# *Besnoitia oryctofelisi* n. sp. (Protozoa: Apicomplexa) from domestic rabbits

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SUMMARY

A species of *Besnoitia* from naturally infected rabbits from Argentina was propagated experimentally in mice, gerbils, rabbits, cats, and cell cultures. Cats fed tissue cysts from rabbits shed oocysts with a prepatent period of nine to 13 days. Sporulated oocysts were infective to gerbils, rabbits, outbred Swiss Webster and interferon gamma gene knockout mice. Bradyzoites were infective orally to gerbils and cats. Tachyzoites were successfully cultivated and maintained *in vitro* in bovine monocytes and African green monkey kidney cells. Schizonts were seen in the lamina propria of the small intestine of cats fed tissue cysts; the largest ones measured 52 × 45 $\mu$m. Schizonts were also present in mesenteric lymph nodes, livers, and other extra-intestinal organs of cats fed tissue cysts. Oocysts were 10–14 × 10–13 $\mu$m in size. This rabbit-derived species of *Besnoitia* resembled *B. darlingi* of the North American opossum, *Didelphis virginiana* with an opossum-cat cycle, but it was not transmissible to *D. virginiana*, and *B. darlingi* of opossums was not transmissible to rabbits. Based on biological, serological, antigenic, and molecular differences between the rabbit and the opossum *Besnoitia*, a new name, *B. oryctofelisi* is proposed for the parasite from domestic rabbits from Argentina.

Key words: *Besnoitia oryctofelisi*, n. sp., *B. darlingi*, rabbits, cats, gerbils, mice, schizonts, cell culture, Argentina.

## INTRODUCTION

*Besnoitia* spp. are coccidians affecting cattle, goats, equines, reindeer, caribou, opossums, rodents, and lizards (Levine, 1973; Frenkel, 1977; Dubey, 1993; Leighton & Gajadhar, 2001; Paperna & Lainson, 2001). There are 7 named species of *Besnoitia*: *B. bennetti* Babudieri, 1932; *B. besnoiti* (Marotel, 1912) Henry, 1913; *B. caprae* Njenga, Bwangamoi, Mutiga, Kangethe & Mugera, 1993; *B. darlingi* (Brumpt, 1913) Mandour, 1965; *B. jellisoni* Frenkel, 1953; *B. wallacei* (Tadros & Laarman, 1976) Frenkel, 1977; and *B. tarandi* (Hadwen, 1922), Levine, 1961. There is, however, a considerable uncertainty regarding identity of some of these species of *Besnoitia* because life-cycles of only 2 species of *Besnoitia* (*B. darlingi* and *B. wallacei*), are known, and morphological differences among other species are poorly defined (Tables 1, 2).

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Recently, a species of *Besnoitia* was found in naturally-infected domestic rabbits from Argentina (Venturini *et al*. 2002). In the present paper, we describe its life-cycle and biology in intermediate and definitive hosts, provide molecular and protein data, and propose a new name, *Besnoitia oryctofelisi*, for this species.

## MATERIALS AND METHODS

### *Origin of* Besnoitia oryctofelisi

*Besnoitia* tissue cysts from a naturally-infected rabbit from a family farm in La Plata, Argentina (Venturini *et al*. 2002) were fed to a 2-month-old cat (cat A) in the laboratory at La Plata, Argentina; the kitten shed *Besnoitia*-like oocysts 13 days later. The oocysts were separated from cat faeces by sugar flotation (Sheather, 1923), washed, and suspended in 2·5% potassium dichromate for 1 week at room temperature. The oocysts were shipped by air to the laboratory in Beltsville, Maryland for identification, where they were again incubated at room temperature on a shaker for 1 week.

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Table 1. Characteristics of 6 *Besnoitia* species

| | *B. bennetti* | *B. besnoiti* | *B. caprae* | *B. jellisoni* | *B. tarandi* | *B. wallacei* |
|---|---|---|---|---|---|---|
| Natural intermediate hosts | Burrows, horse, ass | Cattle, wildebeest, impala, kudu, antelope | Goats | Rodents | Reindeer, caribou, mule deer, muskox | Rats |
| Experimental intermediate hosts | Unknown (not rabbits) | Mice, rabbits hamsters, gerbils, sheep, goats | Unknown (not mice, rabbits, rats) | Mice, rats, hamsters, guinea-pigs, rabbits | Unknown | Mice, rats |
| Definitive host | Unknown | Unknown (not cats) | Unknown (not cats) | Unknown (not cats) | Unknown (not cats) | Cats |
| Geographical distribution | Africa, Europe, Mexico, USA | South Africa, Mediterranean countries, China, USSR | Kenya, Iran | USA, Peru | Alaska, Sweden | Hawaii, Australia, New Zealand, Japan, Africa, Brazil |
| Tachyzoites ($\mu$m) | Unknown | 7–8·6 × 2·3 | Unknown | 7 × 2 | Unknown | Unknown |
| Bradyzoites ($\mu$m) | 5–8 × 1·5–2·0 | Unknown | 9–10 × 1·2 | 8 × 2 | 8–10 × 2–2·6 or 6·5–11·0 × 1·0–1·5 | 11·1 × 2·8 |
| *In vitro* cultivation | No | Yes | No | Yes | No | No |
| References | Bennett (1933); Bigalke (1970); Terrell & Stookey (1973); Foley *et al.* (1990); van Heerden *et al.* (1993); Davis *et al.* (1997) | Pols (1960); Bigalke (1962, 1967, 1968); Bigalke *et al.* (1967); McCully *et al.* (1966); Basson *et al.* (1970); Neuman (1974); Göbel *et al.* (1985); Diesing *et al.* (1988); Shkap *et al.* (1988); Juste *et al.* (1990) | Cheema & Toofanian (1979); Heydorn *et al.* (1984); Bwangamoi *et al.* (1989); Njenga *et al.* (1993, 1995); Ng'ang'a & Kasigazi (1994) | Frenkel (1953, 1977); Jellison *et al.* (1956); Sheffield (1966, 1968); Ernst *et al.* (1968); Fayer *et al.* (1969); Chobotar *et al.* (1970); Sénaud (1969); Sénaud *et al.* (1972, 1974); Scholtyseck *et al.* (1973); Senaud & Mehlhorn (1978) | Levine (1973); Wobeser (1976); Rehbinder *et al.* (1981); Glover *et al.* (1990); Hilali *et al.* (1990); Ayroud *et al.* (1995); Leighton & Gajadhar (2001) | Wallace & Frenkel (1975); Frenkel (1977); Ito *et al.* (1978); Mason (1980); McKenna & Charleston (1980); Loss & Lopes (1992); Ng'ang'a *et al.* (1994) |

Table 2. Summary of *Besnoitia darlingi*-like parasites in animals

| Geographical distribution | Panama | | Belize | Brazil | | | North America |
|---|---|---|---|---|---|---|---|
| Natural intermediate host | Opossum (*Didelphis marsupialis*) | Lizards (*Basiliscus basiliscus*, *Ameiva ameiva*, *A. festiva*, *A. leptophrys*) | Lizard (*Basiliscus vittatus*) | Lizard (*Ameiva ameiva*) | Rodent (*Akodon montensis*) | Opossum (*D. marsupialis*) | Opossum (*D. virginiana*) |
| Experimental intermediate host | Mice, hamsters, opossums, squirrels, marmosets, wooly opossum | Mice, hamsters, marmoset | Unknown | Mice | Mice | Mice, hamsters | Mice, gerbils |
| Transmission to rabbits | Unknown | Not transmitted (1 rabbit used) | Unknown | Unknown | Unknown | Unknown | No |
| Definitive host | Unknown | Unknown | Unknown | Unknown (not cats) | Unknown | Unknown | Cats |
| Tachyzoites ($\mu$m) | $6{\cdot}1 \times 2{\cdot}1$ ($6{\cdot}0$–$9{\cdot}0 \times 1{\cdot}7$–$4{\cdot}0$) | $6{\cdot}7 \times 2{\cdot}3$ ($3{\cdot}6$–$10{\cdot}4 \times 1{\cdot}4$–$2{\cdot}7$) | $<10$ | $6{\cdot}4 \times 1{\cdot}5$ ($4{\cdot}4$–$7{\cdot}4 \times 1{\cdot}5$)* | Unknown | Unknown | $5{\cdot}4 \times 2{\cdot}0$ |
| Bradyzoites ($\mu$m) | Unknown | $7{\cdot}4 \times 1{\cdot}5$ ($7{\cdot}0$–$7{\cdot}7 \times 1{\cdot}4$–$1{\cdot}6$) | Unknown | $8{\cdot}5 \times 1{\cdot}5$ ($6{\cdot}7$–$9{\cdot}0 \times 1{\cdot}5$)* | Unknown | $8{\cdot}8 \times 1{\cdot}4$–$1{\cdot}9$ | $10$–$11 \times 1$ |
| Oocysts ($\mu$m) | Unknown | Unknown | Unknown | Unknown | Unknown | Unknown | $12 \times 10{\cdot}3$ ($11{\cdot}2$–$12{\cdot}8 \times 9{\cdot}6$–$11{\cdot}2$) |
| Cell culture development | Unknown | Unknown | Unknown | Yes | Unknown | Unknown | Yes |
| References | Darling (1910) Schneider (1967*a*, *d*) | Schneider (1965, 1966, 1967*b*, *c*) | Garnham (1966) | Paperna & Lainson (2001) | Grisard *et al.* (1997) | Naiff & Arias (1983) | Stabler & Welch (1961); Conti-Diaz *et al.* (1970); Flatt *et al.* (1971); Jack *et al.* (1989); Smith & Frenkel (1977, 1984); Dubey *et al.* (2002) |

* Air dried, Bouin's fixed, Giemsa-stained preparations. Tachyzoites were from the peritoneal cavity of mice infected i.p. 7 days earlier. Bradyzoites were released from tissue cysts from mice (personal communication from Lainson to J.P.D.).

Table 3. Endogenous stages of the *Besnoitia oryctofelisi* in cats fed with tissue cysts

| Cat no. | Day killed | Oocysts shed | Schizonts in intestine | Schizonts in other organs¶ |
|---|---|---|---|---|
| 666 | 4 | No | Yes | None |
| 667 | 7 | No | Yes | Ml, Li |
| 665* | 12 | Yes† | Yes | Ml, Li |
| 689 | 3 | No | Yes‖ | Ml |
| 687* | 11 | Yes† | Yes | A, B, H, K, Li, Lu, Me, P, Sk, Sp, T |
| 688 | 12 | Yes‡ | Yes | Ml, Lu |
| 709 | 11 | Yes§ | Yes | Ml, Li |

* Methyl prednisolone acetate (Upjohn, Kalamazoo, Michigan, USA) 20 mg/kg body weight, once, i.m.
† Pre-patent period 9 days.
‡ Pre-patent period 10 days.
§ Pre-patent period 11 days.
‖ Only individual zoites seen.
¶ A, adrenal; B, brain; H, heart; K, kidney; Li, liver; Lu, lung; Ml, mesenteric lymph nodes; P, pancreas; Sk, skeletal muscle; Sp, spleen; T, tongue.

### *Infection of laboratory animals*

Laboratory-raised rabbits, gerbils, mice, and cats were infected with *B. oryctofelisi*. The New Zealand White rabbits were 5–8 weeks old and obtained from Covance Research Products Inc., Denver, Pennsylvania, USA. The gerbils were females, 5–8 weeks old and obtained from Charles River Laboratory, Stoneridge, New York, USA. The Swiss Webster (SW) outbred mice were females of 20–25 g, and obtained from Taconic Farms, Germantown, New York, USA. The interferon-gamma gene knockout (KO) mice were 5–10 weeks old and obtained from Jackson Laboratories as previously described (Dubey & Lindsay, 1998); these mice are highly susceptible to intracellular parasites because they lack the capacity to produce the cytokine interferon gamma necessary for intracellular immunity (Dubey & Lindsay, 1998). The cats were from a parasite-free colony (Dubey, 1995).

Eight experiments were performed in animals according to Animal Care Protocols of the US Department of Agriculture. Although controls were not included in each experiment, *Besnoitia* stages have never been detected in hundreds of cats, mice, and numerous rabbits and gerbils used in the senior author's laboratory at Beltsville.

In Exp. 1, oocysts (unknown number) from cat A were fed to 5 gerbils (Nos. 3994–3998). Two gerbils were given sulfadiazine sodium (Sigma, St Louis, Missouri, USA) in drinking water (1 mg/ml) from day 14 to day 21 p.i. and were killed 49 days p.i. The remaining oocysts from cat A were fed to 5 more gerbils (Nos. 4682–4686).

In Exp. 2, the mesenteric lymph node homogenate from gerbil No. 3998 (killed 13 day p.i.) was inoculated i.p. into 3 KO mice (Nos. 4043, 4663, 4665); these mice were killed 7, 11, and 12 days p.i., respectively. Tachyzoites obtained from the peritoneal exudate of a KO mouse (No. 4665) were inoculated s.c. into 5 gerbils (Nos. 3989–3993); these gerbils were killed 53 days p.i.

In Exp. 3, rabbit No. 1 was inoculated i.m. with culture-derived tachyzoites (see *in vitro* cultivation, below) obtained from KO mouse No. 4043 from Exp. 2. The rabbit was inoculated with many ($>10^6$) organisms on days 0, 3, 18, 31, and 45 and killed 9 days after the last inoculation (day 54 after the first inoculation).

### *Endogenous development of* B. oryctofelisi *in cats* (*Exp. 4*)

In the first trial, three 70-day-old littermates (Nos. 665–667) were fed tissues from experimentally infected gerbils (Nos. 3989–3993 from Exp. 2). In the second trial, three 64-day-old cats (Nos. 687–689) were fed tissues from the rabbit No. 1 (from Exp. 3) containing thousands of tissue cysts; cats were fed infected tissues for a period of 2–3 days. The cats were killed 3–12 days after feeding infected rabbit tissues (Table 3). Thus, cat No. 689 was killed day 3 after the initial meal (day 1 after the ingestion of the last infected meal) (Table 3). For study of intestinal stages, the entire small intestine was divided into 5 equal parts and fixed in 10% neutral buffered formalin. From each intestinal region, 6–8 sections were embedded in paraffin for histological sections; thus 30–40 sections were examined from the intestine of each cat. Pieces of mesenteric lymph nodes, liver, spleen, kidneys, adrenals, heart, lungs, skeletal muscle, eyes, and brain of each cat were also fixed in formalin for histology.

### *Comparative infectivity of* B. oryctofelisi *oocysts to rabbits, gerbils, KO and SW mice* (*Exp. 5*)

To study comparative infectivity of *B. oryctofelisi* oocysts to rabbits, gerbils, KO and SW mice, oocysts from pool A (from cat 665, see Table 3) were used (Table 4). The total number of oocysts in pool A was approximately 10 000/ml (total volume about 120 ml). Initially, 50 ml of the pool (approximately 500 000 oocysts) was centrifuged, and the oocysts washed in water by further centrifugation to remove potassium dichromate; the sediment was suspended in 10 ml of water. Five ml of the pool (250 000 oocysts) was fed to rabbit No. 2, 0·05 ml (2500 oocysts) to rabbit No. 3, and 0·5 ml (25 000 oocysts) to each of 5 SW mice and 3 gerbils (not shown in Table 4). Because the inoculum was lethal to gerbils (see results), pool A was diluted further ($10^{-1}$ to $10^{-4}$) and an aliquot from each dilution was fed to SW and KO mice and gerbils indicated in Table 4.

To trace the development of *Besnoitia*, an additional 10 SW mice were fed undiluted suspension

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Table 4. Comparative infectivity of *Besnoitia oryctofelisi* oocysts to mice and gerbils (Exp. 5)

| Oocyst dilution (No. of oocysts) | SW mice | | | KO mice | | | Gerbils | | |
|---|---|---|---|---|---|---|---|---|---|
| | No. fed | No. died | No. infected | No. fed | No. died | No. infected | No. fed | No. died | No. infected |
| Undiluted (10 000) | 5 | 0 | 4 | 4 | 2 (8)* | 4 | N.D. | N.A. | N.A. |
| $10^{-1}$ (1000) | 5 | 0 | 4 | 4 | 2 (10) | 4 | 5 | 5 (8–12) | 5 |
| $10^{-2}$ (100) | 5 | 0 | 2 | 2 | 2 (13, 17) | 2 | 5 | 1 (16) | 5 |
| $10^{-3}$ (10) | N.D. | N.A. | N.A. | 2 | 2 (14, 16) | 2 | N.D. | N.A. | N.A. |
| $10^{-4}$ (1) | 5 | 0 | 0 | 2 | 0 | 0 | 5 | 0 | 1 |

* Day of death.
N.D., Not done; N.A., Not applicable.

of oocysts from pool A and killed 1, 3, 5, 7, 9, 11, 14, 19, 25, and 28 days later (not shown in Table 4).

### *Oral infectivity of* B. oryctofelisi *tissue cysts to gerbils and cats* (*Exp. 6*)

To determine if *Besnoitia* tissue stages are infectious orally to animals, lungs of a gerbil fed approximately 100 *Besnoitia* oocysts 25 days previously were used for passage. After ascertaining the presence of tissue cysts by microscopical examination, the lungs of this gerbil were homogenized in 0·85% NaCl (saline) in a pestle and mortar and fed to 5 gerbils. The recipient gerbils were killed day 37 p.i. (1 gerbil) or day 73 p.i. (4 gerbils) and their tissues were examined histologically. Tissues of the 4 gerbils killed on day 73 p.i. were also fed to cat No. 709 for oocyst shedding (Table 3).

### *Attempted cross-transmission of* B. darlingi *of opossum to rabbits and of* B. oryctofelisi *to opossums* (*Exp. 7, 8*)

*B. oryctofelisi* morphologically resembled *B. darlingi*, therefore, cross-transmission experiments were performed. In Exp. 7, attempts were made to transmit *B. darlingi* to laboratory-raised rabbits using oocysts or culture-derived organisms. In the first trial, an unknown number of oocysts of *B. darlingi* pooled from cats 496 and 570 (Dubey *et al.* 2002) were fed to a rabbit (No. 4) and the rabbit was killed 50 days later. In the second trial, 2 rabbits (Nos. 5 and 6) were inoculated s.c. and i.m. with $>10^7$ tachyzoites on days 0, 14, 17, 21 and killed 62 and 63 days after the first inoculation. In the third trial, 2 rabbits (Nos. 7 and 8) were inoculated s.c. with $>10^7$ tachyzoites on days 0, 6 and 10 and killed 57 days after the first dose. A complete post-mortem was made on all 5 rabbits and samples of tissues were studied histologically. In addition, about 500 g of tissues from peritoneal and pleural cavities, muscles, and brain of rabbits Nos. 7 and 8 were fed to 2 parasite-free 3-month-old cats, the faeces of which were examined for oocysts for 1 month after ingesting rabbit tissues. The viability of the tachyzoites given to rabbits was confirmed by inoculating 1000-fold diluted inocula into gerbils and KO mice.

In Exp. 8, transmission of *B. oryctofelisi* to 4 laboratory-raised opossums (*Didelphis virginiana*) was attempted. The opossums were 1–1·5 years old when used in the experiment and were obtained as infants in their mother's pouch. The opossums remained with their mothers until they were weaned, and were housed individually at the University of Florida Animal Care Facility, Gainesville, Florida, USA. Each opossum was microchipped for identity. The opossums were fed only dry dog food, and had never consumed meat. Sporulated *B. oryctofelisi* oocysts (25 ml of pool A, see Exp. 5) and infected gerbil tissues were shipped cold by overnight mail from Beltsville, Maryland to Gainesville, Florida for feeding to opossums. The 2 gerbils had been inoculated orally 31 days previously with *B. oryctofelisi* oocysts and had demonstrable tissue cysts as determined by microscopical examination of lung squashes. For feeding to opossums, oocysts were poured over 5–10 g of dry dog food that was quickly consumed. For feeding gerbil tissues, opossums were fasted overnight and they consumed gerbil tissues within a few min of presentation. One opossum fed oocysts died of unrelated causes 11 days later. The remaining 3 opossums were killed by i.p. injection of 4 ml of pentobarbital sodium (Somlethal, J. A. Webster, Sterling, Massachusetts, USA) 67 days after feeding oocysts or infected tissues. Tissues of all 4 opossums were fixed in 10% formalin and shipped to Beltsville for histological examination. Tissues of the 3 opossums killed 67 days p.i. were also shipped unfixed to Beltsville where they were fed to two 3-month-old cats.

### In vitro *cultivation of* B. oryctofelisi

Tachyzoites obtained from the peritoneal exudate of KO mouse No. 4043 (see Exp. 2) were inoculated onto bovine monocytes and African green monkey (CV-1) cells are previously described (Dubey *et al.* 2002).

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Table 5. Indirect fluorescent antibody titres in rabbits against homologous and heterologous *Besnoitia* antigens

| Rabbit no. | Exp. no. | Inoculum | Route | Day p.i. | Antibody titre against | |
|---|---|---|---|---|---|---|
| | | | | | *B. darlingi* | *B. oryctofelisi* |
| 1 | 3 | *B. oryctofelisi** | i.m., s.c. | 54 | 1:16 000 | 1:32 000 |
| 4 | 7 | *B. darlingi*† | oral | 50 | 1:8000 | 1:4000 |
| 7 | 7 | *B. darlingi** | i.m., s.c. | 57 | 1:64 000 | 1:16 000 |

* Tachyzoites derived from culture.
† Oocysts.

*Histological examination*

Samples of brain, lung, heart, tongue, liver, kidneys, intestines, mesenteric lymph nodes, urinary bladder, and muscles from limbs of rabbits, gerbils, mice and cats were fixed in 10% formalin. Paraffin-embedded sections were cut at 5 $\mu$m, and examined after staining with haematoxylin and eosin (H&E) or periodic acid Schiff (PAS) reaction and counter stained with haematoxylin.

*Morphological comparison of stages of* B. darlingi *and* B. oryctofelisi

Cell culture-derived tachyzoites of *B. darlingi* (Dubey *et al*. 2002) and *B. oryctofelisi* were measured using identical conditions in 72 h preparations in CV-1 cells after fixation in Bouin's fluid and staining with Giemsa's stain. Bradyzoites of *B. darlingi* were measured from 1 $\mu$m Toluidine blue stained sections of 6 tissue cysts from the livers of naturally infected opossums (Dubey *et al*. 2002). Bradyzoites of *B. oryctofelisi* released from tissue cysts from rabbit No. 1 were measured in smears from methanol-fixed, Giemsa's-stained preparations and in 1 $\mu$m Toluidine blue stained sections of the same rabbit. Oocysts of *B. oryctofelisi* from experimentally infected cats from Table 3 were compared with measurements of *B. darlingi* oocysts reported by Smith & Frenkel (1977). Schizonts of *B. darlingi* (Dubey *et al*. 2002) and *B. oryctofelisi* were compared in H&E stained sections of intestines of experimentally infected cats.

*Serological examination*

Sera from 3 rabbits were examined using an indirect fluorescent antibody test (Table 5). Culture-derived tachyzoites of *B. darlingi* and *B. oryctofelisi* were spotted on slides and used as antigens. Sera from all 3 rabbits (Nos. 1, 4, and 7) were serially diluted 2-fold from 1:1000 in PBS and added to homologous and heterologous antigen spots. Pre-infection serum from rabbit No. 7 was used as the negative control serum at 1:100 and 1:200 dilutions. The slides were incubated for 45 min at 37 °C and washed twice in PBS. Fluorescein isothiocyanate labelled, goat anti-rabbit IgG (Kirkgaard and Perry, Gaithersburg, Maryland, USA) at 1:50 dilution was then added to the spots and the slides were incubated for 45 min at 37 °C. The slides were then washed as above and examined under a fluorescent microscope. The last dilution of serum producing visible peripheral fluorescence of the whole tachyzoites was considered end-titre.

*Comparison between* B. darlingi *and* B. oryctofelisi *by Western blotting*

Antigens were prepared from the *in vitro*-cultivated tachyzoites of *B. oryctofelisi*, *B. darlingi*, and host (CV-1) cells. The tachyzoites were harvested from culture supernatant and filtered through 5 $\mu$m polycarbonate membranes (Millipore Corporation, Bedford, Massachusetts, USA). Host cells were collected from a confluent monolayer, washed in PBS, and pelleted by centrifugation. The tachyzoite and cell pellets were subjected to 2 freeze-thaw cycles and sonicated at 20 amps for 3–5 sec pulses. Protein was extracted from the sonicate by the addition of reducing PAGE sample buffer (2 mM 2-mercaptoethanol, 1% SDS, 50% glycerol, pH 8·0) and submersion in boiling water for 2 min. The samples were centrifuged and the supernatant was recovered, and the protein content was estimated using a modified Bradford protein assay (BioRad, Hercules, California, USA) and same concentration of protein was used for all blots. Samples were electrophoresed on a 4–12% NuPage Bis-Tris gradient gel (Invitrogen, Carlsbad, California, USA) in 50 mM pH 8·0 MOPS buffer at 200 volts along with a marker. For Western blotting, samples were run as above with Benchmark pre-stained ladder (Invitrogen), electroblotted onto a Immobilon-P$^{sq}$ PVDF membrane (Millipore Corporation, Bedford, Massachusetts, USA), blocked in non-fat dry milk, and lanes were incubated overnight in antisera from rabbit No. 1 (*B. oryctofelisi* infected rabbit from Exp. 3), rabbit No. 7 (infected with *B. darlingi* from Exp. 7), or pre-inoculation serum of rabbit No. 7 as negative control serum. Horseradish peroxidase-conjugated goat anti-rabbit IgG was used

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as the second step antibody, and bands were visualized using 4-chloro-1-naphthol and peroxide as the substrate (Kirkegaard and Perry, Gaithersburg, Maryland, USA). The lanes were analysed and molecular weights were calculated using Labworks Image Acquisition and Analysis Software.

### *Random amplified polymorphic DNA-polymerase chain reaction* (*RAPD-PCR*)

The DNA from *B. darlingi* (Dubey *et al.* 2002) and *B. oryctofelisi* were analysed by RAPD-PCR. Tachyzoites were derived from culture in CV-1 cells and were purified by filtration through 5 $\mu$m membrane filters. DNA was extracted by digestion in DNAzol as per the manufacturer's instructions (MRC, Cincinnati, Ohio, USA) followed by alcohol precipitation.

The RAPD-PCR reactions were prepared in 25 $\mu$l volumes in 0·2 ml thin-walled PCR tubes. The reaction mixture contained 2 $\mu$l (20 ng) of template DNA, 2·5 $\mu$l of 10 × PCR buffer (Invitrogen) 0·75 $\mu$l (1·5 mM) of 50 mM $MgCl_2$, 0·2 $\mu$l (1 U) of *Taq* DNA polymerase (Invitrogen) 0·625 $\mu$l of 10 mM dNTP mixture (Invitrogen) and 0·5 $\mu$l of primer (100 pmol) and autoclaved distilled water to 25 $\mu$l. The sequences of the random primers used were GTG-ATCGCAG (AP7), CCGGTGTGGG (AP10), CGGACGTCGC (AP15), TCGTAGCCAA (AP17) and CTGAGACGGA (AP22). DNA from host (CV-1) cells was used as a control with each primer.

Cycling conditions included an initial denaturation at 94 °C for 5 min, followed by 45 cycles of 1 min denaturation at 94 °C, 45 sec annealing at 36 °C and 1 min elongation at 72 °C, followed by a final extension for 5 min at 72 °C. The appropriate volume of DNA electrophoresis loading buffer was added to each tube, and 15–18 $\mu$l of the product were run on 1·5% agarose gels in TAE running buffer containing ethidium bromide. Standard molecular size markers were run with the samples. Electrophoresis was performed at 5 volts/cm (90–100 V) and resulting bands were photographed using the ProExpress Imaging system (Perkin Elmer, Gaithersburg, Maryland, USA).

Comparative data from each isolate-primer combination were totalled and the similarity coefficient between the isolates was calculated using the formula: $F = 2N_{xy}/(N_x + N_y)$ where '$N_x$' and '$N_y$' are the numbers of DNA segments amplified in isolates '$x$' and '$y$', respectively, and $N_{xy}$ are the numbers of DNA segments shared by '$x$' and '$y$' (Nei & Li, 1979).

### *Molecular characterization of* B. oryctofelisi

*Gene sequencing*. DNA was extracted from *B. oryctofelisi* stages in cell culture by means of proteinase K digestion, ethanol precipitation, and purification using Qiagen DNAeasy columns according to the manufacturer's protocol. Aliquots, along with extraction negatives, were subsequently used as templates in polymerase chain reactions (PCR). The ITS-1 rDNA was amplified using primers 69 and 70 of Tanhauser *et al.* (1999), and the beta subunit of the plastid-encoded RNA polymerase (rpoB) was amplified with degenerate primers newly designed from available apicomplexan homologues (f1 5′-GCG GTC CCA AAG GGT CAG TGG ATA TGA TWT WTG AAG ATG C and r3 5′-GCG GTC CCA AAA GGG TCA GTC CTT TAT KTC CAT RTC T). Each of these primers contains a 5′ linker region, intended to normalize their annealing kinetics, adjacent to the 3′, gene-specific sequence. PCR products were directly sequenced in both directions using BigDye fluorescent chemistries and an ABI 377 automated sequencer, and chromatograms were edited using Sequencher software (Genecodes Corp., Ann Arbor, Michigan, USA).

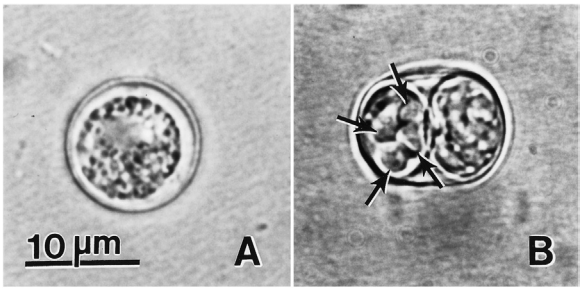


Fig. 1. Unstained oocysts of *Besnoitia oryctofelisi* from cat No. 709 fed tissues of infected gerbils. (A) Unsporulated; (B) sporulated. Note 4 sporozoites (arrows) in 1 sporocyst.

## RESULTS

### *Oocyst shedding in cats*

Most of the oocysts shed by the Argentinian cat did not sporulate, and only a few oocysts were shed by cats experimentally infected at Beltsville. Only a few sporulated oocysts were available, therefore, for experimentation. The oocysts from the Argentinian cat were structurally similar to the oocysts shed by cats at Beltsville. The description of oocysts is from faeces of experimentally infected cats at Beltsville. Oocysts were shed unsporulated in cat faeces (Fig. 1).

### *Infection in gerbils*

The gerbils fed 10 or more infective *B. oryctofelisi* oocysts became ill about 6 days p.i. and died within 3 weeks unless treated with sulfadiazine sodium when they became ill and until they were clinically normal. *B. oryctofelisi* tachyzoites initially caused necrosis of the cells of the lamina propria of small intestines

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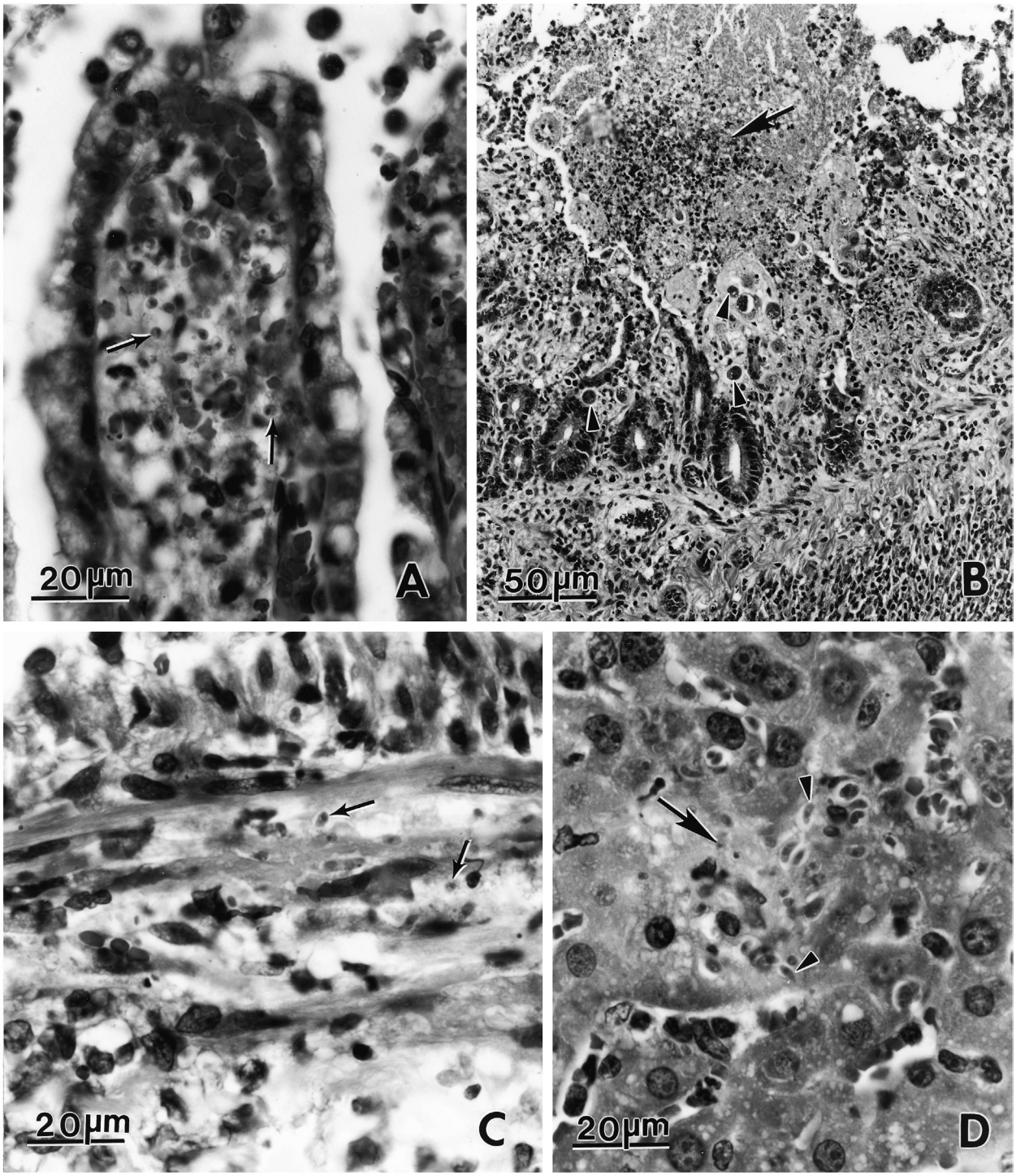


Fig. 2. (A–D) Lesions and parasites in rodents fed *Besnoitia oryctofelisi* oocysts. H&E stain. (A) Small intestinal villus of gerbil No. 4685, 8 days p.i. Note tachyzoites (arrows) destroying host cells in the lamina propria. Enterocytes in the surface epithelium are unaffected. (B) Necrosis and ulceration (arrow) of small intestine of gerbil No. 3995, 13 days p.i. *Besnoitia* stages (arrowheads) in the lesion. (C) Necrosis of the muscular layers of small intestine of KO mouse, 17 days p.i. Note tachyzoites (arrows) destroying host cells. (D) Liver of KO mouse 8 days p.i. Note focal necrosis (arrow) and tachyzoites (arrowheads).

(Fig. 2A, B). Tachyzoites multiplied in virtually all cells of the intestinal lamina propria but not in enterocytes (Fig. 2A). Infection extended from the lamina propria to the serosal layer of intestines. Extensive parasitization led to formation of ulcers (Fig. 2B), peritonitis, and death. At 4 days post-feeding oocysts (p.f.), all elements of mesenteric lymph nodes were severely parasitized with tachyzoites. In gerbils that died 7–13 days p.f., enteritis, mesenteric lymph node necrosis, and peritonitis

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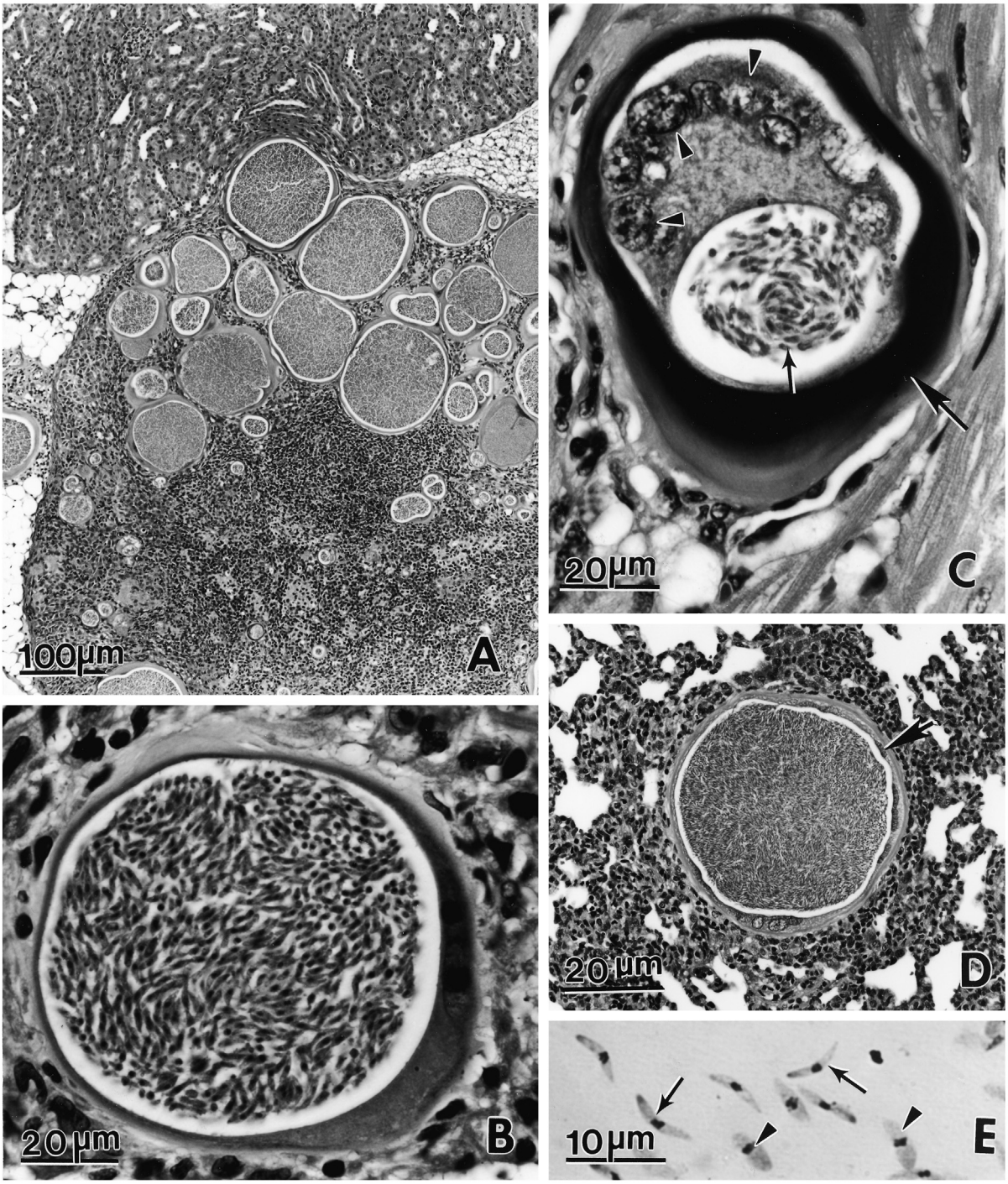


Fig. 3. (A–E) *Besnoitia oryctofelisi* tissue cysts and bradyzoites. H&E stain. (A) Section of adrenal gland of gerbil No. 3996, 49 days post-feeding (p.f.) oocysts. Note numerous tissue cysts. (B) A tissue cyst in section of heart of gerbil No. 3997, 49 days p.f. (C) Section of heart of SW mouse No. 755, 30 days p.f. Note host cell nuclei (arrowheads), bradyzoites in parasitophorous vacuole (small arrow) and dark staining tissue cyst wall (large arrow). (D) Section of lung of rabbit No. 1, 54 days p.i. Note a large tissue cyst (arrow). (E) Bradyzoites released from a tissue cyst from the fascia of rabbit No. 1, 54 days p.i. Slender (arrows) and plump (arrowheads) forms can be seen. Smear, methanol-fixed, Giemsa's stain.

were observed. Among the extra-intestinal organs, adrenal glands, hearts, lungs, skeletal muscles, livers, brains, and kidneys were parasitized in decreasing order of frequency and intensity as judged by microscopical examination of histological sections. Beginning the third week after feeding oocysts, tissue cysts were seen in the adrenal gland (Fig. 3A), intestines, heart (Fig. 3B), skeletal muscle, lungs, and the kidneys. In the heart, tissue cysts were in the pericardium, myocardium, endocardium and in the mitral valves. Most tissue cysts were unaccompanied by inflammation. Mononuclear cells infiltrated around a few intact tissue cysts and a few degenerating ones.

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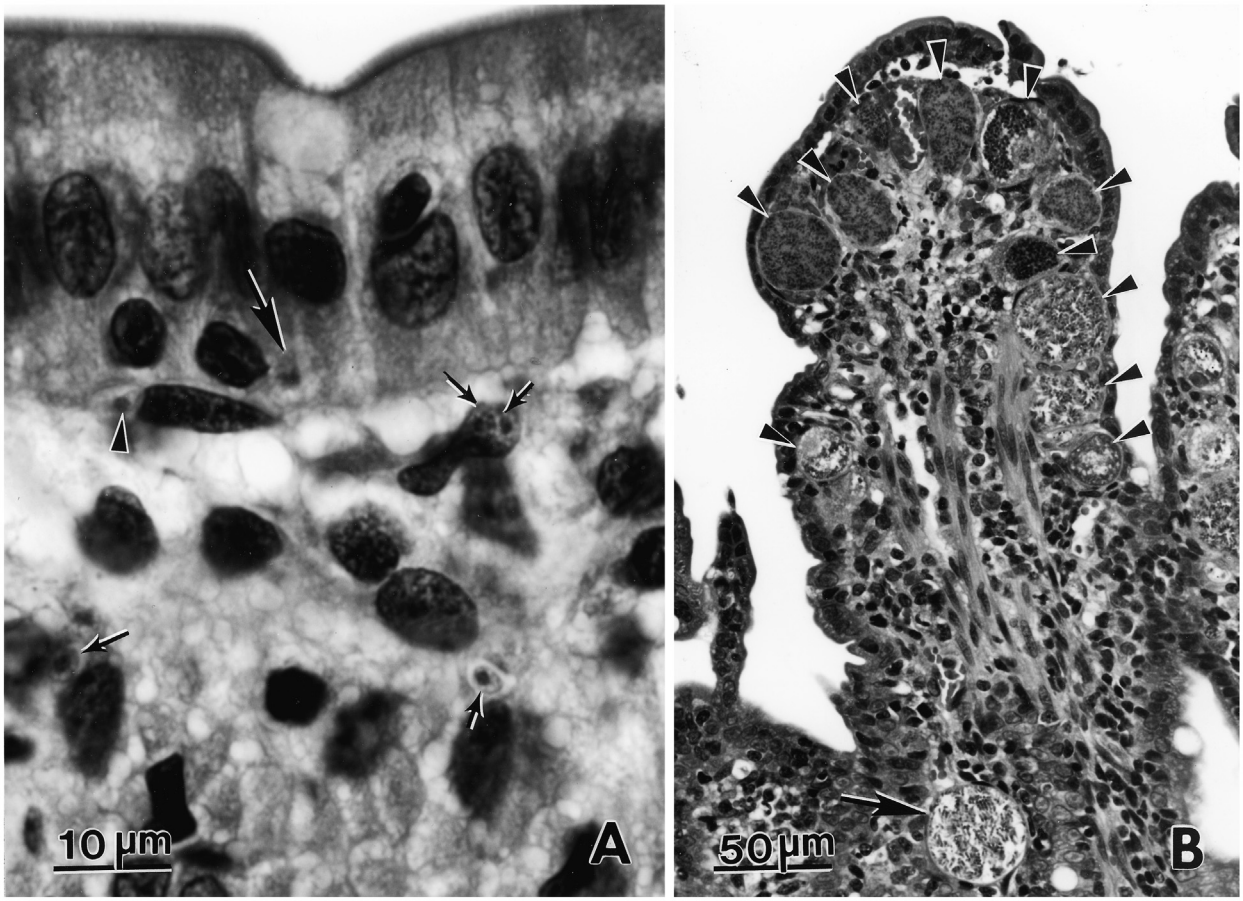


Fig. 4. (A–B) *Besnoitia oryctofelisi* stages in the jejunum of cats fed tissue cysts. H&E stain. (A) Individual zoites in an enterocyte (large arrow), just below the epithelium (arrowhead), and in the cells of the lamina propria (small arrows). Cat No. 689, 3 days p.i. (B) A heavily infected villus with 11 schizonts (arrowheads) located in the upper half and one located deeper (large arrow), in the intestine of cat No. 688, 12 days p.i.

The 5 gerbils (Nos. 3989–3993) inoculated s.c. with tachyzoites in Exp. 2 became infected and tissue cysts were found in their hearts, lungs, mesenteric lymph nodes and brains 42 days p.i.

The gerbils fed *Besnoitia* from the lungs of an experimentally infected gerbil (Exp. 6) remained asymptomatic and tissue cysts were found in their lungs when killed 37 or 73 days later. The cat (No. 709) fed tissues of 4 gerbils shed oocysts starting day 11 (Table 3).

### *Infection in outbred SW mice*

Feeding of *B. oryctofelisi* oocysts produced inconsistent results in SW mice. Initially, all 5 mice fed approximately 25 000 oocysts became ill and 4 died of acute besnoitiosis 7 to 9 days p.f. (not shown in Table 4). The fifth mouse survived and was asymptomatic when killed 30 days p.f. In total, 27 tissue cysts were found in the sections of the heart (Fig. 3C) and 1 in the skeletal muscle; all had thick cyst walls with an extra layer of dark staining material (Fig. 3C). Two tissue cysts were found in its brain and they were degenerating. Subsequently, all 20 SW mice fed oocysts (Table 4) remained asymptomatic; most infected mice had only solitary tissue cysts.

The 10 SW mice each fed approximately 10 000 oocysts remained asymptomatic and *Besnoitia* stages were not seen in the sections of tissues of mice killed 1, 3, 9, 14, 19, 25 or 28 days p.f. Tachyzoites were seen in the sections of mesenteric lymph nodes of mice killed 5, 7 or 11 days p.f. One tissue cyst was found in the unstained brain squash of the mouse killed 25 days p.f. but no parasites were seen in histological sections.

### *Infection in KO mice*

The KO mice fed infective oocysts became ill and died by 18 days p.f. (Table 4); oocysts in the last dilution, estimated to be fewer than 10, were not infective. The predominant lesions in KO mice were enteritis (Fig. 2C) mesenteric lymph node necrosis, and hepatitis (Fig. 2D). The 3 KO mice inoculated i.p. with tachyzoites developed besnoitiosis-associated peritonitis.

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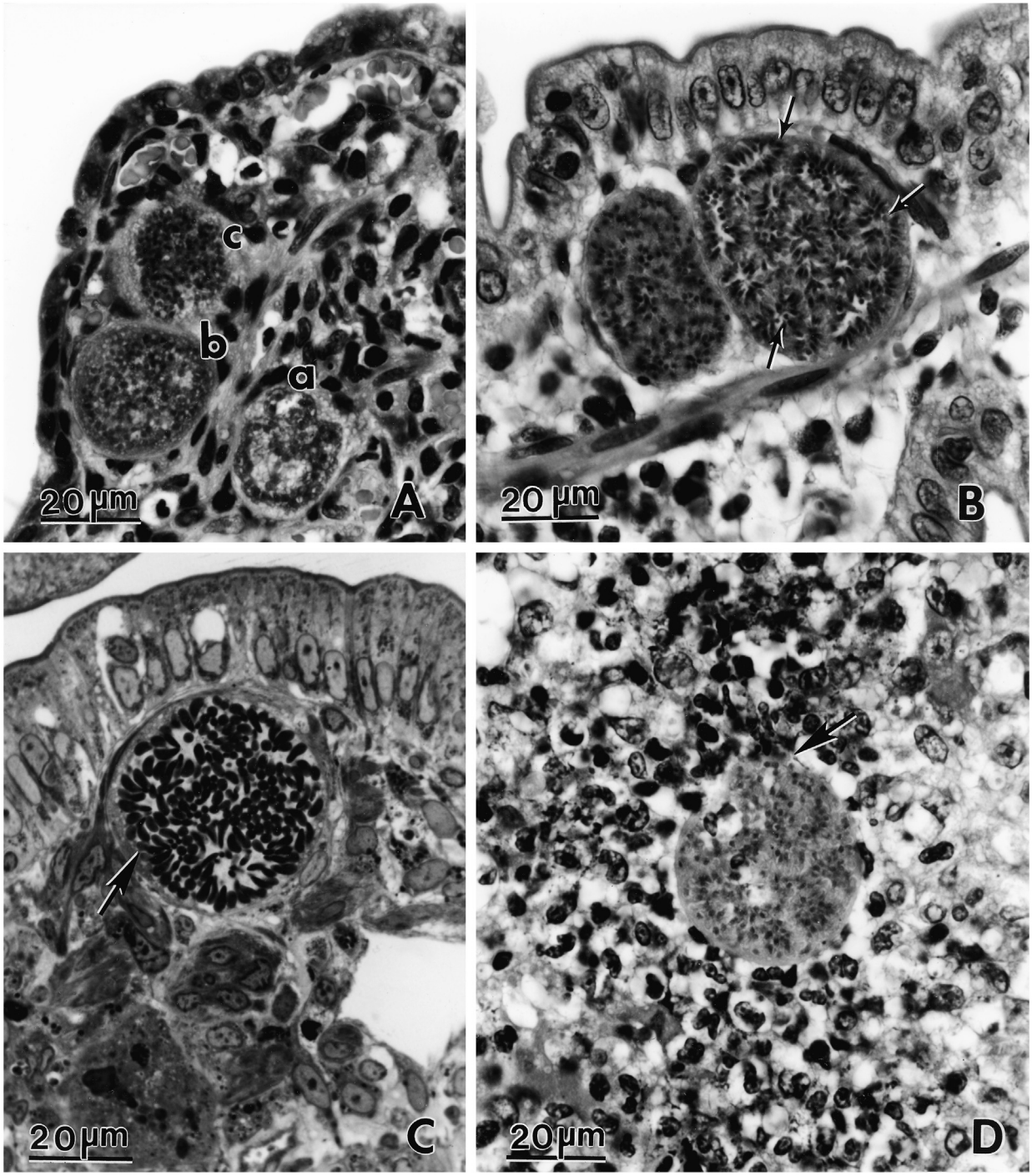


Fig. 5. (A–D) Schizonts in the lamina propria of jejunum of cats fed *Besnoitia oryctofelisi* tissue cysts. (A) Three schizonts (a–c) in order of their development. The nucleus in 'a' has not yet differentiated into individual nuclei. Cat No. 688, 12 days p.i. H&E stain. (B) Two schizonts. Merozoite formation has started in the schizont on the left. Merozoites have already formed in the schizont on the right and they are arranged in separate groups (arrows). Cat No. 688. H&E stain. (C) Mature schizont with merozoites (arrow). Cat No. 665, 12 days p.i. Toluidine blue stain. (D) Ruptured schizont (arrow) surrounded by inflammatory cells. Cat No. 688, 12 days p.i. H&E stain.

*Infection in rabbits*

The rabbit (No. 1) inoculated with culture-derived *B. oryctofelisi* and the 2 rabbits (Nos. 2, 3) fed oocysts of *B. oryctofelisi* remained healthy. Numerous small (<1 mm in diameter) tissue cysts were seen in the skeletal muscles and fascia between muscles of rabbit No. 1, 54 days p.i.; fewer tissue cysts were seen in tissue parenchyma than in the fascia. Among the tissues parasitized, more tissue cysts were found in adrenals and lungs (Fig. 3D) than in other tissues. Tissue cysts were barely visible to the naked eye in the 2 rabbits (Nos. 2 and 3) killed 34 or 37 days post-feeding oocysts.

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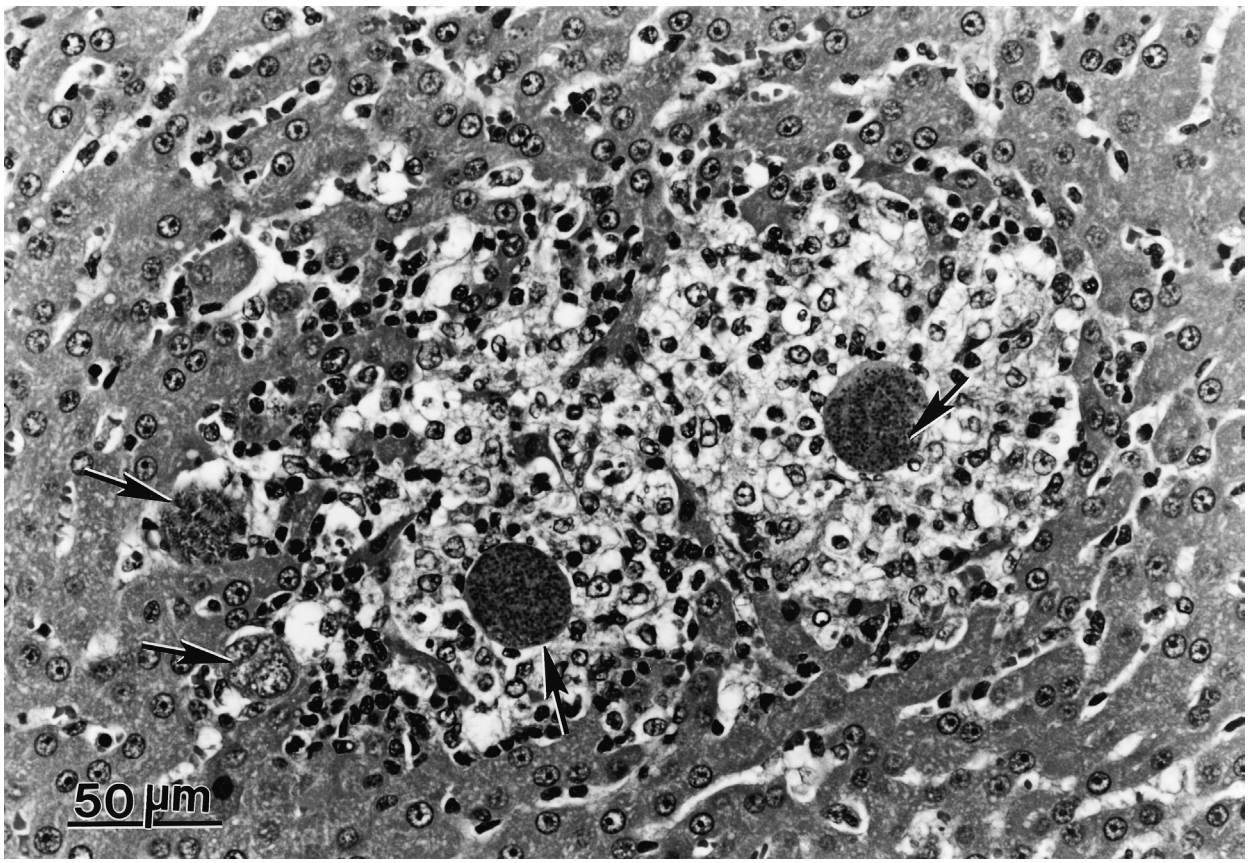


Fig. 6. Section of liver of cat No. 688, 12 days p.i. Note necrosis and infiltration with mixed leucocytes around 4 schizonts (arrows). H&E stain.

*Infection in cats*

Cats were asymptomatic for 7 days p.i. Beginning on the eighth day, 2 cats Nos. 687 and 688 (Table 3) appeared ill but were eating and defaecating. Individual zoites and immature and mature schizonts were seen in sections of intestinal and extra-intestinal organs (Figs 4–6). The host tissue around hepatic schizonts was necrotic and infiltrated with mixed leukocytes (Fig. 6).

*Cross-transmission experiments*

*Besnoitia* stages were not found in tissues of the 5 rabbits inoculated with *B. darlingi*; cats fed tissues of 2 of these rabbits did not shed oocysts. The inocula given to rabbits was infective for KO mice and gerbils. *Besnoitia* stages were not found in tissues of the 3 opossums fed *B. oryctofelisi*; the cats fed tissues of the opossums died the next day probably because of toxaemia.

*Growth in cell culture*

Parasites grew for several generations in bovine monocytes before they were transferred to CV-1 cells. By 24 h, the parasites had divided, often with simultaneous endodyogeny, forming rosettes of 8 tachyzoites (Fig. 7A,C, D). Occasionally tachyzoites multiplied in the nucleus of the host cell (Fig. 7B). By 72 h, more than 30 tachyzoites could be seen in a single vacuole. Spontaneous release of tachyzoites into the extracellular space without rupture of the host cell was noticed even from small rosettes containing only 8 tachyzoites. Extracellular forms were more crescentic and the nucleus appeared pyknotic suggesting degeneration (Fig. 7E). After 96 h, a few rosettes were found to have a central mass resembling a residual body (Fig. 7D). In general, parasites did not survive long extracellularly in cell cultures that contained healthy host cells. Cultures of bovine monocytes and CV-1 cells were destroyed by parasites by 3 weeks p.i. Tissue cysts were not identified.

*Serological examination*

Antibody titres in rabbits to homologous antigens were 2-fold higher than with heterologous antigens of *Besnoitia* (Table 5). Antibodies to *B. darlingi* and *B. oryctofelisi* were not found in 1:100 dilution of pre-inoculation serum of rabbit No. 7.

*Western blotting*

Protein profiles of the 2 *Besnoitia* species revealed numerous bands that appeared unique to *B. darlingi* and *B. oryctofelisi* which were not due to host cell

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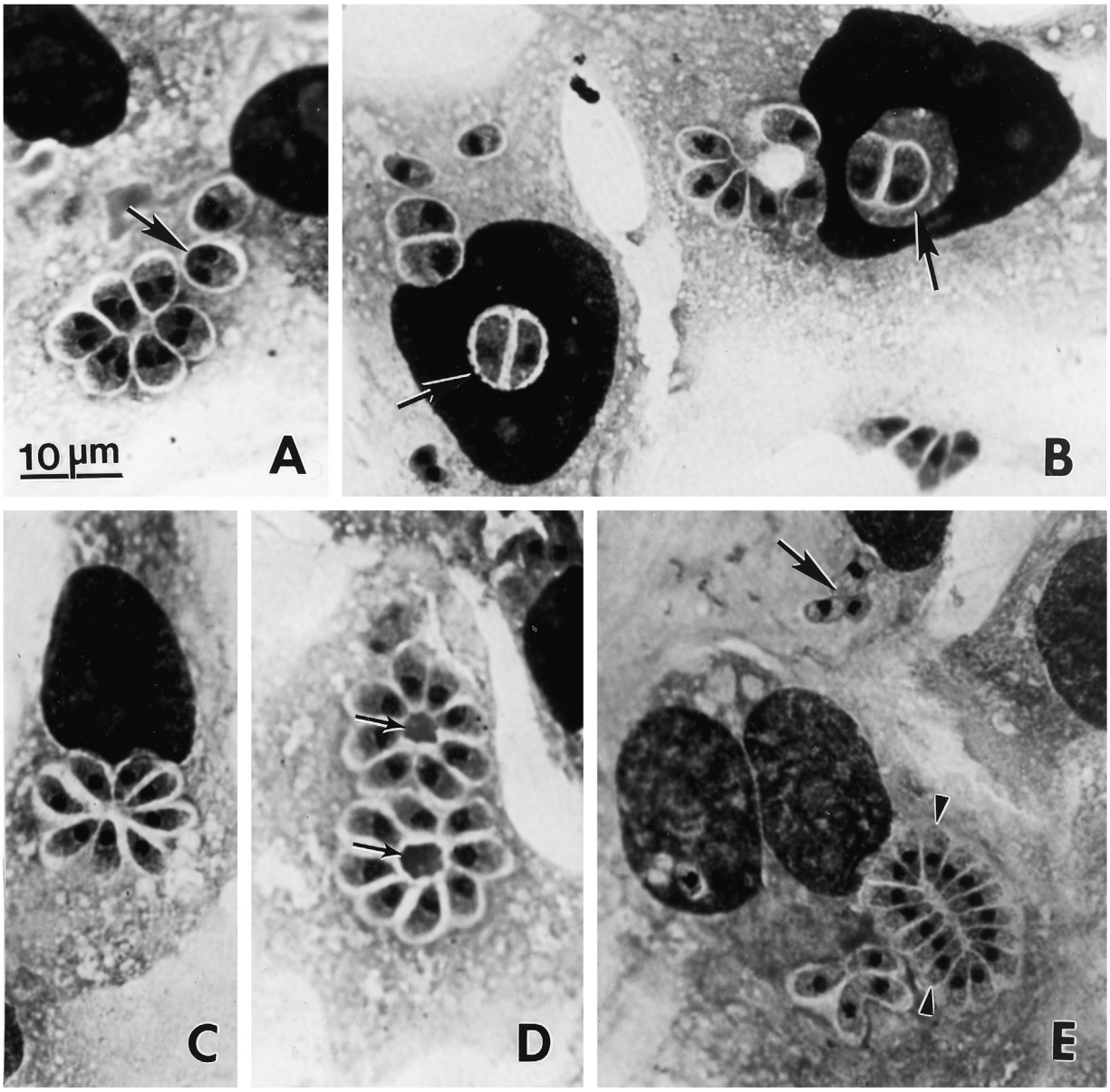


Fig. 7. (A–E) *Besnoitia oryctofelisi* tachyzoites in cell culture. Bouin's fixed, Giemsa's stain. Bar in (A) applies to all figures. (A) Tachyzoites in simultaneous endodyogeny (arrow). (B) Paired tachyzoites (arrows) in parasitophorous vacuoles in host cell nucleus. (C) Rosette of 8 tachyzoites. (D) Two rosettes of 8 organisms, each with a central mass resembling residual body (arrows). (E) Rosette of 16 organisms (arrowheads) and 3 extracellular organisms with pyknotic nuclei (arrow).

contamination (Fig. 8). When the lanes were probed with serum raised against *B. oryctofelisi*, 4 prominent common bands (94, 64, 40 and 30 kDa) were revealed. In addition to this, bands unique to *B. oryctofelisi* (76, 74, 48 and 46 kDa) and *B. darlingi* (75, 73, 49 and 45 kDa) were also seen (Fig. 9).

Rabbit serum against *B. darlingi* recognized more proteins than that of *B. oryctofelisi*. There were 2 more common bands (114 and 22 kDa) in addition to the four seen above. While bands in the molecular weight range of 203, 84, 78, and 29 kDa were unique to *B. darlingi*, 89 and 27 kDa bands distinguished *B. oryctofelisi* (Fig. 9). No bands of similar size were found to be stained in the control lanes containing protein extracted from CV-1 host cells, or with the negative control serum.

### *Molecular characterization*

All 5 random primers used in the RAPD analysis amplified fragments from *Besnoitia* DNA (Fig. 10) and were found to produce polymorphic fingerprint patterns differentiating the 2 *Besnoitia* species. The primers each produced a fingerprint pattern from the CV-1 host cell DNA which was distinct from the fingerprint produced using *B. darlingi* or *B. oryctofelisi* DNA as the target (data not shown). The similarity coefficient obtained using the combined

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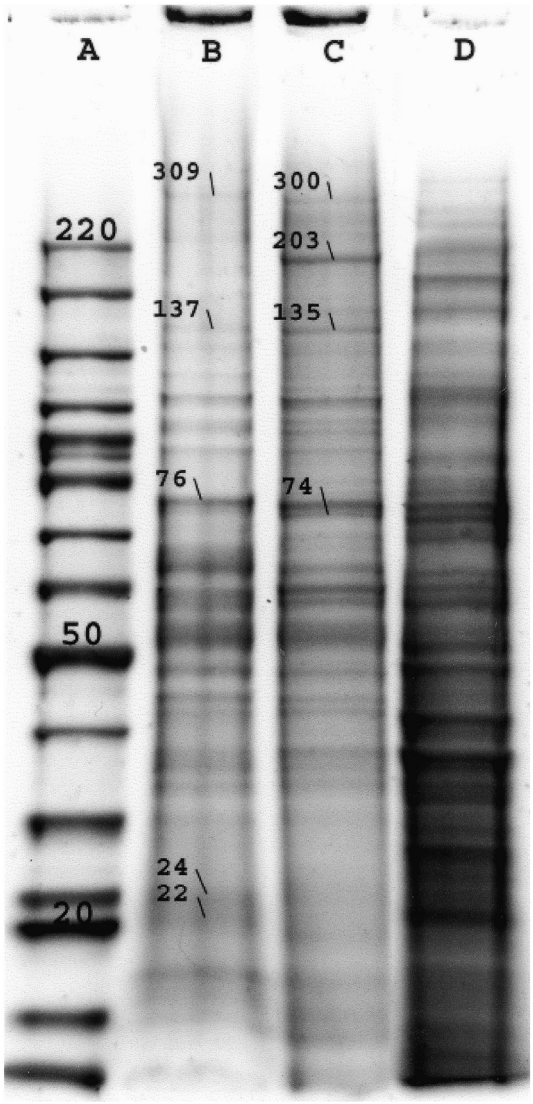


Fig. 8. Polyacrylamide gel electrophoresis of molecular weight markers (lane A), and extracted proteins from *Besnoitia oryctofelisi* (lane B), *B. darlingi* (lane C), and CV-1 host cell (lane D); protein bands visualized with Sypro Ruby stain and photographed with ProExpress proteomic imager. The approximate molecular weights of unique bands are indicated.

data from the 5 primers for *B. darlingi* and *B. oryctofelisi* was found to be 62%.

Each of 2 genetic loci were sequenced from *B. oryctofelisi* and compared to other available homologues. Three ITS-1 nucleotide substitutions were evident between *B. oryctofelisi* and *B. darlingi*. These 2 species, then, resemble each other more greatly at this locus than either does to the next most similar sequence presently available for comparison, that of *B. jellisoni*, from which they differ by over 4%. Positional homology of the *B. oryctofelisi* ITS-1 can be established with no other homologue presently available for comparison, including that of *B. besnoiti* (Ellis *et al.* 2000). A single nucleotide substitution differentiates the rpoB sequences of *B. oryctofelisi* from that representing *B. darlingi* (Dubey *et al.* 2002), but no other homologues from this genus are presently available for comparison. These sequences have been deposited to GenBank as accessions AY181998 (*B. darlingi* rpoB gene), AY181999 (*B. oryctofelisi* rpoB gene), and AY182000 (*B. oryctofelisi* ITS-1).

### *Description of* Besnoitia oryctofelisi *n. sp.*

(*Figs 1–7, Table 6*)

Tachyzoites and tissue cysts were present in intermediate hosts. Tachyzoites were lunate to tear drop shaped, $5{\cdot}3 \times 2{\cdot}0\ \mu$m in size (Figs 2 and 7). They divided into 2 by endodyogeny. Tissue cysts were up to 1 mm in diameter, glistening white and present in connective tissue and parenchymal cells. The tissue cyst wall enclosed host cell nuclei (Fig. 3C). Bradyzoites were slender to plump depending on the fixation and phase of their division (Fig. 3E). The nucleus was located towards the middle of the bradyzoite (Fig. 3E). Bradyzoites that were released from a tissue cyst from rabbit No. 1 were $9{\cdot}7 \times 1\ \mu$m ($9–10 \times 1{\cdot}0–1{\cdot}5$; $n=100$) in size; bradyzoites in tissue sections from the rabbit were $9{\cdot}7 \times 1{\cdot}2\ \mu$m ($9–10 \times 1{\cdot}0–2{\cdot}0$; $n=100$) in size.

Schizonts and oocysts were present in the definitive host, cats. Schizonts were present in intestinal lamina propria and in extra-intestinal tissues of cat (Figs 4–6). The jejunum was the most heavily parasitized region. Individual zoites, seen at 3 and 4 day p.i., were small (2–3 $\mu$m long), often globular, and had a vesicular nucleus (Fig. 4A). Most schizonts were located towards the villar tips but some were also seen in the deeper parts of the villus (Fig. 4B). The schizonts appeared to be in the capillary endothelium. The host cell nucleus was sometimes hypertrophied and indented (Fig. 5). Some schizonts ruptured in the lamina propria, leading to local infiltration by neutrophils around the free merozoites (Fig. 5D). Schizonts and merozoites in cats were PAS-negative. Schizonts varied in size; the largest schizont measured $52 \times 45\ \mu$m (Fig. 5B), and contained many distinct nuclei (Fig. 5A). Merozoites were approximately $5 \times 1\ \mu$m. Merozoites of some intact schizonts appeared slightly larger in size than others but they appeared to belong to one generation. Merozoites were arranged in distinct groups within a schizont (Fig. 5B). Gamonts were not seen.

Unsporulated oocysts were $11{\cdot}7 \times 11{\cdot}5\ \mu$m ($10–13 \times 10–13\ \mu$m; $n=50$) in size with a length–width ratio of 1:1·02 (Fig. 1A). Micropyle and polar granules were absent. Sporulated oocysts were $12{\cdot}2 \times 11{\cdot}3\ \mu$m ($11–14 \times 10–13\ \mu$m; $n=67$) in size with a length–width ratio of 1:1·08 (Fig. 1B). Each oocyst contained 2 sporocysts that were $8{\cdot}7 \times 5{\cdot}7\ \mu$m ($8–9 \times 5–7\ \mu$m; $n=54$) in size with a length–width ratio of 1:1·53. Each sporocyst contained 4 sporozoites (Fig. 1B) and a dispersed or compact sporocyst residuum. Stieda body and oocyst residuum were absent.

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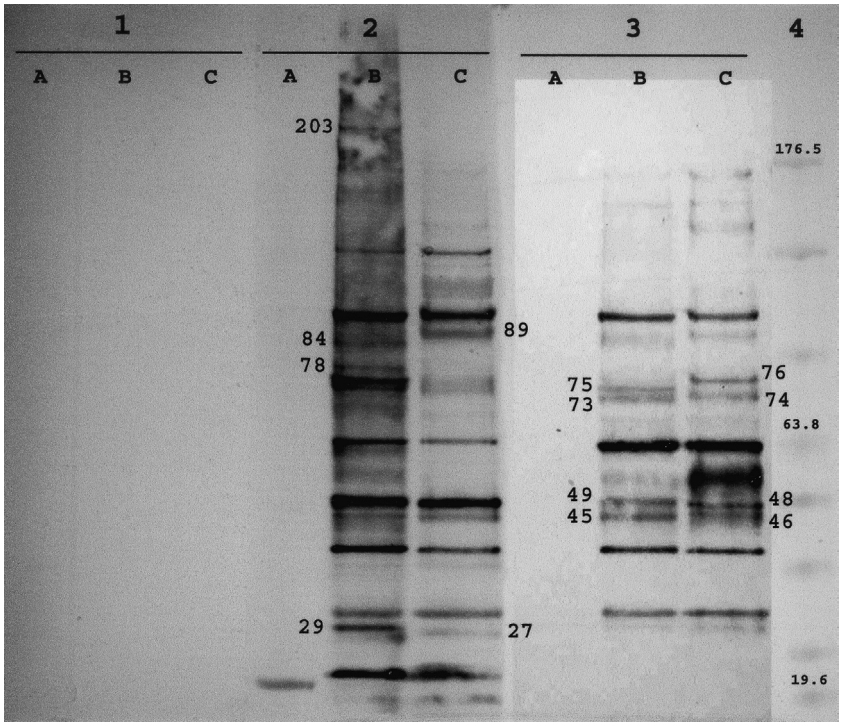


Fig. 9. Western blot of *Besnoitia* proteins; lane A, CV-1 host cells; lane B, *B. darlingi*; lane C, *B. oryctofelisi* exposed to panel 1 is negative control serum (rabbit No. 7 pre-inoculation serum); panel 2 is anti-*B. darlingi* serum (rabbit No. 7 p.i. serum); panel 3 is anti-*B. oryctofelisi* serum (rabbit No. 1 p.i. serum). Panel 4 is pre-stained molecular weight marker.

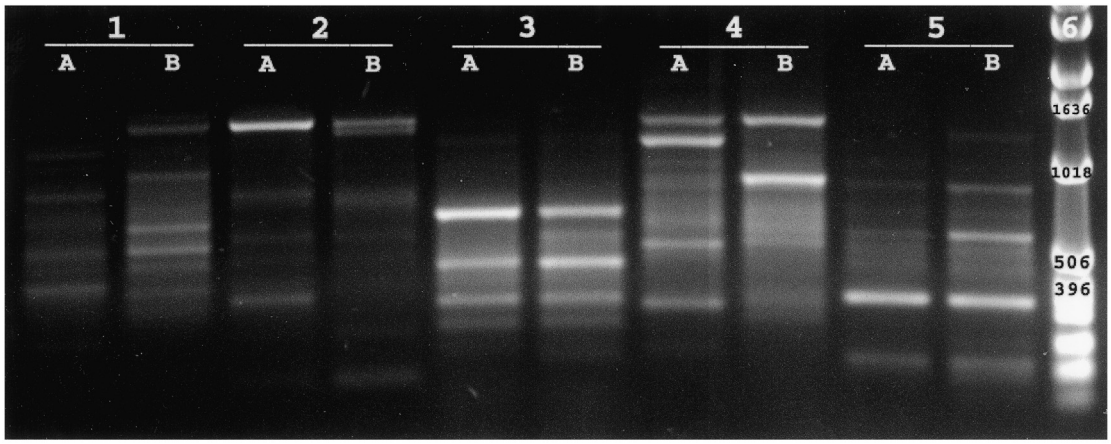


Fig. 10. RAPD-PCR fingerprint patterns of *Besnoitia darlingi* (A) and *B. oryctofelisi* (B) using primer AP22 (1), AP17 (2), AP15 (3), AP10 (4) and AP7 (5). Lane 6-1 Kb DNA marker.

TAXONOMIC SUMMARY

*Intermediate type host*: Domestic rabbit (*Oryctolagus cuniculus*)
*Definitive host*: Cat (*Felis domesticus*)
*Locality*: Argentina
*Specimens (hapantotypes) were deposited in the United States National Parasite Collection (USNPC), United States Department of Agriculture, Beltsville, Maryland, USA*
(1) Histological sections of tissue cysts stained with H&E in uterus of a naturally-infected rabbit (No. 92547) and (2) an experimentally infected rabbit (No. 92548). (3) Schizonts in small intestine of an experimentally infected cat (No. 92549). (4) Tachyzoites in small intestines of an experimentally infected gerbil (No. 92550). (5) Tachyzoites in cell culture preparations of CV-1 cells stained with Giemsa's stain (No. 92551). (6) Sporulated oocysts from cat 688 fixed in 10% formalin (No. 92552). (7) Phototypes of unsporulated and sporulated oocysts as shown in Fig. 1 (No. 92553).

Live cultures of tachyzoites of *B. oryctofelisi* were deposited in the American Type Culture Collection (ATCC), Manassas, Virginia 20108, USA, ATCC Accession No. 50999).

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Table 6. Comparison of *Besnoitia darlingi* and *B. oryctofelisi*

| Character | *B. darlingi** | *B. oryctofelisi*§ |
|---|---|---|
| Distribution | United States† | Argentina |
| Intermediate host | | |
| Natural | Opossum (*D. virginiana*)† | Rabbit |
| Experimental | Mice, gerbils, not rabbits†‡ | Mice, gerbils, not opossums |
| Tachyzoites (μm) (uninucleate in CV-1 cells $n=75$) | 5·4 × 2·0 (5–6 × 2·0)§ | 5·3 × 2·0 (5–6 × 2·0) |
| Bradyzoites (μm) (longitudinally cut in tissue sections, $n=100$) | 10·2 × 1·0 (10–11 × 1·0–1·2)§ | 9·7 × 2·0 (9–10 × 1–2) |
| Definitive host | | |
| Natural | Unknown | Unknown |
| Experimental | *Felis domesticus*† | *Felis domesticus* |
| Intestinal schizonts | Up to 55 μm long‡ | Up to 52 μm long |
| Merozoites (μm) | ~5 × 1·0‡ | 5 × 1·0 |
| Extra-intestinal schizonts | Liver, mesenteric lymph nodes‡§ | Many tissues |
| Oocysts (μm) | | |
| Unsporulated | 11·9 × 12·3 (10·8–12·8 × 11·2–12·8, $n=25$)† | 11·7 × 11·5 |
| Sporulated | 10·3 × 12·0 (9·6–11·2 × 11·2–12·8, $n=20$)† | 12·2 × 11·3 |
| Western blot | | |
| Unique bands (kDa) | | |
| With *B. oryctofelisi* serum | 75, 73, 49, 45§ | 76, 74, 48, 46 |
| With *B. darlingi* serum | 203, 84, 78, 29§ | 89, 27 |
| Molecular | | |
| GenBank Accession No. | | |
| rpoB gene | AY181998§ | AY19999 |
| ITS-1 | AF489696‡, AF489697‡ | AY182000 |

* From the North American opossum, *D. virginiana*.
† Smith & Frenkel (1977).
‡ Dubey *et al.* (2002).
§ Present study.

## DISCUSSION

*B. oryctofelisi* proposed in the present study most closely resembles *B. darlingi* from the North American opossum, *D. virginiana* (Table 6). *B. darlingi* was initially observed and named for the parasite in the Central and South American opossum, *D. marsupialis* and later thought to occur in other hosts in Americas (Darling, 1910; Frenkel, 1977). However, it is uncertain if *B. darlingi* from *D. marsupialis* from Central and South America is the same as the parasite in *D. virginiana* from North America. Paperna & Lainson (2001) did not find oocysts in cats fed the *B. darlingi*-like parasite from lizards from Brazil, and *B. darlingi*-like parasite from lizards from Panama was not transmissible to rabbits (Schneider, 1965). In the present study, *B. oryctofelisi* was not transmissible to *D. virginiana* and *B. darlingi* from *D. virginiana* was not transmissible to rabbits.

In the present study, polymorphism was appreciable from the RAPD profiles as illustrated by variation in amplicon size from each of the *Besnoitia* samples. These variations may have resulted from deletions, mutations, or insertions of DNA sequences. The RAPD data show that all 5 informative primers produced fingerprints that clearly differentiated *B. oryctofelisi* from *B. darlingi*.

Limited genetic variation in the present study provides a molecular basis to differentiate *B. oryctofelisi* from *B. darlingi*. Whether these sequence differences diagnose fixed, interspecific differences cannot be known until a wide sampling of parasites from these and other hosts is available. The reported sequence differences should serve, then, as a basis by which to test whether such small amounts of accumulated genetic change, such as those observed here, have accrued among parasite taxa that differ markedly in their natural and experimental host range. A reasonable alternative hypothesis would instead hold that the genetic variation we observe here represents allelic polymorphism that does not strictly correlate with parasite phenotypes such as host range. Ellis *et al.* (2000) compared sequences of the ITS-1 locus from *B. besnoiti* of cattle, *B. jellisoni* of rodents, and *B. caprae* of goats, and found *B. jellisoni* to be distinct from *B. besnoiti* but *B. caprae* identical to *B. besnoiti*.

*B. oryctofelisi* shares some antigens and proteins with *B. darlingi*. Western blotting showed that while many bands were shared between the two, they could be differentiated by specific bands with both anti-*B. darlingi* and anti-*B. oryctofelisi* sera. In the indirect fluorescent antibody test, antibody titre to homologous antigens was higher than that with

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heterologous antigens. Finding of antibodies to *Besnoitia* in rabbit No. 4 fed *B. darlingi* oocysts indicates that sporozoites penetrated rabbit tissues providing antigenic stimulus although the rabbit did not develop a patent infection.

In conclusion, 2 morphologically similar parasites differing in their experimental host range may also be distinguished by genetic and antigenic criteria. We advocate application of these diagnostic means to recognize 2 distinct, but closely related parasite species, *B. darlingi* and *B. oryctofelisi*. Examining the attributes of more such material will further clarify the diversity and relationships among such parasites, and is particularly needed in the case of the *Besnoitia* sp. initially named from *Didelphis marsupialis*.

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