *et al*. 2013), which originate from fibroblasts during tissue repair (Hinz, 2007). Interestingly, many tissue cysts may contain dead bradyzoites or even appear empty (Dubey *et al*. 2013). Multifocal white pinpoint tissue cysts in the scleral conjunctiva are pathognomonic, and, together with those located in the vulvar region, nasal mucous tissues may be detected upon clinical examination as early as 8 wpi (Fig. 2A). Mature cysts are spherical to sub-spherical in shape and measure up to $390 \, \mu$m in diameter, containing approximately 200 000 packed bradyzoites (Bigalke, 1981). According to Bigalke (1968), an estimation of the infection age can be based on cyst size, although it is dependent on the incubation period. Interestingly, tissue cyst disintegration might be observed from 30 dpi onward (Basson *et al*. 1970). With the disappearance of oedemas, a progressive thickening, folding or wrinkling of the skin and scrotal skin, alopecia, hyperkeratosis, scleroderma, scars and nodules on udders, atrophy and induration of testes are observed (Fig. 2B and C). Skin lesions may be explained by the presence of numerous tissue cysts in the dermal and stratum papillare, accompanied by a granulomatous reaction and fibrosis surrounding the cysts. These events, together with vascular lesions that interfere with the blood supply, are responsible for alopecia (Bigalke, 1968).

Tissue cysts frequently appear in the same tissues where the initial proliferation in vessels occurred (Neuman, 1962; McCully *et al*. 1966; Kumi-Diaka *et al*. 1981; Nobel *et al*. 1981). In general terms, tissue

cysts are more likely to be found in the skin (dermis and subcutis), sclera, upper respiratory tract, testes and epididymes in males and in the vestibulum vaginae and vagina in females. In accordance with these results, Frey *et al*. (2013) recently assessed the intra-organ parasite distribution in cattle sub-clinically infected with *B. besnoiti* using histological and molecular tools and determined that the most frequent cyst locations were the upper respiratory tract (rhinarium, larynx/pharynx) followed by the distal genital tract (vulva/vagina) and the skin of the neck. The skin of the face, upper eyelid, tip of the tail and breech harbour the highest parasite load (Basson *et al*. 1970). Tissue cysts can also be present in other locations, such as connective tissues, skeletal muscles and tendons of the limbs, the periosteum of metatarsal and metacarpal bones, and sometimes in the connective tissue and muscles of the tongue, lymph nodes, spleen, liver, lung and heart (McCully *et al*. 1966; Basson *et al*. 1970; Nobel *et al*. 1977; Rommel, 1978; Kumi-Diaka *et al*. 1981; Rostaher *et al*. 2010; Olias *et al*. 2011; Dubey *et al*. 2013; Frey *et al*. 2013).

McCully *et al*. (1966) studied the distribution of *Besnoitia* spp. cysts in the cardiovascular system of chronically infected cattle, reporting that small veins of the limbs, head (saphenous, cephalic and facial veins) and tail are the most parasitized blood vessels. Moreover, cysts were frequently observed in the valves and cysts attached to the endocardium. Cysts were also found in the arteries of the limbs. The cysts located under the endothelium may protrude (or not) into the lumen, and, in response to this, the endothelium becomes hyperplasic (McCully *et al*. 1966). Tissue cysts located in the three histological layers of the blood vessels – intima, media and adventitia – may cause sclerosis. Interestingly, no cysts were observed in large veins and arteries (McCully *et al*. 1966). In this study, the presence and position of cysts is reported, which are clearly dependent on the vascularity of the peripheral tissues. Therefore, cysts located in skin appear more numerous in the vascular stratum papillare and subcutis compared with the stratum reticulare. Respiratory system cysts were more frequently found in the nasal mucosa, paranasal sinuses, pharynx and larynx followed by the trachea, bronchi and lungs (McCully *et al*. 1966).

The presence of tissue cysts in different locations of the male genital organs may lead to permanent sterility as a result of several sequential events (Neuman, 1962; Kumi-Diaka *et al*. 1981; Dubey *et al*. 2013). The scrotum and its contents are the most strongly affected organs, in contrast to the vasa deferentia, which are less affected (Neuman, 1962; Kumi-Diaka *et al*. 1981). Tissue cysts have tropism for vascular walls, particularly those within the pampiniform plexus, leading to reduction of the blood supply to the testes (Kumi-Diaka *et al*. 1981).

In addition, scattered cysts are located under the epithelial cells in the parenchyma between the seminiferous tubules and are surrounded by lymphocytic infiltration and extensive fibrosis (Dubey *et al*. 2013). These cysts cause direct pressure that leads to necrosis and mineralization with subsequent testicular and epididymal atrophy (Kumi-Diaka *et al*. 1981) (Fig. 3). Coagulative necrosis with dystrophic calcification may also be found in seminiferous tubules (Basson *et al*. 1970). Moreover, poor heat exchange through the thickened scrotum can contribute to permanent azoospermia and infertility. The testicles appear fibrotic, and the seminiferous tubules are degenerated or even calcified. Moreover, cysts were also detected in the testes and epididymes (interstitial tissue and seminiferous tubules) as well as in the tunica dartos, vaginalis and albuginea, containing some peritoneal exudate. In addition, in accordance with the observations made by Schulz (1960), fibrosis and inflammatory cells were detected with diffuse interstitial lymphoplasmacytic orchitis associated with the presence of cysts (Dubey *et al*. 2013). Consequently, there may be an absence of spermatogenic activity; alternatively, even if the lesions are not very extensive, 50% of the spermatozoa may be dead, and the remainder may be morphologically abnormal. The spermiogram is consequently altered, with an absence of tissue cysts but many giant cells, epithelial cells and medusa cells present in the semen (Kumi-Diaka *et al*. 1981). Further investigations are needed to elucidate the incidence of sub-fertile bulls.

Concerning the female reproductive tract, Nobel *et al*. (1981) assessed the complete genital tract of 16 chronically infected cows with visible tissue cysts in the skin and eyes. However, only six cows with more than 50 cysts per skin/eye section had cysts in their genital tracts, with the uterus being the most affected organ. In this work, granulomas were observed around approximately 30% of the cysts. However, it was stated that neither the presence of cysts nor the development of these granulomas interfered with the normal functioning of the affected organs. Interestingly, cysts were not detected in the placenta. In contrast to the results reported by Nobel *et al*. (1981), Frey *et al*. (2013) were only able to find the parasite in the distal genital tract of sub-clinically infected cows. Moreover, vertical transmission has not been proven. Indeed, chronically infected cows may become pregnant and successfully give birth to healthy calves that develop normally (Shkap *et al*. 1994), which leads us to hypothesize that vertical transmission is unlikely to occur.

The degree of inflammation surrounding the tissue cysts can be extremely variable, and in some cases, inflammation may not even be detected. Some cysts associated with a significant non-purulent inflammatory infiltrate and normal cysts surrounded by granulomatous reactions without evident
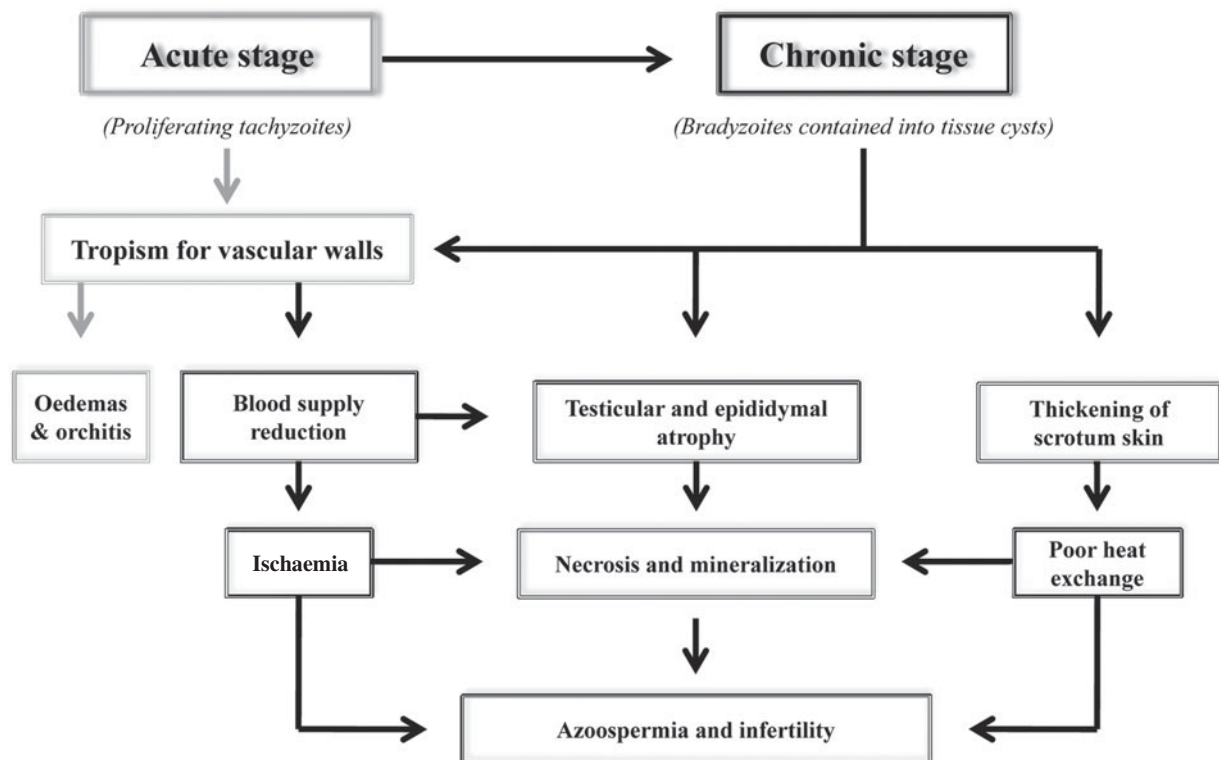
Fig. 3. Sequential pathogenic events associated with *B. besnoiti* infection in the male reproductive tract.

degeneration or rupture of the cyst wall can be detected. Inflammation could be observed in all three blood vessel layers, and moreover, necrotizing inflammatory or mild granulomatous reactions were observed mainly around degenerated and/or ruptured cysts, as previously reported by Besnoit and Robin (1912) and Schulz (1960).

Death may occur in both stages of the disease regardless of the sex of the animal, and animals may be culled due to poor body condition (Pols, 1960; Schulz, 1960).

In an infected herd, cattle showing clinical signs either of acute or chronic infection appear to be only the tip of the iceberg in both endemic and epidemic situations. Moreover, only a small portion of infected animals may exhibit macroscopic tissue cysts in the conjunctiva or vulvar region. However, a higher percentage of cattle comprises sub-clinically infected but seropositive animals, thus representing an important risk for disease transmission (Fernández-García *et al*. 2010; Basso *et al*. 2013; Frey *et al*. 2013).

Parasite and host factors associated with B. besnoiti *infection*

Many fundamental variables related to both the parasite and the host were studied between the 1960s and 1980s under different experimental conditions, making it difficult to compare the results. Indeed, there was a lack of harmonization in experimental design without a commonly accepted procedure for

evaluation of the parameters, including experimental infection/inoculations, sampling of animals, number of animals per group (in many cases, only one animal per group was employed) and a variable experimental duration. Moreover, in the past, experiments were mainly based on clinical inspection and histopathology, serological tests were rarely employed, and molecular tests were not available. Despite these issues, experimental inoculations in bovines were carefully described, and the authors pointed out the difficulty of producing a characteristic clinical case of bovine besnoitiosis (Bigalke, 1968; Basson *et al*. 1970; Diesing *et al*. 1988; Shkap and Pipano, 1993).

In the studies carried out by Bigalke and co-workers in South Africa, three parameters were considered for infection criteria: detection of a distinct febrile reaction, demonstration of *Besnoitia* tachyzoites and immunity to challenge, as infected animals seemed to be resistant to re-infections (Bigalke, 1968).

Parasite-dependent factors. Information available to date concerning parasite-dependent factors, such as virulence of the parasite isolate, parasite stage and inoculation dose, has been obtained from a few previously mentioned studies (summarized in Tables 1 and 2). Most of the acute cases described were characterized by fever and, in a few cases, by localized oedemas in extremities and the head. A few exceptional cases were described by Basson *et al*. (1970), in which a generalized and profound oedema restricted

to the head and extremities was developed, resulting in the death of animals at 1–2 wpi.

Parasite isolates. Different parasite isolates obtained in various laboratories were employed in various experimental inoculations (between the 1960s and the 1980s) (Tables 1, 2 and 3). They were initially isolated from either acute or chronic cases of besnoitiosis in cattle and were maintained in the laboratory by successive inoculations of blood from donor cattle into rabbits. In some cases, the parasite isolates were maintained *in vitro* through passaged cell culture systems (Tables 1 and 3). The bovine isolates obtained in Israel and, more recently, in Europe were directly isolated from biopsies from chronically infected cattle and further maintained and propagated in cell cultures (Table 3; Fig. 1A).

The isolate appears to be a key parasite-dependent factor according to differences in virulence observed among isolates. Two *B. besnoiti* isolates were obtained from cattle and other ruminant species, including chronically infected blue wildebeest and impala antelopes in South Africa. At the time of the study, it was assumed that these isolates were *B. besnoiti* species due to the similarities of the clinical signs and tissue cysts observed. In addition, it was possible to induce cross-immunity between the cattle and antelope isolates. Moreover, differences in virulence were reported, and it was speculated that the higher virulence observed in cattle could be explained by a recent adaptation of the parasite to the host, compared with a more ancient form of besnoitiosis in antelopes. In particular, when the impala and blue wildebeest strains were inoculated into rabbits, they produced milder infections compared with the bovine isolates (Basson *et al*. 1970). The blue wildebeest strain was not virulent in cattle and showed viscerotropism compared with dermatotropism of bovine strains and was later employed as a live vaccine (Bigalke *et al*. 1967, 1974). Furthermore, a different topographical distribution of tissue cysts was observed between the bovine and antelope strains: the antelope strains were usually found in the jugular veins compared with cattle strains, whose cysts were more abundant in superficial veins of the head (McCully *et al*. 1966). Another significant difference was the position of the tissue cysts of cattle strains in the three histological layers of blood vessels whereas in the antelope tissue cysts were attached to the intima (McCully *et al*. 1966). However, whether it is a strain- or host-dependent finding remains to be elucidated. The Israeli bovine and the South African wildebeest strain were compared using isoenzyme electrophoresis to clarify the relationship between cattle and antelope strains. The banding patterns of six enzymes were used to distinguish between the two strains. The results indicated clear genetic differences, thus supporting the hypothesis of Bigalke *et al*. (1967) and Tadros and Laarman (1982) that cattle

and antelope strains are distinct (Le Blancq *et al*. 1986). The bovine isolate of *B. besnoiti* employed by Basson *et al*. (1970) was more pathogenic to rabbits than the isolate obtained from an antelope, which was unable to cause lesions in the skin and testes. Either tachyzoites or bradyzoites isolated from natural bovine cases or even isolates maintained *in vitro* for 2–13 passages in cell culture were able to induce the formation of a high number of cysts in rabbits (Pols, 1954, 1960; Bigalke *et al*. 1967, Bigalke, 1968). This finding contrasts with the finding that a high passage-level rabbit isolate (the Fuls isolate) was considerably less pathogenic, and it was hypothesized that this strain may have lost its cyst-forming capacity (Basson *et al*. 1970). In the experimental infections carried out by Shkap, a bovine isolate from Israel proved to be of low virulence and has been employed as a live vaccine for the last two decades (Shkap, 1986). The degree of pathogenicity of various *B. besnoiti* isolates has not yet been addressed and therefore requires further research. Basson *et al*. (1970) suggested that differences in the parasite isolate or bovine host might influence the cyst growth rate and the parasite load. Intra-specific strain/isolate variability should be further investigated, as the panel of available *B. besnoiti* isolates might differ and is likely to have changed from the 1960s to the present day (Table 3).

Parasite stage. Both parasite stages described to date – tachyzoite and bradyzoite – have been used for inoculations, and the results showed that both stages are infective for both cattle and rabbits (Pols, 1960; Bigalke *et al*. 1967, Bigalke, 1968). However, different inoculation routes and variable ranges of inoculation doses and types of inoculum were employed (Tables 1 and 2).

The clinical outcome of experimental infection in cattle varied from complete absence of clinical signs to severe acute cases with respiratory disorders and even death with parasitaemia. There were also infected animals that showed early development of tissue cysts and skin lesions. Tissue cysts were only found in the veins of the extremities in the mildest chronic cases, while as the severity of the disease increased, cysts were widely distributed and were observed in the conjunctiva, upper respiratory tract and subcutis. Severely affected chronic cases showed thickened skin and partial alopecia, mainly around the muzzle, ears, eyes and neck. In general, most infected animals developed mild disease characterized by fever with no oedema development; a few cases developed tissue cysts. Infections with bradyzoites were less severe, and the most successful infections were obtained upon immunosuppression treatments with cortisone (Diesing *et al*. 1988). However, further comparative experimental infections are needed to determine the ability to induce clinical signs when bradyzoites *vs* tachyzoites from the same isolate are inoculated in cattle.

Table 1. Experimental infections with *B. besnoiti* tachyzoites in cattle

	Parasite-dependent factors				Host-dependent factors				Clinical signs		Presence of parasite (location)	Serology (test)	
Isolate	Species of origin	Inoculum	Dose	Route of administration	Sex	Breed	Age	Immuno suppression	Acute[a]	Chronic[b]			Reference
Lamprechts A and B strains	Cattle	Viscera of cortisonized rabbits	ND	Oral	Male	Holstein	2–3 years	No	Mild	Mild	Yes (cysts in sclera conjunctiva)	ND	Bigalke (1968)
Low passage-level strain	Cattle	Rabbit testes suspension	$2 \cdot 7$–$14 \cdot 1 \times 10^7$	IV	Male	ND	3 years	No	Mild	Mild	Yes (blood, sclera conjunctiva and veins)	ND	Bigalke (1968)
Low passage-level strain	Cattle	Rabbit testes suspension and blood	ND	IV	Female	Hereford	ND	No	Moderate	Severe	Yes (blood, sclera conjunctiva, skin, veins)	ND	Bigalke (1968); Basson *et al.* (1970)
Low passage-level strain	Cattle	Lamb kidney culture	104×10^6	IV	Female	Hereford	7 years	Yes[c]	Severe	No	Yes (endothelial cells)	ND	Basson *et al.* (1970)
Low passage-level strain	Cattle	Lamb kidney culture	$984 \cdot 9 \times 10^6$ (volume: 42 mL)	IV	Male	South Devon-Afrikaner	1 year	No	Mild	Mild	Yes (blood, sclera conjunctiva, skin, veins)	ND	Basson *et al.* (1970)
Low passage-level strain	Cattle	Lamb kidney culture	1068×10^6 (volume: 42 mL)	IV	Male	Hereford	3 years	Yes[c]	Moderate	Severe	Yes (blood, sclera conjunctiva, skin, veins)	ND	Bigalke (1968); Basson *et al.* (1970)
Low passage-level strain	Cattle	Blood of cattle	ND (volume: 3·5–4 L)	IV	Male	Hereford	2–3 years	No	Moderate	Moderate	Yes (blood, sclera conjunctiva, skin, veins)	ND	Bigalke (1968); Basson *et al.* (1970)
High passage-level strain (Fuls strain)	Cattle	Lamb kidney culture	$46 \cdot 75$–$66 \cdot 4 \times 10^6$	IV	Male	ND	2 years	No	Mild	No	No	ND	Bigalke (1968)
High passage-level strain (Fuls strain)	Cattle	Lamb kidney culture	$106 \cdot 56 \times 10^6$	SC	Male	ND	3 years	No	Mild	No	No	ND	Bigalke (1968)
High passage-level strain (Fuls strain)	Cattle	Rabbit testes suspension	$141 \cdot 75 \times 10^6$ (45 mL)	IV	Male	Hereford	3 years	No	Severe	No	Yes (blood)	ND	Bigalke (1968); Basson *et al.* (1970)
High passage-level strain	Cattle	Blood of cattle	ND (volume: 4 L)	IV	Male	Hereford	3 years	No	Mild	Mild	Yes (skin and veins)	ND	Bigalke (1968); Basson *et al.* (1970)
Bb-Israel	Cattle	Vero cell culture	10^6–$7 \cdot 6 \times 10^9$	IV & IM & SC	Male	Holstein	>1 year	No	Mild	No	No	Positive (IFAT)	Shkap (1986)
Bb-Israel	Cattle	Vero cell culture	1–$2 \cdot 5 \times 10^9$	IV	Male	Holstein	>1 year	Yes[d]	Mild	No	No	Positive (IFAT)	Shkap (1986)
Bb-Israel	Cattle	Blood of cattle	ND (volume: 1 L)	IV	Male	Holstein	>1 year	Yes[d]	Mild	No	No	Positive (IFAT)	Shkap (1986)
Bb-Wildebeest	Wildebeest	Blood of rabbit	ND (volume: 50 mL)	IV	Male	Hereford	3–6 years	No	No	No	No	ND	Bigalke *et al.* (1967)
Bb-Wildebeest	Wildebeest	Blood of sheep	ND (volume: 40–80 mL)	SC & IV	Male	Hereford	3–6 years	No	No	No	No	ND	Bigalke *et al.* (1967)
Bb-Wildebeest	Wildebeest	Cell culture	1×10^6	SC	ND	ND	3 months	No	ND	ND	ND	Positive (IFAT)	Janitschke *et al.* (1984)

ND	Cattle	Cell culture	3×10^6	IV	ND	ND	3 months	No	ND	ND	ND	Positive (IFAT)	Janitschke et al. (1984)
ND	Cattle	Peritoneal lavage of gerbil	2×10^7	IV	ND	Holstein	3–6 months	No	No	No	No	ND	Diesing et al. (1988)
ND	Cattle	Peritoneal lavage of gerbil	2×10^7	IV	ND	Holstein	3–6 months	Yes[d]	Mild	No	Yes (skin)	ND	Diesing et al. (1988)
ND	Cattle	MDBK cell culture	$3 \cdot 7 \times 10^8$	SC	ND	Holstein	3–6 months	No	Mild	No	No	ND	Diesing et al. (1988)
ND	Cattle	MDBK cell culture	$3 \cdot 7 \times 10^8$	SC	ND	Holstein	3–6 months	Yes	Mild	No	No	ND	Diesing et al. (1988)
ND	Cattle	MDBK cell culture and peritoneal lavage of gerbil	$2 \times 10^8 + 5 \times 10^7$	IP	ND	Holstein	3–6 months	Yes	No	No	No	ND	Diesing et al. (1988)
ND	Cattle	MDBK cell culture and peritoneal lavage of gerbil	$2 \times 10^8 + 5 \times 10^7$	IP	ND	Holstein	3–6 months	No	Mild	No	No	ND	Diesing et al. (1988)
ND	Cattle	Peritoneal lavage of hamster	4×10^7	IP	ND	ND	>1 year	Yes[c,d]	Severe	No	Yes (blood)	ND	Diesing et al. (1988)

ND: not determined.
[a] Acute clinical signs: mild (fever)/moderate (fever, oedema, weakness)/severe (death).
[b] Chronic clinical signs: mild (visible tissue cysts in scleral conjunctiva)/moderate (hyperkeratosis, alopecia and tissue cysts)/severe (loss body condition and death).
[c] Splenectomy.
[d] Corticoids.

Table 2. Experimental infections with *B. besnoiti* bradyzoites in cattle

Parasite-dependent factors				Host-dependent factors				Clinical signs		Presence of parasite (location)	Serology (test)	Reference
Species of origin	Inoculum	Dose	Route of administration	Sex	Breed	Age	Immuno suppression	Acute[a]	Chronic[b]			
Cattle	Tissue cysts	ND	SC&IV	Female	ND	ND	No	No	No	No	ND	Besnoit and Robin (1912)
Wildebeest/ Impala	Tissue cysts	$1{\cdot}8–5{\cdot}4\times10^7$	SC & IV	ND	Cross breed	<1 year	No	Mild	No	Yes (blood)	ND	Bigalke et al. (1967)
Cattle	Tissue cysts	ND	Oral	Male & Female	ND	ND	No	No	No	No	ND	Bigalke (1968)
Cattle	Tissue cysts	$2{\cdot}2–4{\cdot}2\times10^6$	Intranasal	Male	ND	2–3 years	No	Mild	Mild	Yes (cysts in sclera conjunctiva and veins)	ND	Bigalke (1968)
Cattle	Tissue cysts	$2{\cdot}3\times10^6$	SC & IV	ND	ND	2 years	No	Mild	No	No	ND	Bigalke (1968)
Cattle	Bites of tsetse flies	ND	SC	Male	ND	ND	No	Mild	Mild	Yes (cysts in sclera conjunctiva)	ND	Bigalke (1968)
Cattle	Bites of tsetse flies	ND	SC	Male	ND	ND	No	Moderate	No	No	ND	Bigalke (1968)
Cattle	Bites of *Stomoxys* (n = 145,725)	ND	SC	Male	ND	2 years	No	Mild	Mild	Yes (cysts in sclera conjunctiva and skin)	ND	Bigalke (1968)
Cattle	Bites of 2 tabanids	ND	SC	Male	ND	3 years	No	No	No	No	ND	Bigalke (1968)
Cattle	Bites of 6 tabanids (n = 25)	ND	SC	ND	ND	ND	No	Moderate	Mild	Yes (cysts in sclera conjunctiva and skin)	ND	Bigalke (1968)
Cattle	Tissue cysts	2×10^7	IP	ND	Holstein	3–6 months	Yes[c]	Moderate	Moderate	Yes (connective tissues)	ND	Diesing et al. (1988)
Cattle	Tissue cysts	2×10^7	IP	ND	Holstein	3–6 months	No	Mild	No	Yes (connective tissues)	ND	Diesing et al. (1988)
Cattle	Tissue cysts	$1{\cdot}4\times10^7$	SC & IV	ND	Holstein	3–6 months	Yes[c]	Moderate	Moderate	Yes (connective tissues)	ND	Diesing et al. (1988)
Cattle	Tissue cysts	$1{\cdot}5\times10^7$	IP	ND	Holstein	3–6 months	No	No	No	No	ND	Diesing et al. (1988)
Cattle	Tissue cysts	3×10^8	SC & IV	ND	Holstein	3–6 months	Yes[c,d]	Severe	No	Yes (blood)	ND	Diesing et al. (1988)

Host	Parasite stage	Dose	Route	Sex	Breed	Age	Corticoids/Splenectomy	Clinical signs		Parasite (blood)	Serology	Reference
Cattle	Tissue cysts	2×10^8	SC	Male	ND	Adult	Yes[c,d]	Moderate/Severe	No	Yes (blood)	ND	Diesing et al. (1988)
Cattle	Tissue cysts	2×10^8	IV	Male	ND	Adult	Yes[c,d]	Severe	No	Yes (blood)	ND	Diesing et al. (1988)
Cattle	Tissue cysts	2×10^8	IP	Male	ND	Adult	Yes[c,d]	Moderate/Severe	No	Yes (blood)	ND	Diesing et al. (1988)
Cattle	Tissue cysts	2×10^7	IP	ND	ND	Adult	Yes[c,d]	Severe	No	Yes (blood)	ND	Diesing et al. (1988)
Cattle	Tissue cysts	3×10^7	SC	Male	Holstein	>1 year	No	Mild	No	No	Positive (IFAT)	Shkap (1986)

[a] Acute clinical signs: mild (fever)/moderate (fever, oedema, weakness)/Severe (death).
[b] Chronic clinical signs: mild (visible tissue cysts in scleral conjunctiva)/moderate (hyperkeratosis, alopecia and tissue cysts)/severe (loss body condition and death).
[c] Corticoids.
[d] Splenectomy.
ND: not determined.

There are several concerns that need to be addressed with regard to the inocula employed for both parasite stages. Acutely and chronically infected animals usually served as parasite donors throughout infection via blood transfusions and skin tissues, respectively. On some occasions, blood from other acutely infected species (e.g. rabbits and sheep) as well as rabbit testes suspensions was employed in the inoculations to provide proliferating organisms (tachyzoites). The main limitations included the variable inoculation volumes, undetermined parasite doses, number of *in vivo* rabbit passages, preparation of inocula that could compromise parasite viability and unknown virulence of the isolates. Moreover, parasites propagated in cell culture were also inoculated. However, on some occasions, the number of *in vitro* passages and the parasite viability prior to inoculations were not determined (Tables 1 and 2).

Dose. A variable range of tachyzoite- and bradyzoite-based inocula doses were employed (Tables 1 and 2). The dose is expected to play an important role in producing disease. Based on the clinical findings in experimentally infected cattle, the inocula dose might be highly dependent on the parasite viability and virulence of the isolate. According to the results obtained, it appears that this variable might be an important determinant in prioritizing a severe *vs* a mild infection.

Host-dependent factors. The most robust data on host-dependent factors have been obtained from natural infections. The limitations of the experimental infections mentioned above make it difficult to infer conclusive results regarding sex, aptitude and breed, age and immunity. Moreover, the animals employed in the experimental inoculations were not previously serologically tested in most cases, and immunity to re-infections has been reported.

Host species. Regarding ruminant species, other similar *Besnoitia* spp. infections have also been reported in reindeer and caribou, caused by *B. tarandi* (Dubey *et al.* 2004), and in goats, caused by *B. caprae* (Njenga *et al.* 1993; Oryan and Azizi, 2008). The ability of these species to infect cattle has not yet been investigated; thus, it is not known whether these species provide cross-immunity to bovine *B. besnoiti*. This important issue deserves further attention to determine the possible role of these ruminants in the epidemiology of bovine besnoitiosis. With regard to this question, Pols (1960) showed that sheep and goats were susceptible to *B. besnoiti* infections. When these small ruminant species received parasitized blood from rabbits, both developed fever, and skin tissue cysts could be detected in goats.

Concerning laboratory animals, rabbits were inoculated repeatedly with *B. besnoiti* because both the acute and chronic stages of the disease could be

Table 3. *B. besnoiti* isolates

Isolate	Country	Location	Species	Acute/chronic infection	*In vitro* culture	Employed in experimental infections	Reference
Impala strain[a]	South Africa	Pretoria	Impala	Chronic infection	No	Yes (rabbits)	Bigalke *et al.* (1967); Basson *et al.* (1970)
Bb-Fuls strain[a]	South Africa	Pretoria	Cattle	Acute infection	Lamb kidney culture	Yes (cattle, rodents and rabbits)	Bigalke (1968); Basson *et al.* (1970)
Bb-Schoeman strain[a]	South Africa	Pretoria	Cattle	Acute infection	No	Yes (rabbits and rodents)	Bigalke (1968)
Bb-Lamprechts A strain[a]	South Africa	Pretoria	Cattle	Acute infection	No	Yes (cattle, rodents and rabbits)	Bigalke (1968)
Bb-Lamprechts B strain[a]	South Africa	Pretoria	Cattle	Chronic infection	No	Yes (cattle, rodents and rabbits)	Bigalke (1968)
Bb-Wildebeest[a]	South Africa	Pretoria	Wildebeest	Chronic infection	Vero culture	Yes (cattle, rodents and rabbits)	Bigalke (1968); Basson *et al.* (1970)
Bb-Israel	Israel	ND	Cattle	Chronic infection	Vero culture	Yes (cattle and rodents)	Dubey *et al.* (2003*a*)
Bb1-Evora03	Portugal	Evora	Cattle	Chronic infection	Vero culture	No	Cortes *et al.* (2006*b*)
Bb-Spain 1	Spain	Guadalajara	Cattle	Chronic infection	MARC-145 culture	No	Fernández-García *et al.* (2009)
Bb-Ger1	Germany	Babaria	Cattle	Chronic infection	Vero culture	Yes (cats, dogs, rabbits, rodents and guinea pigs)	Schares *et al.* (2009)
Bb-French isolate	France	French Pyrenees	Cattle	Chronic infection	Vero culture	Yes (blood of cattle)	Liénard *et al.* (2013)
Bb-Italy1	Italy	Emilia-Romagna	Cattle	Chronic infection	MARC-145 culture	No	Gentile *et al.* (2012)
Bb-IPZ-1-CH	Switzerland	Thurgau	Cattle	Chronic infection	HFF culture	No	Basso *et al.* (2013)
Bb-IPZ-2-CH	Switzerland	Lucerne	Cattle	Chronic infection	HFF culture	No	Basso *et al.* (2013)
Bb-IPZ-3-CH	Switzerland	Thurgau	Cattle	Chronic infection	HFF culture	No	Basso *et al.* (2013)

[a] At present not available isolates.

successfully reproduced in this animal species, which served as a source of parasites for experimental inoculations in cattle. These studies also provided some clues about the role of parasite-dependent factors in the pathogenesis of the disease (Pols, 1960; Bigalke, 1968). Unfortunately, many other rodent species, such as Syrian hamsters (*Mesocricetus auratus*), guinea pigs (*Cavia porcellus*), gerbils (*Meriones unguiculatus*), common voles (*Microtus arvalis*) and NMRI mice have also been inoculated with either *B. besnoiti* tachyzoites or bradyzoites; however, the persistence of the parasite could only be demonstrated in voles, and none of the species developed clinical signs (Bigalke, 1968; Basso *et al*. 2011). Thus, information regarding other potential intermediate hosts aside from bovids is still scarce. Moreover, the possible role of different mammal species, including cats, different wild felid species, and dogs, in addition to birds and snakes as definitive hosts was investigated by administering tachyzoites and tissue cysts containing bradyzoites from chronically infected cattle via an oral route; however, oocysts were not shed in the faeces (Diesing *et al*. 1988; Basso *et al*. 2011).

Inoculation route. The inoculation route is also a critical factor in infection. In fact, the incubation period in cattle is dependent on the route of infection, and the shortest period (less than 2 days) was observed when tachyzoites were intravenously inoculated. Consequently, the route of inoculation is associated with the time required to develop mature tissue cysts (Bigalke, 1968; Basson *et al*. 1970). Subcutaneous and intravenous inoculations appear to more properly mimic the natural transmission route of the parasites, as they were the most successful with respect to inducing the characteristic clinical signs of bovine besnoitiosis (Tables 1 and 2).

Interestingly, the oral and nasal routes were also employed in experimental cattle infections (Bigalke, 1968). When inoculations were successful, the incubation period lasted between 9 and 14 days. When viscera of experimentally infected rabbits harbouring most likely only tachyzoites were given orally to an ox, a pronounced febrile reaction lasting for 5 days was observed and a few tissue cysts were detected at 80 dpi. In contrast, when cysts were administered via an oral route, the typical bovine besnoitiosis signs were absent. Finally, the author suggested that bradyzoites are able to penetrate mucous membranes when cysts are administered via a nasal route, causing a mild infection.

Breed. All breeds of cattle appear to be susceptible to besnoitiosis. The infection has been described in a wide variety of breeds, such as Africander, Hereford, Shorthorn, Bonsmara, Nguni, Jersey, Holstein, Alpine brown, Pyrenean, Cross-breeds, Charolaise and Limousine (among others), and significant differences appear to be related with breed aptitude and, consequently, husbandry conditions that may facilitate parasite transmission, as higher prevalence rates have been detected in beef cattle breeds (Bigalke, 1968; Goldman and Pipano, 1983; Liénard *et al*. 2011; Álvarez-García *et al*. 2014).

Aptitude. Differences in prevalence have been detected between beef and dairy cattle, which appear to be associated with different management systems rather than different breed susceptibilities. In Israel, a seroprevalence rate of 10% for dairy cattle *vs* 50% for beef cattle was reported when 1700 animals were tested. Moreover, the highest antibody titres corresponded to beef cattle (Goldman and Pipano, 1983). Similarly, a recent study conducted in a traditionally endemic northern province in Spain (Navarra) with mountainous areas showed a significant association between the prevalence of *B. besnoiti* infection and cattle management systems, reporting 16% seroprevalence in beef cattle *vs* 0% in dairy cattle (Álvarez-García *et al*. 2014). In beef cattle systems, parasite transmission might be favoured by different factors, such as natural mating, communal pastures outdoors, and exposure to wild ruminants (red deer and roe deer) and blood-sucking arthropods, which may explain the differences in prevalence rates observed between beef and dairy cattle (Frank *et al*. 1977; Goldman and Pipano, 1983; Fernández-García *et al*. 2010; Álvarez-García *et al*. 2014).

Nevertheless, bovine besnoitiosis has also been described in dairy cattle. Liénard *et al*. (2011) carried out a longitudinal study in an affected dairy herd located in a region of France, where the disease is endemic and clinical cases have been recorded for many years. An increase in seroprevalence from 30 to 89·5% over a period of 14 months was reported, similar to findings reported in beef cattle in other regions (Cortes *et al*. 2004). The prevalence of scleral conjunctiva cyst detection did not vary between the first and last clinical inspections (10·53 and 12·3%, respectively), with a peak in the middle of study (22·81%) lasting for more than a year. Most risky practices (e.g. natural mating and communal pastures outdoors) carried out in extensive husbandry systems are irrelevant under intensive husbandry, and animal trade is considered to be the main entryway for the infection into dairy herds. Moreover, because artificial insemination is predominant in dairy herds, the most important consequences of the infection might be loss of weight, decreased milk production and occasional abortion in pregnant dams, likely caused by the high fever of short duration that occurs in the acute phase of the disease (unpublished result in a *B. besnoiti*-infected dairy herd in northern Spain).

Age. The disease has been mainly reported in animals older than 6 months. Moreover, an increase in seropositivity with age has been reported, which

might be explained by the efficient horizontal transmission. This was also observed when bulls from Israel were serologically examined. Older animals have a greater chance of becoming infected due to longer periods of exposure; these animals are more often serologically positive and clinically diseased (Goldman and Pipano, 1983; Fernández-García *et al*. 2010; Gutiérrez-Expósito *et al*. in press).

Clinical disease is more often observed among 2–4-year-old adults than among those older than 4 years (Legrand, 2003; Alzieu, 2007). This could indicate that many of the infected animals are able to develop an immunity (most likely non-sterile) during the years following the infection, thus reducing the severity of clinical signs of a chronic infection. The clinical incidence was lower in calves and heifers compared with bulls and cows (Bigalke, 1968). Schulz (1960) reported a peak between 3–4 years and noticed that calves are susceptible from the age of 6 months. Later, Bigalke (1981) and Janitschke *et al*. (1984) reported that clinical cases rarely occur in calves under 6 months of age. It has been speculated that calves younger than 6 months might be more resistant to the infection or even less exposed to the infection; however these issues remain to be clarified. The most feasible explanation could be related with the presence of colostral antibodies that might be protective (Shkap *et al*. 1994). However, the disease was recently confirmed in a 4-month-old calf (unpublished data), demonstrating that animals younger than 6 months can also be infected (Ferrié, 1984; Legrand, 2003; Alzieu, 2007).

Sex. Regarding clinical signs, Pols (1960) pointed out that there was no sex susceptibility. Data obtained from several prevalence studies, initially reported in Israel (Goldman and Pipano, 1983) and more recently in Spain, showed that both sexes may be equally infected. In Spain, Fernández-García *et al*. (2010) did not observe significant differences during an outbreak in central Spain. In accordance with a recent study carried out in an area where the disease is endemic – the Spanish Pyrenees – both sexes were similarly infected, with 49·2 and 52·3% prevalence rates in males and females, respectively (Gutiérrez-Expósito *et al*. in press).

With regard to the prevalence of clinically affected animals, several authors have reported that males were more susceptible than females, as they appeared to show more acute signs and a higher mortality rate; these results were in agreement with local reports provided by veterinarians in Spain (Schulz, 1960; reviewed by Jacquiet *et al*. 2010). However, these observations might be biased by the fact that breeding bulls are the most valuable animals in a herd and, consequently, may attract the attention of the veterinarians more easily (only based on clinical inspection).

Immune response. There are many unanswered questions concerning acquired humoral and cellular immune responses elicited against *B. besnoiti* infection in cattle. Nevertheless, the most striking finding is the field evidence showing that cattle that have contracted the disease, either clinically or sub-clinically, become immune against re-infections. This finding was supported by Bigalke (1968), who conducted a cohabitation experiment with chronically infected and naïve cattle. Cohabitation led to a 100% transmission rate with unapparent infections. This finding set the basis for later vaccination studies. The efficacy of the first live vaccine based on a blue wildebeest strain with naturally low pathogenicity was assessed by Bigalke *et al*. (1974). The field trial showed that vaccination induced protective immunity against the clinical form of the disease in 100% of vaccinated animals. Subsequently, vaccinations with tachyzoites of bovine isolates from Israel passaged *in vitro* for years produced variable increases in specific antibody levels at 30–40 dpi without any clinical signs. Immunized calves did not develop a clinical response to tachyzoite challenge with large numbers ($>2 \times 10^9$ per dose) of parasites (Shkap, 1986). Interestingly, following infection, partial clinical recovery with an appreciable decrease in visible cysts has been observed in chronically infected animals (Frey *et al*. 2013).

Although the role of the humoral response during *B. besnoiti* infection remains to be clarified, it is known that animals develop specific antibody levels at 2–3 wpi that remain detectable for a long period of time, and the majority of chronically infected cattle with tissue cysts are seropositive (Fernández-García *et al*. 2010). In fact several authors reported higher antibody levels in animals with tissue cysts in their scleral conjunctiva than in animals without cysts (Liénard *et al*. 2011; Schares *et al*. 2013). Interestingly, Frey *et al*. (2013) described a significant increase in antibody levels related to the presence of ruptured cysts, which may have favoured the re-exposure of the immune system to parasite antigen. The existence of parasite reactivation and subsequent recrudescence of infection remains to be clarified.

The critical question of whether colostral antibodies are protective requires further research. It is known that colostral antibodies may still persist at 4 months of age. These specific anti-*B. besnoiti* antibodies were observed by indirect immunofluorescence in four calves born from two chronically infected dams with IFAT titres ranging from 1 : 64 to 1 : 1024. Although the protective effect against infection was not studied, these calves did not contract the infection and developed normally (Shkap *et al*. 1994).

The cell-mediated immune response is likely to play a crucial role similar to its role in the defence against other intracellular obligate and closely related parasites (*N. caninum* and *T. gondii*). Indeed, a T-cell response has been detected around several tissue

cysts. Immunohistochemical studies have provided details regarding the immune reaction around the cysts in naturally infected cattle. Frey *et al*. (2013) showed that cysts were associated with a significant non-purulent inflammatory infiltrate consisting predominantly of T lymphocytes and activated cells of the monocyte/macrophage lineage. Interestingly, some cysts appeared to be degenerated, as previously indicated by others (Bigalke *et al*. 1966; Basson *et al*. 1970). Bradyzoites were not clearly identified, and some inflammatory cells were infiltrating the degenerated cyst. The effect of cyst rupture has been studied by Frenkel (1955) in *B. jellisoni* infection. This study showed that organisms liberated by cyst rupture were found to be poorly stained and subject to phagocytosis. The lesions were circumscribed, resembling a granuloma; however, there was no evidence that *Besnoitia* liberated from cysts entered new cells and proliferated (Frenkel, 1955). Nevertheless, during immunosuppression, *B. besnoiti* proliferation might be facilitated. Indeed, the administration of high doses of corticosteroids to hamsters chronically infected with *B. jellisoni* triggered relapse in 7–14 days, with an increase in parasites (Frenkel and Lunde, 1966). Regarding the role of immunosuppression during *B. besnoiti* infection, Bigalke (1968) described a severe reaction with fatal termination in a 3-year-old splenectomized ox inoculated intravenously with 1×10^9 tachyzoites from cell culture. Shkap (1986) showed that cattle infected intravenously with different numbers of tachyzoites from cell culture (Table 1) did not develop tissue cysts under treatment with high levels of corticosteroids ($0.6 \, \text{mg kg}^{-1}/24$ days), which may be explained by the fact that the isolate employed had naturally low pathogenicity. In contrast, when Diesing *et al*. (1988) inoculated bradyzoites in cattle clinical signs were observed upon immunosuppression treatment with cortisone.

CONCLUDING REMARKS

Bovine besnoitiosis is a re-emergent disease in Europe, and this parasitic disease is expected to have a noticeable economic impact in beef cattle raised under extensive conditions but this impact has not yet been quantified. Moreover, there are still many gaps related to the pathogenesis of the disease that deserve further research. In this context, the development of a well-established experimental bovine model should be prioritized to explore the role of host- and parasite-dependent factors that may be involved in parasite virulence and to evaluate the safety and efficacy of tools for parasite control. Thus, harmonized experimental designs and validated serological, histological and molecular techniques should be employed to successfully reproduce the disease and to study the elicited immune response in depth.

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