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Isolation of *Besnoitia besnoiti* from infected cattle in Portugal

H. Cortes [a,*], Y. Reis [b,c], H. Waap [b], R. Vidal [d], H. Soares [c,e], I. Marques [e], I. Pereira da Fonseca [f], I. Fazendeiro [f], M.L. Ferreira [f], V. Caeiro [a], V. Shkap [g], A. Hemphill [h], A. Leitão [b]

[a] *Laboratório de Parasitologia, ICAM, Núcleo da Mitra, Universidade de Évora, Ap 94, Évora 7002-554, Portugal*
[b] *Instituto de Investigação Científica Tropical, CIISA, Lisboa, Portugal*
[c] *Instituto Gulbenkian de Ciência, Oeiras, Portugal*
[d] *Faculdade de Farmácia, Laboratório de Engenharia Genética, Universidade de Lisboa, Portugal*
[e] *Escola Superior de Tecnologia da Saúde de Lisboa, Portugal*
[f] *Faculdade de Medicina Veterinária de Lisboa (CIISA), Portugal*
[g] *Division of Parasitology, Kimron Veterinary Institute, Bet Dagan, Israel*
[h] *Institute of Parasitology, Vetsuisse Faculty, University of Bern, Bern, Switzerland*

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Abstract

Besnoitia besnoiti, an obligate intracellular protozoan parasite belonging to the phylum apicomplexa, ~~and~~ is the causative agent of bovine besnoitiosis. Besnoitiosis is responsible for significant losses in the cattle industry of Africa and Mediterranean countries due to the high morbidity rate, abortion and infertility in males. The acute stage of disease is associated with the proliferative forms (tachyzoites) and is characterized by fever, whimpery, general weakness and swelling of the superficial lymph nodes. During the following chronic stage, a huge number of cysts are formed mainly in the subcutaneous tissues. This process is non-reversible, and chronic besnoitiosis is characterized by hyper-sclerodermia, hyperkeratosis, alopecia and, in bulls, atrophy, sclerosis and focal necrosis that cause irreversible lesions in the testis.

In this paper we report on the identification of large cysts in the skin of a cow and a bull in Portugal, which presented loss of hair and enlargement and pachydermis all over the body. The observation of a two-layered cyst wall within the host cell, the encapsulation of the host cell by a large outer cyst wall, and the subcutaneous localization of the cysts within the host, were characteristic for *B. besnoiti*. The parasites were isolated from the infected animals and successfully propagated in Vero cells without prior passages in laboratory animals. Morphological characterization of *B. besnoiti* tachyzoites and the amplification of the 149 bp segment from the internal transcribed spacer 1 (ITS1), aided with specific primers, confirmed the identification of *B. besnoiti*.

Keywords: *Besnoitia besnoiti*; Bovine besnoitiosis; Portugal; Cyst; *In vitro* cell culture

1. Introduction

Besnoitia besnoiti, the causative agent of bovine besnoitiosis, is an obligate intracellular parasite, belonging to the Sarcocystidae family. This protozoan parasite was first described in France by Besnoit and

* Corresponding author. Tel.: +351 965017554; fax: +351 268639667.
E-mail address: hcec@uevora.pt (H. Cortes).

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Robin (1912). Three years later Franco and Borges (1915) published a study on the occurrence of bovine besnoitiosis in Portugal, based on animals rejected in a slaughterhouse between 1887 and 1915. All these animals came from Alentejo, south of Portugal (Franco and Borges, 1915). Subsequently, the occurrence of the disease in Europe received little attention until the last decade of the 20th century when it was reported in Spain (Juste et al., 1990), Portugal (Cortes et al., 2003; Cortes et al., 2005) and France (Bourdeau et al., 2004).

A herd study on clinical cases of bovine besnoitiosis showed that the fatality rate is usually about 10% (Pols, 1960) despite the fact that 70–90% of animals had specific antibodies directed against *B. besnoiti* (Bigalke, 1968). Thus, most animals die during the chronic stage of infection. Only few studies have been carried out on the early stage mechanisms of *B. besnoiti* infection and immune pathways (Basson et al., 1970; Bigalke et al., 1974; Bigalke, 1967, 1968, 1960).

Due to the isolation of *B. besnoiti* from farm animals and the development of in vitro culture, further investigations on the transmission and epidemiology of besnoitiosis (Bigalke, 1968; Janitschke et al., 1984) were possible. In addition, *in vitro* culture of *B. besnoiti* allowed the production of attenuated parasites, which were then used as an experimental vaccine in South Africa (Bigalke et al., 1974) and Israel (Pipano, 1997), and this has lead to the beginning of studies on the immunology (Shkap et al., 1989, 1990, 2002; Shkap and Pipano, 1993), chemotherapy (Elsheikha and Mansfield, 2004; Shkap et al., 1987b), phylogeny, and ultrastructure of *Besnoitia* (Dubey et al., 2003; Ellis et al., 2000; Shkap et al., 1988).

So far *B. besnoiti* has been isolated after several passages in laboratory animals, namely rabbits in South Africa (Pols, 1954) and gerbils in Israel (*Meriones tristrami* Shawii) (Neuman, 1974), prior to adaptation to *in vitro* culture. Subsequently, additional isolates were produced in South Africa (Bigalke, 1968) and in Israel (Shkap et al., 1987a) using the same approach. No isolates from other geographical areas have been reported to date.

The present paper describes two *B. besnoiti* isolates from Portugal. These isolates described in this report could be propagated under *in vitro* conditions without the need of laboratory animals for merozoite adaptation. These results will contribute to the research on this etiological agent of a potentially significant disease in cattle.

2. Material and methods

2.1. Identification of cattle potentially infected with Besnoitia

Subsequently to a case of bovine besnoitiosis identified by Malta and Silva in Portugal (1984, data not published), veterinarians were actively contacted in order to obtain material for further studies *B. besnoiti*. Cattle presenting chronic manifestations of skin disease were subjected to a skin biopsy and serum collection. Skin biopsies were performed using biopsy punch (Ø 8 mm), were fixed in 10% formalin, embedded in paraffin, and 3–5 μm sections were processed for hematoxilin/eosin (H/E) staining. *B. besnoiti* tissue cysts were identified by light microscopy.

2.2. Culture of Vero cells

Vero cells (ATCC-CCL81) were cultured in T-25 tissue culture flasks, and were maintained in Dulbecco's modified eagle culture medium (D-MEM with 100 U penicillin/ml, 100 μg/streptomycin/ml and 0.25 μg amphotericin B/ml) in a humidified incubator with 5% CO_2 atmosphere. Confluent monolayers were passaged routinely every 6 days.

2.3. Isolation and tissue culture of B. besnoiti

One 4 years old cow from Sallers' breed, and a 6 years old Charolais bull, from two separate farms in Évora region (south-east of Portugal), both exhibiting clinical features reminiscent for besnoitiosis were culled, and subcutaneous tissues from these animals were collected, stored at 4 °C, and transported to the laboratory. With a scalpel, tissue pieces showing cysts of *B. besnoiti* were collected in a Petri dish containing PBS plus 100 U penicillin/ml, 100 μg streptomycin/ml, and 0,25 μg amphotericin B/ml, and were washed twice with this solution. The endozoites were freed from the large cysts by scattering the tissue with an 18 G needle.

The PBS with liberated bradyzoites was collected and centrifuged at 770 × *g* for 15 min at 4 °C. The pellets were resuspended in tissue culture medium (DMEM 10%) and *B. besnoiti* bradyzoites were counted in a Neubauer chamber in PBS containing 10% trypan blue. Monolayers of Vero cells in 25 cm^2 flasks were inoculated with 5×10^6 *B. besnoiti* bradyzoites. Infected cultures were passaged every 6 days.

At 48 h after inoculation, the medium was changed to DMEM 2% FCSI with medium changes every 3 days.

Infected cultures were inspected daily using an inverted microscope for the presence of free tachyzoites.

Once free tachyzoites were detected, cryopreservation of infected Vero cell cultures was done at the following passage, by resuspending infected Vero cells in FCSI containing 10% DMSO, and freezing and storage in liquid nitrogen.

2.4. Infection of rabbits with B. besnoiti bradyzoites and tachyzoites

Two rabbits were inoculated intraperitoneally with 10^7 *B. besnoiti* bradyzoites isolated from the Sallers' cysts, and were regularly inspected for the occurrence of skin lesions during 1 year. After 12 months, the same rabbits were inoculated with 10^7 tachyzoites obtained from the respective *in vitro* culture, boosted with the same amount of tachyzoites 21 and 50 days later and were euthanized at 90 days post-inoculation. Serum and tissue specimens were collected for serology and histopathology, respectively. Animals were handled according to the legal stipulations of animal welfare.

2.5. Indirect imunofluorescence antibody test (IFAT) for the detection of anti-Besnoitia antibodies

Paraformaldehyde fixed *B. besnoiti* suspension (2×10^6 ml^{-1}) was obtained from cultured *B. besnoiti* tachyzoites from Israel (Kimron Veterinary Institute, Bet Dagan). Parasites were applied to microscopy slides in 6 μl, dried and fixed with cold acetone (−20 °C) for 10 min, as described (Shkap et al., 2002). Serial two fold dilutions of serum samples in PBS were added and after 45 min at 37 °C, slides were washed with distilled water (three times, 10 min) and droplets were covered with FITC conjugated rabbit anti-bovine IgG in PBS, incubated and washed as above. Bound antibodies were detected under 200× amplification using an UV light microscope Olympus BX50.

2.6. Identification of B. besnoiti by PCR

B. besnoiti infected Vero cell monolayers were scraped from the tissue culture flask using a rubber policeman. The parasites were separated from Vero cells and debris by passage through a Whatman CF-11 cellulose column as described (Shkap et al., 1984). Parasites were centrifuged at 770 × *g* during 15 min at 4 °C. The pellet was resuspended in PBS, and the tachyzoites were counted in a Neubauer chamber. A 10^8 tachyzoites were centrifuged (10,000 × *g* at 4 °C for 1 min) and resuspended in 200 μl TE, followed by the addition of 1 ml of lysis buffer (10 mM Tris–Cl (pH 8.0); 0.1 M EDTA (pH 8.0); 0.5% (w/v) SDS; 20 μg/ml RNase) to the cell suspension and a 1 h incubation at 37 °C. For tissue digestion, 6 μl of proteinase K (20 mg/ml) were added to the lysate, mixed gently and incubated in a water bath for 3 h at 50 °C, followed by phenol extraction as described by Sambrook and Russell (2001). DNA was solubilized in TE (pH 8.0) and stored over night at 4 °C. DNA concentration was measured spectrophotometrically at λ = 260 nm (DU 68 Beckman Fullerton, USA).

Primers for polymerase chain reaction (PrCR) reaction were designed with primer 3™ software (Rozen and Skaletsky, 2000) using the published ITS1 partial DNA sequence of *B. besnoiti* (GenBank™ accession number AF076859). The forward primer (5′-GGGTGCATTCGAGAAGTGTG-3′) and reverse primer (5′-TCCGTGATAGCAGAGTGAGGAGG-3′) were used for amplification of the *B. besnoiti* ITS1 sequence by PCR in an Eppendorf Mastercycler gradient Thermal Cycler (Hamburg, Germany), applying the following conditions: 3 min at 94 °C followed by 30 cycles of 30 s at 94 °C, 30 s at 65.5 °C, and 2 min at 72 °C. A final extension of 5 min at 72 °C was used. PCR products were separated on 2% agarose gels and stained with 0.3 μg/ml ethidiumbromide.

2.7. Transmission electron microscopy (TEM) and scanning electron microscopy (SEM)

Both, small tissue pieces containing *B. besnoiti* cysts obtained from infected animals as well as *B. besnoiti* isolates (tachyzoites) were inspected by TEM. Samples were fixed in 2.5% glutaraldehyde in 100 mM sodium cacodylate buffer (pH 7.3) for 12–24 h at 4 C, were washed in cacodylate buffer and subsequently postfixed in 2% OsO4 in cacodylate buffer for 4 h at room temp. Following extensive washing in water, specimens were left in 1% uranyle acetate for 1 h, were washed in water, and were dehydrated in a graded series of ethanol. Specimens were embedded in Epon 812 resin, and sections were cut on a Reichert and Jung ultramicrotome. Sections were stained with uranyle acetate and lead citrate as described (Hemphill and Croft, 1997).

For SEM analysis, specimens were dehydrated by sequential incubations in increasing concentrations of ethanol (50–70%), and were finally immersed in hexamethyl-disilazane and air-dried under a fume hood. They were then sputter-coated with gold, and inspected on a JEOL 840 scanning electron microscope operating at 25 kV.

3. Results

As a prerequisite to this study, cattle in Portugal were surveyed for the occurrence of clinical signs of besnoitiosis. As there are other diseases with similar signs (burns; mange; fungus infection), it was necessary to confirm *B. besnoiti* infections serologically by detection of anti-*B. besnoiti* antibodies and by IFAT, and by direct histopathological detection of the parasite in the skin, isolation of tachyzoites in cell culture, and molecular confirmation of its identity by PCR. Confirmed clinical cases are reported from a large area in the south of Portugal (Alentejo).

In two animals presenting severe skin lesions reminiscent of *B. besnoiti* infection (Fig. 1A), indirect diagnosis of besnoitiosis was conducted by IFAT and revealed a high antibody titer (>1024), while direct detection of the parasite tissue cysts by histophatology from skin biopsies (Fig. 1B) confirmed the diagnosis. TEM of skin biopsies showed, that parasites were surrounded by a massive, two-layered cyst wall (Fig. 1C). A large number (>500) of parasites were found to be located within a parasitophorous vacuole that is delineated by an intracellular tissue cyst wall and the parasitophorous vacuole membrane, followed distally by an outer cyst wall (Fig. 1C). Closer inspection of parasites by TEM revealed typical features of bradyzoite stage parasites, including a nucleus located in the posterior region of the cell, and a large number of micronemes at the anterior part (Fig. 1D and E). Bradyzoites in the periphery (Fig. 1D), near the inner cyst wall, appeared to release small vesiculated structures, which were mostly found in the vicinity of the tissue cyst wall. These vesicles were absent in the matrix surrounding the parasites located in the interior region of the tissue cyst (Fig. 1E). Interior bradyzoites were embedded in a granular matrix that fills out the intercellular spaces. The molecular nature of this material is still unknown. Inoculation of isolated *B. besnoiti* from both animals into Vero cell culture (Fig. 2A and C) showed that bradyzoites were moving over, under and around the Vero cells monolayer during the 5 days subsequent to the inoculation. During this time, bradyzoites were motile and employed movements such as circular gliding, upright twirling and helical gliding (data not shown). After these initial 5 days, the bradyzoites were not visible anymore by phase contrast microscopy. However, the presence of the parasite was confirmed in *in vitro* cultures initiated from both animals by the amplification of the ITS1 partial sequence DNA of 149 bp, which was absent in material obtained from uninfected control cultures (Fig. 2B). The amplified products revealed 100% identity with the DNA fragment reported by Ellis et al. (2000) (GenBank™ accession number AF076859).

At 30 and 40 days post-infection characteristic forms of *B. besnoiti* tachyzoites of both the Sallers' and the Charolay's isolate were visible. Parasites formed small plaques, gradually destroyed the Vero cell layer, and large number of tachyzoites were released into the culture medium (Fig. 2A and C). Continuation of the culture on the same monolayer resulted in complete destruction of host cells due to continuous tachyzoite proliferation within the next 2–3 days. TEM (Fig. 2D and E) showed that these parasites proliferated within a parasitophorous vacuole, surrounded by a distinct parasitophorous vacuole membrane, and tachyzoites exhibited typical features of apicomplexan tachyzoite stage parasites of other species such as anterior conoid, micronemes, rhoptries and dense granules. In contrast to bradyzoites, the mitochondria were clearly visible, and tachyzoites were much more densely packed during their intracellular phase, thus a granular cyst matrix like in bradyzoites was not discernable. The two isolates were named Bb1Evora03 (from the 4 years old Sallers cow) and Bb2Evora03 (from the 6 years old Charolais bull). They were further passaged on Vero cells, and were cryopreserved in liquid nitrogen.

The two rabbits that had been initially inoculated initially with *B. besnoiti* bradyzoites isolated from the Sallers' cysts did not exhibit any clinical signs of disease during the following 12 months. Subsequent inoculation of cell culture-derived tachyzoites did also not result in any clinical manifestation of besnoitiosis. At necropsy, no lesions were found and no cysts were observed by histopathology. Both animals presented a high serum titer of >2048 in the IFAT test using slides sensitized with the Israel isolate of *B. besnoiti* (data not shown).

4. Discussion

This paper reports on the isolation and description of two new isolates (Bb1Evora03 and Bb2Evora03) of *B. besnoiti*, obtained from two naturally infected cattle from Alentejo, south of Portugal. Although at present we consider them as separate isolates, there is, at present, no evidence that these two isolates differ in any way with regard to morphological, structural or molecular features. Ongoing studies should clarify this point in the future. Only few isolates have been obtained so far in South Africa and Israel, thus our report on the

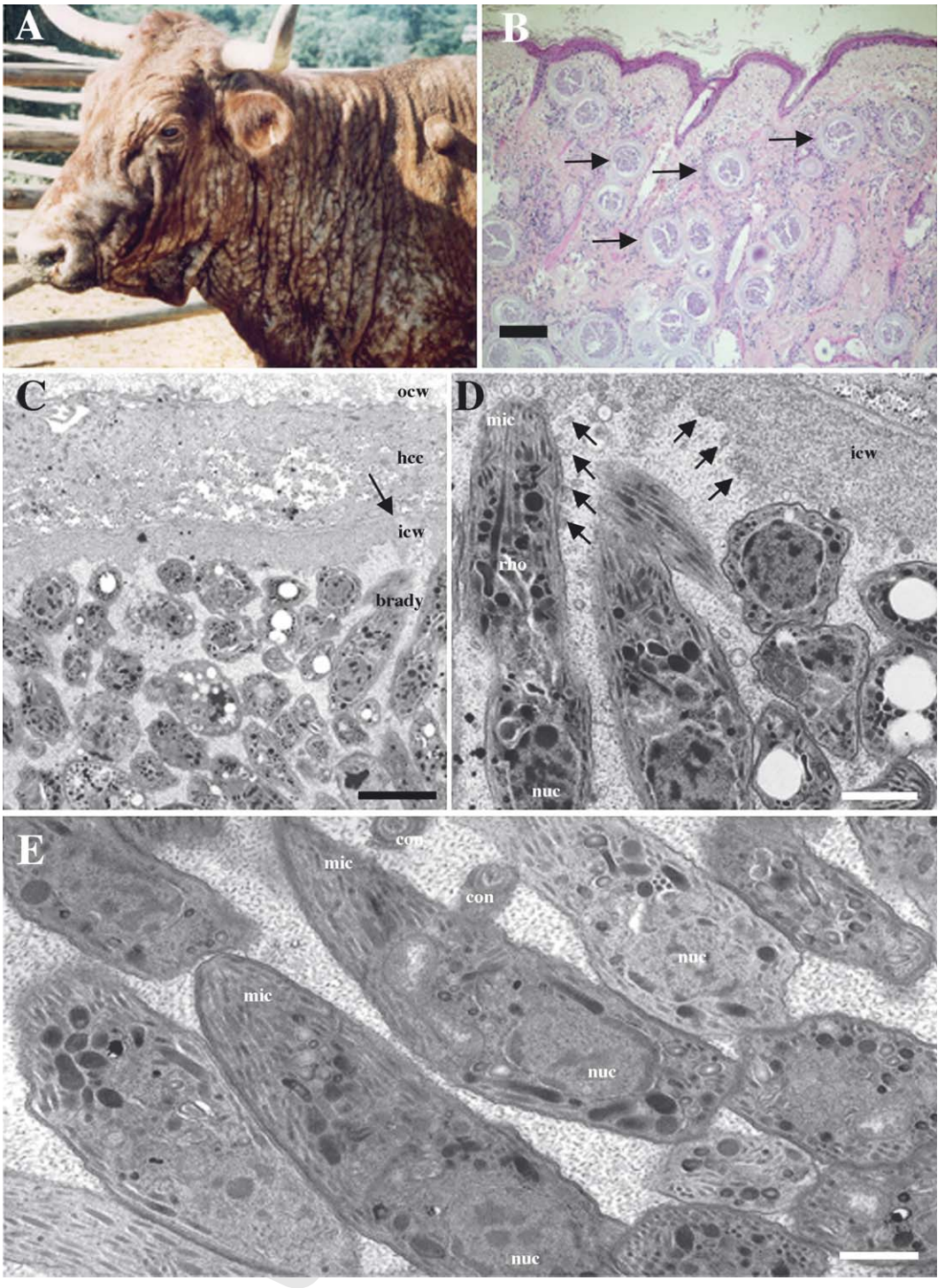

Fig. 1. Besnoitiosis in cattle. (A) A case of besnotiosis in a cow presenting severe besnoitiosis skin lesions. (B) Histopathology (paraffin section) of a skin lesion, stained with hematoxylin/eosin. The double-layered cysts are indicated with arrows. Bar = 200 μm. (C) TEM of *Besnoitia* cyst. Bradyzoites (brady) are located within an intracellular cyst, delineated by an intracellular cyst wall (icw) and the parasitophorous vacuole membrane (indicated by an arrow). Distally to the parasitophorous vacuole membrane, a portion of the host cell cytoplasm (hcc) and the outer cyst wall (ocw) is seen. Bar = 1.9 μm. (D) TEM of a *Besnoitia* cyst showing the peripheral region with parasites adjacent to the inner cyst wall (icw). Note presence of small vesiculated structures emanating from the parasites and incorporated into the cyst wall (arrows). nuc: nucleus, rho: rhoptries; bar = 0.9 μm. (E) TEM of the central portion of a *Besnoitia* cyst, showing numerous bradyzoites embedded in a granular matrix; nuc: nucleus; mic: micronemes; co: conoid; bar = 0.5 μm.

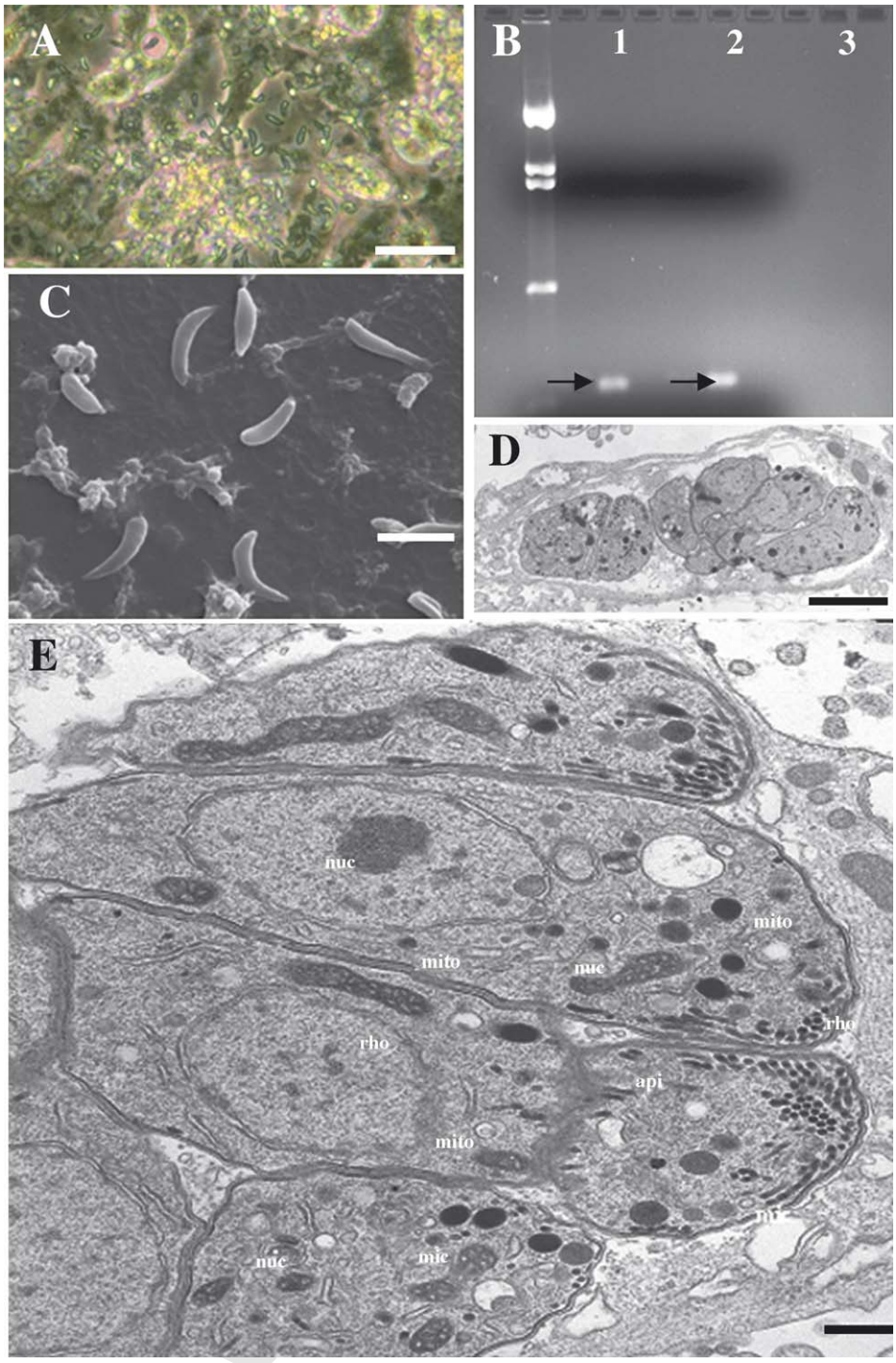

Fig. 2. In vitro culture of *B. besnoiti*. (A) Phase contrast micrograph of *B. besnoiti* in Vero cells, with tachyzoites emanating from host cells. (B) Molecular identification of *B. besnoiti* by PCR. ITS1 fragments of the isolates Bb1Evora03 (lane 1) and Bb2Evora03 (lane 2) by amplification of the 149 bp ITS1 fragments (arrows), while uninfected control cultures (lane 3) were negative. Molecular weight markers (MW) are pBR322 DNA BstN I digest Biolabs®. (C) Scanning electron micrograph of *Besnoitia* tachyzoites in an infected Vero cell monolayer; bar = 12 μm. (D) TEM of infected Vero cell, showing tachyzoites situated within a parasitophorous vacuole; bar = 10 μm. (E) TEM of *Besnoitia* tachyzoites in Vero cell culture. Note that parasites are tightly packed, and consequently a cyst matrix is not visible. Nuc: nucleus; mito: mitochondria; mic: micronemes; api: apicoplast; bar = 0.3 μm.

first European isolates may contribute to future studies on this barely known parasite with its increasing economical relevance.

While the *B. besnoiti* isolates obtained so far have been obtained by passage through laboratory animals, we are the first to describe the isolation of *B. besnoiti* directly by inoculation into cell culture. The fact that none of the two rabbits inoculated in this work showed any clinical signs of infection or lesions, as it was observed occasionally by others (Pols, 1960; Bigalke, 1968), reinforces the advantages of isolating these organisms directly in cell cultures. In the adaptation phase to *in vitro* conditions, meaning the first 30 days, the parasites were not identifiable by light microscopy, but clearly detectable by ITS1-based PCR. Thus, although there is a high geographical distance between our isolates (Bb1Evora03 and Bb2Evora03) from Portugal and those from South Africa on which the molecular phylogeny was published by Ellis et al. (2000), there is no difference in the available ITS1 sequences. This clearly suggests a high similarity between isolates from different geographical areas, and highlights the suitability of the primer pairs for future molecular confirmation of further *B. besnoiti* isolates.

The numbers of actual and new notifications of bovine besnoitiosis have grown in Europe (Cortes et al., 2003, 2004, 2005; Juste et al., 1990). Recently, *Besnoitia tarandi* has been isolated from reindeer in Finland (Dubey et al., 2004), and clinical besnoitiosis in roe deer (*Capreolus capreolus*) in Spain has been described (De Luco et al., 2000). These studies emphasize that infection by protozoans of the genus *Besnoitia* occurs in Europe more frequently than previously thought. The impact relative to sick animals at a farm level is related to 10% of the herd (Pols, 1960) and to a high number of infected animals, usually more than 80% (Bigalke, 1968).

Although our investigations are by no means comparable to an epidemiological study, we have demonstrated the presence of the disease in the south of Portugal, where beef production is the predominant output. In fact, in the majority of cases the disease had never been described in the farm, suggesting some dynamic of bovine besnoitiosis. Due to the overall skin lesions as shown in Fig. 1, and due to the secondary infections in wounds on areas of high elasticity demand, which cause a severe limitation to movement, animals end up in a severely impaired body condition. In addition, disease leads to painful breast feeding and increased abortion incidence in females and, in the males, to severe necrotizing orchitis and permanent infertility (Basson et al., 1970; Ferreira et al., 1982; Cortes et al., 2005). In our field observations, during the initial phase of infection of a given herdaround 10% of animals die while in the acute stage of infection or in the chronic stage due to starvation. In addition, a large portion of animals are being culled due to the fact that they do not represent any commercial value anymore. After this dramatic, initial scenery on a herd, sporadic clinical cases, usually lower than 1%, will occur. This just illustrates that basic biological questions regarding the life cycle, infection dynamics, and the host-parasite relationship more research on the infection biology of *B. besnoiti* is needed, and the isolation of this parasite will aid in those future investigations.

~~Uncited references~~

~~Frixione et al. (1996) and Sheffield (1968).~~

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