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Rapid communication

Redescription of *Neospora caninum* and its differentiation from related coccidia

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Abstract

Neospora caninum is a protozoan parasite of animals, which before 1984 was misidentified as *Toxoplasma gondii*. Infection by this parasite is a major cause of abortion in cattle and causes paralysis in dogs. Since the original description of *N. caninum* in 1988, considerable progress has been made in the understanding of its life cycle, biology, genetics and diagnosis. In this article, the authors redescribe the parasite, distinguish it from related coccidia, and provide accession numbers to its type specimens deposited in museums. Published by Elsevier Science Ltd. on behalf of Australian Society for Parasitology Inc.

Keywords: *Neospora caninum*; Redescription; Tachyzoites; Tissue cysts; Oocysts; Life cycle; *Hammondia heydorni*; *Toxoplasma gondii*

1. Introduction

Neospora caninum Dubey, Carpenter, Speer, Topper and Uggla, 1988 was proposed for a protozoan found associated

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with a severe neuromuscular disease in dogs (Dubey et al., 1988a). Historically, Bjerkås et al. (1984) reported a protozoan (Fig. 1A, B) causing severe encephalomyelitis in seven Norwegian dogs that had no antibodies to *Toxoplasma gondii*; the dogs originated from three litters from a single bitch. Ultrastructurally, the organism was different from *T. gondii* (Bjerkås and Presthus, 1988). Dubey et al. (1988a) found a similar parasite (Fig. 1C, D, E) in formalin-fixed tissues from 10 dogs in the USA and named it *N. caninum*. These animals had died several months earlier from what was considered to be toxoplasmosis and no frozen or live materials were available for further studies. Three characters based on the information available separated *N. caninum* from *T. gondii* and related coccidia. First, the clinical entity with paralysis, particularly of the hind limbs, was a syndrome not observed in other species diagnosed to have toxoplasmosis. Secondly, tissue cysts of this parasite appeared morphologically distinct. Thirdly, antibodies to *T. gondii* were not present in these dogs and the parasites did not react to *T. gondii* antibodies in immunohistochemical tests. The *N. caninum* tissue cysts (Fig. 1A) in dogs reported by Bjerkås et al. (1984) and in the original description of the parasite (Fig. 1C) by Dubey et al. (1988a) had walls 1–4 μm thick and were restricted to neural tissues. *Toxoplasma gondii* tissue cysts (Fig. 1G) have thinner walls (<0.5 μm) and may be found in many organs. A new genus, *Neospora* was proposed to accommodate the species *N. caninum* because it was considered that the parasite did not fit into existing genera.

Soon after the description of *N. caninum*, a *N. caninum*-like parasite (Fig. 2) was isolated in cell culture (Dubey et al., 1988b) inoculated with tissues from puppies in a litter of congenitally infected dogs suffering from identical clinical syndrome as originally described by Bjerkås et al. (1984); thick-walled tissue cysts were found in these dogs (Fig. 1F). At the time of inoculation of cell cultures, Swiss–Webster immunocompetent laboratory mice had been inoculated with neural tissues of these dogs, and the subsequent finding of thick-walled tissue cysts in their brains (Fig. 3A) made it possible to link the newly isolated parasite in cell culture to the parasite described from the fixed material. A serological test (Dubey et al., 1988b) and an immunohistochemical method (Lindsay and Dubey, 1989a) were developed that further distinguished *N. caninum* from *T. gondii*. A morphological and immunohistochemical comparison of the parasites in fixed material from the dogs in the report of Bjerkås et al. (1984) and Dubey et al. (1988a) demonstrated that the parasites in these reports were indistinguishable from each other (Bjerkås and Dubey, 1991). The sexual phase of *N. caninum* was not discovered until recently when McAllister et al. (1998) demonstrated *N. caninum* oocysts in faeces from dogs fed *N. caninum* thick-walled tissue cysts from experimentally infected mice. *Neospora caninum* was subsequently transmitted experimentally to cattle, sheep, goats, pigs, dogs, cats, and rodents, and *N. caninum*-like tissue cysts were found naturally in cattle, sheep, goats, deer, and horses (Figs. 3–5).

Recently, concerns have been raised about whether *N. caninum* is a separate species, a strain of *T. gondii*, or another parasite previously called *Isospora bigemina* because of the morphological similarities of their oocysts (Mehlhorn and Heydorn, 2000; Heydorn and Mehlhorn, 2002). These authors suggested that the original description of *N. caninum* was incomplete and that no specimens had been deposited in a museum (Heydorn and Mehlhorn, 2002) as now required by the International Code of Zoological Nomenclature.

The objective of this article is to redescribe *N. caninum* and to report the deposition of specimens in museums. We also review available information concerning *I. bigemina* and *Hammondia heydorni* and differentiate *N. caninum* from related coccidia.

2. Redescription of *N. caninum*

Details of its morphology were given by Dubey et al. (1988a), Dubey and Lindsay (1996), Speer et al. (1999), and Lindsay et al. (1999b). The tachyzoites of *N. caninum* are ovoid, lunate or globular and measure 3–7 × 1–5 μm, depending on the stage of division; undividing tachyzoites are approximately 7 × 2 μm. They may be present in many cell types and individual infected host cells may each contain many tachyzoites (Fig. 1D). They are located within a parasitophorous vacuole in the host cell cytoplasm and each tachyzoite may contain 6–16 rhoptries (Figs. 2 and 4) with electron-dense contents, some extending posterior to the nucleus. Tachyzoites contain most organelles that are found in coccidian merozoites although micropores are difficult to detect (Fig. 4B). The tissue cysts of *N. caninum* are found primarily in neural tissues (Fig. 1A, C). The cyst wall is up to 4 μm thick, often with a wavy contour in tissue sections, but without protrusions (Figs. 3A–C, 5A, B). Septa and a secondary tissue cyst wall are absent. The bradyzoites located within the tissue cysts are elongate with a subterminal nucleus and measure approximately 8 × 2 μm. Bradyzoites contain the organelles typically found in other coccidian zoites including large and small dense granules, rhoptries, and micronemes, the latter often arranged perpendicularly to the plasmalemma. Rhoptries in bradyzoites are 6–12 in number with electron-dense contents. Oocysts of *N. caninum* are 11.7 × 11.3 μm (10.6–12.4 × 10.6–12.0) with a length–width ratio of 1.04. Oocyst wall, which is colourless, and 0.6–0.8 μm thick encompasses two sporocysts each 8.4 × 6.1 μm (7.4–9.4 × 5.6–6.4) with a length–width ratio of 1.37. Each sporocyst contains four sporozoites and a residuum. Sporozoites are elongate, 6.5 × 2.0 μm (5.8–7.0 × 1.8–2.2). The entero-epithelial stages in the dog intestines are currently unidentified.

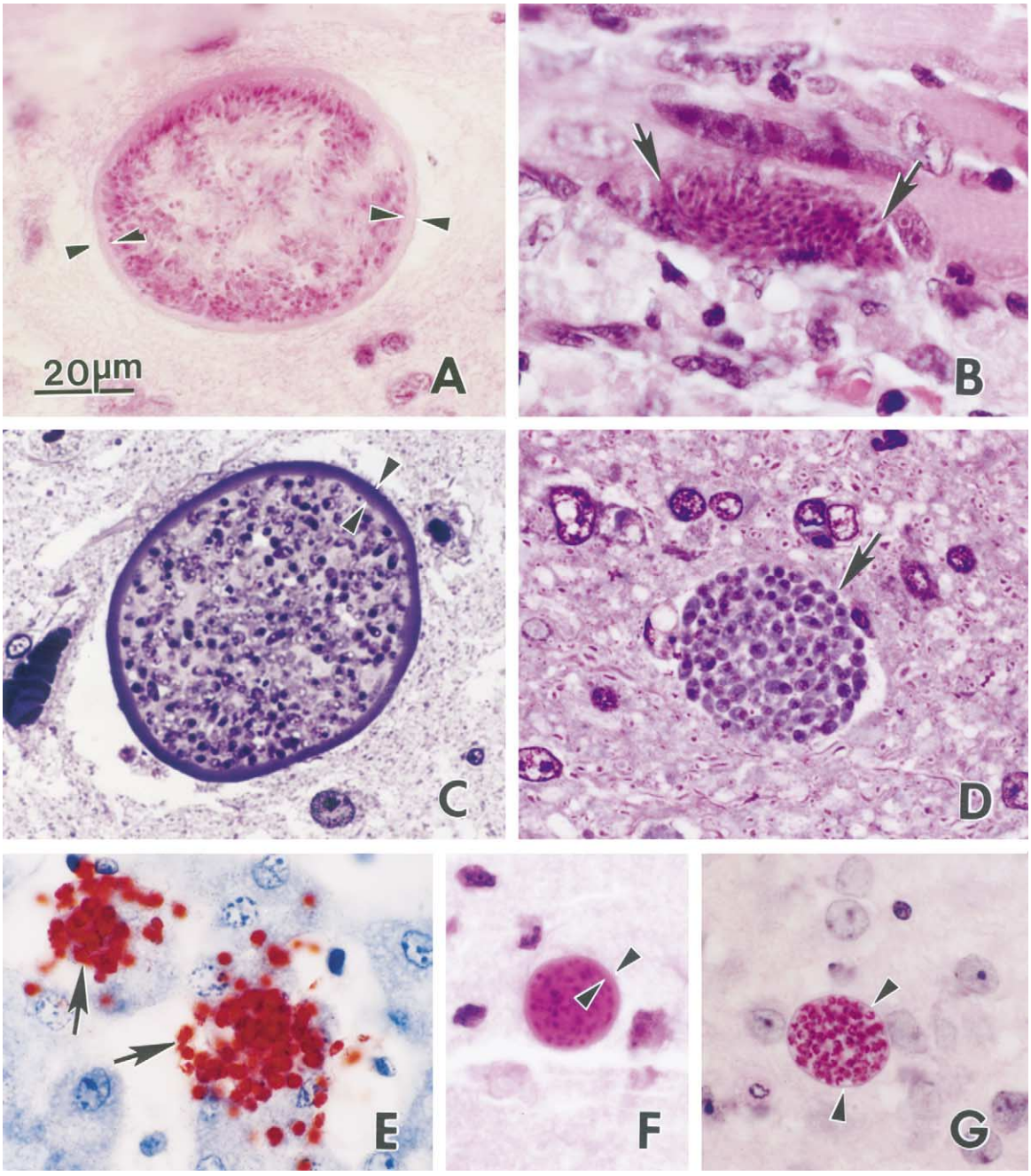

Fig. 1. *Neospora caninum* (A–F) tachyzoites and tissue cysts in histologic sections of tissues of naturally infected dogs compared with tissue cysts of *Toxoplasma gondii* (G). The thickness of the tissue cyst wall is indicated by arrowheads. Bar applies to all parts. (A) Tissue cyst from the cerebellum of a dog from Bjerkås et al. (1984). H&E. (B) A large group of tachyzoites (arrows) in skeletal muscle of the same dog as in Fig. 1A. H&E. Note absence of a cyst wall. (C) Tissue cyst from the brain of dog No. 6 of Dubey et al. (1988a). Toluidine blue stain. (D) A group of tachyzoites (arrow) in the brain of dog No. 7 of Dubey et al. (1988a). Note absence of a cyst wall. Giemsa stain. (E) Tachyzoites (arrows) from the liver of dog No. 6 of Dubey et al. (1988a) stained with polyclonal antibodies to *N. caninum*. (F) Tissue cyst in the brain of a dog from Dubey et al. (1988b). The NC-1 strain of *N. caninum* was isolated from this dog. H&E. (G) Tissue cyst in the brain of an experimentally infected mouse, 3 months p.i. Periodic acid Schiff (PAS) reaction counter stained with haematoxylin. PAS-positive bradyzoites are enclosed in a thin PAS-negative cyst wall.

3. Designation of hosts and deposition of materials

Because in 1988 the only known host for *N. caninum* was the dog, type host and locality were not defined. Because the entire type series were from the dogs the type host for *N. caninum* is *Canis familiaris*. The dog is presently the only definitive host known for *N. caninum*. Intermediate hosts for *N. caninum* are now known to be cattle (*Bos taurus*), dogs, sheep (*Ovis aries*), and probably other warm-blooded animals. The type locality is therefore, north-eastern U.S. to include the collection areas of hosts of the type series of *N. caninum*.

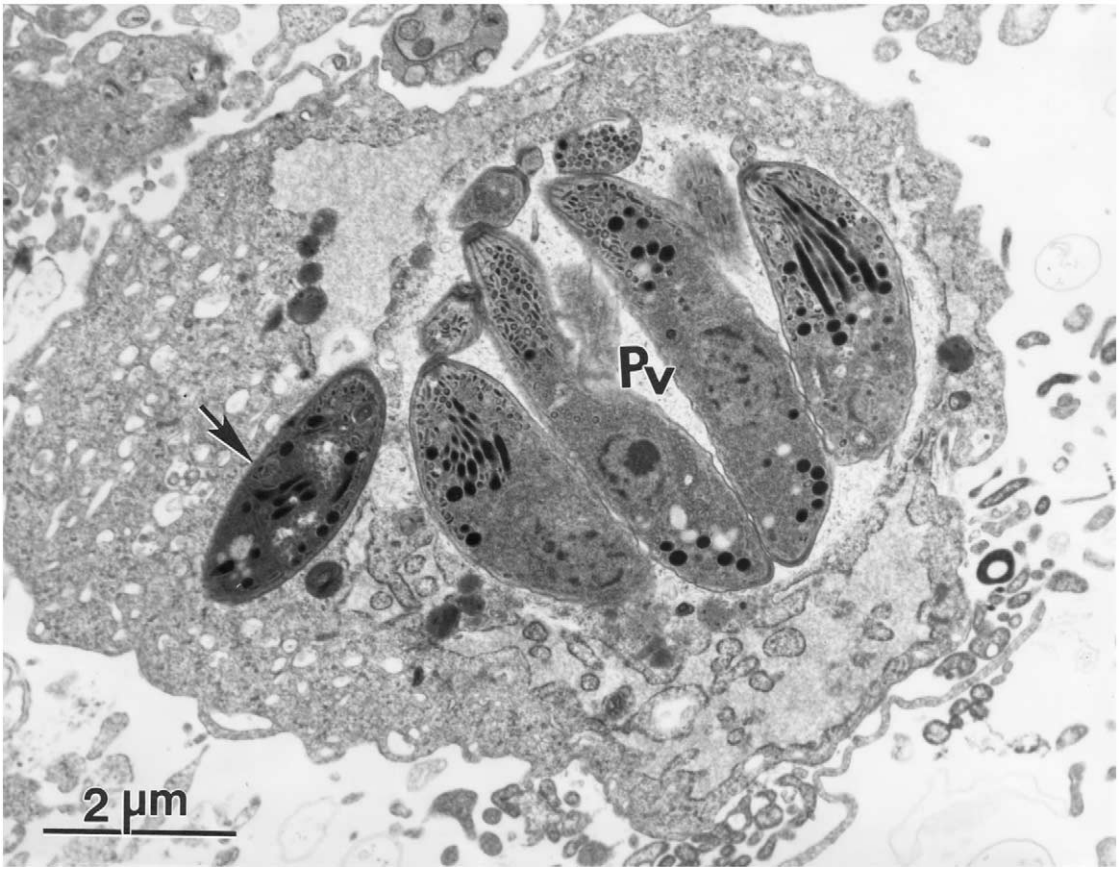

Fig. 2. TEM of tachyzoites in HS68 cells in culture, 2 days after inoculation with the NC-2 isolate (Hay et al., 1990) of *N. caninum*. Note a group of tachyzoites in a parasitophorous vacuole (Pv). One tachyzoite (arrow) is located in the host cell cytoplasm without any visible surrounding parasitophorous vacuole.

Both the asexual and the sexual phases of the life cycle can be completed in the dog. Hosts may become infected by ingestion of oocysts and/or by the ingestion of infected tissues. The parasite is transmitted transplacentally in some animals. Tachyzoites can be maintained indefinitely in cell culture (Dubey et al., 2002).

As stated earlier, *N. caninum* was named for the parasites found in 10 dogs that had been necropsied at the Angel Memorial Animal Hospital (AMAH), Boston, USA. After publication of the article in 1988, formalin-preserved tissues, all paraffin blocks, and most stained slides were deposited in the Department of Pathology, AMAH (Dubey et al., 1988a). However, this material was recently discarded by the staff of AMAH without our knowledge. Materials deposited in the present study include 2 slides of tachyzoites and preserved brain tissue from those specimens of the type series (syntype) that were retained at the laboratory of J.P.D.

3.1. Live parasite culture deposited

The NC-1 strain of *N. caninum* isolated from dogs by Dubey et al. (1988b) has been deposited in American Type Culture Collection (ATCC), Manassas, VA 20108, USA (ATCC Accession No. 50977).

3.2. Tissue sections deposited

Histological sections of tissues have been deposited in the United States National Parasite Collection (USNPC), Beltsville, MD 20705, USA under USNPC No. 088804.00. (1) Tissue cysts in the brain of a dog from Bjerkås et al. (1984). Accession No. 92224 (Fig. 1A). (2) Tachyzoites in the musculature from the same dog as in Fig. 1A, Accession No. 92224 (Fig. 1B). (3) Tachyzoites in the brain of dog No. 7 (syntype) from Dubey et al. (1988a). Accession No. 92225 (Fig. 1D). (4) Tachyzoites in the liver of dog No. 6 (syntype) from Dubey et al. (1988a) labelled by an immunochemical method using anti-*N. caninum* antibodies. USNPC Accession No. 92226 (Fig. 1E). (5) Tissue cyst in the brain of a dog from Dubey et al. (1988b); NC-1 strain of *N. caninum* was isolated from this dog. Accession No. 92227 (Fig. 1F). (6) A tissue cyst in the brain of a cat inoculated with tachyzoites of the NC-1 strain of *N. caninum*, 55 days p.i. (Dubey et al., 1990a). Accession No. 92228 (Fig. 5A). (7) A tissue cyst in the brain of a naturally infected deer (Dubey et al., 1996). Accession No. 92229 (Fig. 5D). (8) A tissue cyst in the spinal cord of a naturally infected calf (Dubey et al., 1989a). Accession No. 92230 (Fig. 5E). (9) A tissue cyst in the spinal cord of the natu-

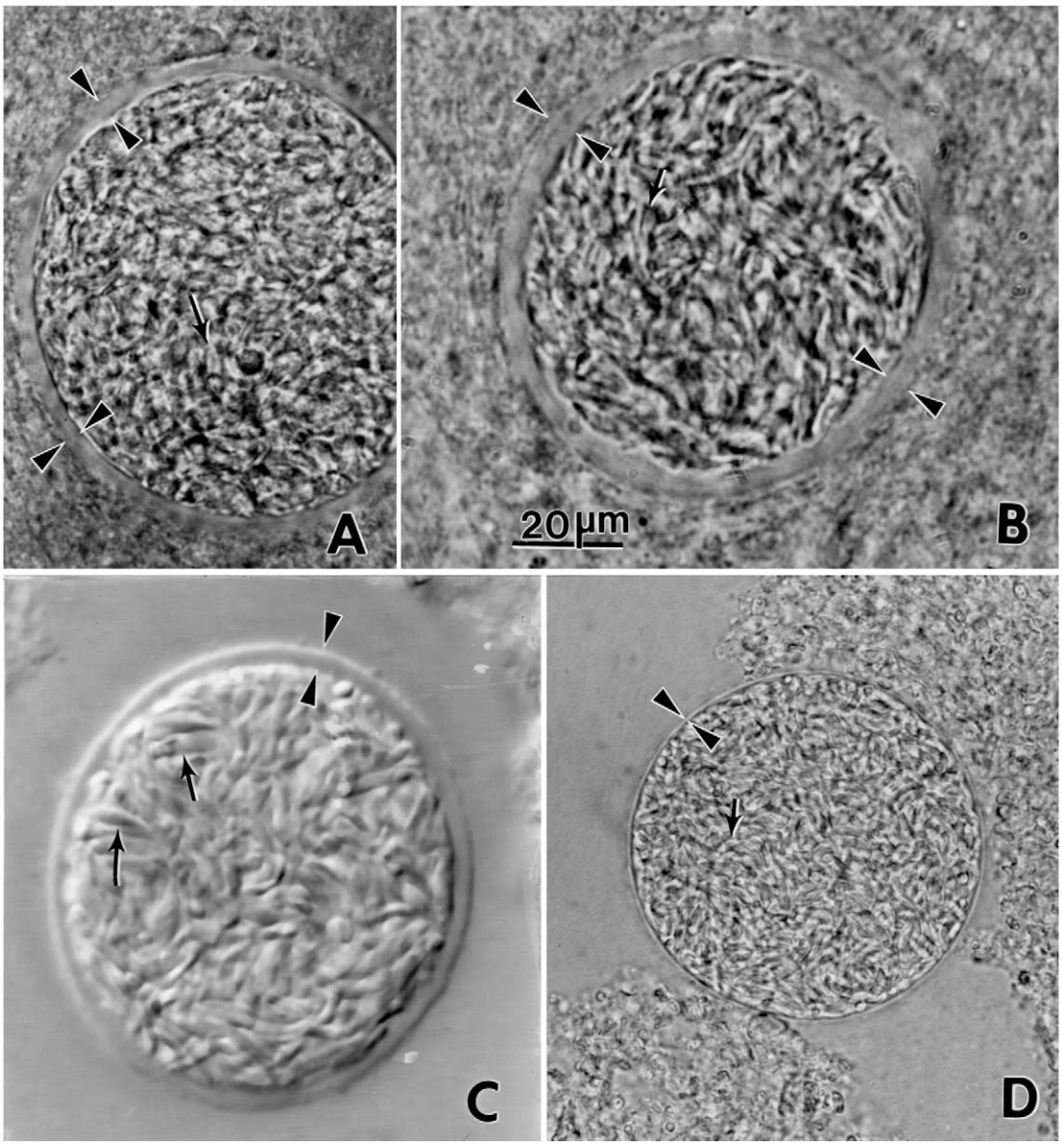

Fig. 3. *Neospora caninum* (A–C) thick-walled tissue cysts compared with a thin-walled *Toxoplasma gondii* (D) tissue cyst. Unstained. Bar applies to all parts. Arrowheads point to thickness of the tissue cyst wall. Arrows point to bradyzoites. (A) Tissue cyst in the brain squash of Swiss–Webster laboratory mouse, 91 days after inoculation with tissue homogenates of naturally infected dogs (Dubey et al., 1988b). This strain was designated as the NC-1 strain of *N. caninum*. (B) Tissue cyst in the brain squash of a gerbil 99 days after inoculation with tachyzoites of the NC-beef isolate of *N. caninum* (McAllister et al., 1998; Dubey et al., 2001). (C) Tissue cyst in homogenate of brain of a mouse inoculated with tachyzoites of the NC-beef isolate of *N. caninum* (McAllister et al., 1998; Dubey et al., 2001), >2 months p.i. (D) Tissue cyst in homogenate of brain of a mouse inoculated with *T. gondii*, 3 months p.i. Note thin cyst wall.

rally infected neonatal sheep from Dubey et al. (1990b). Accession No. 92231 (Fig. 5H). (10) Tissue cyst in the brain of a naturally infected goat (Dubey et al., 1992). Accession No. 92232 (Fig. 5F, G).

3.3. Wet tissued deposited

Formalin-preserved wet brain tissue from dog No. 6 (syntype) of Dubey et al. (1988a). Accession No. 92233.

4. Comments on the modification of the original description of *N. caninum* of Dubey et al. (1988a)

4.1. Intraneural tissue cysts

In the original description of *N. caninum* by Dubey et al. (1988a), tissue cysts were stated to have cyst walls 1–4 μm thick and were found only in the central nervous system (CNS). Since then, thick-walled *N. caninum* tissue cysts have been produced experimentally in the CNS of mice

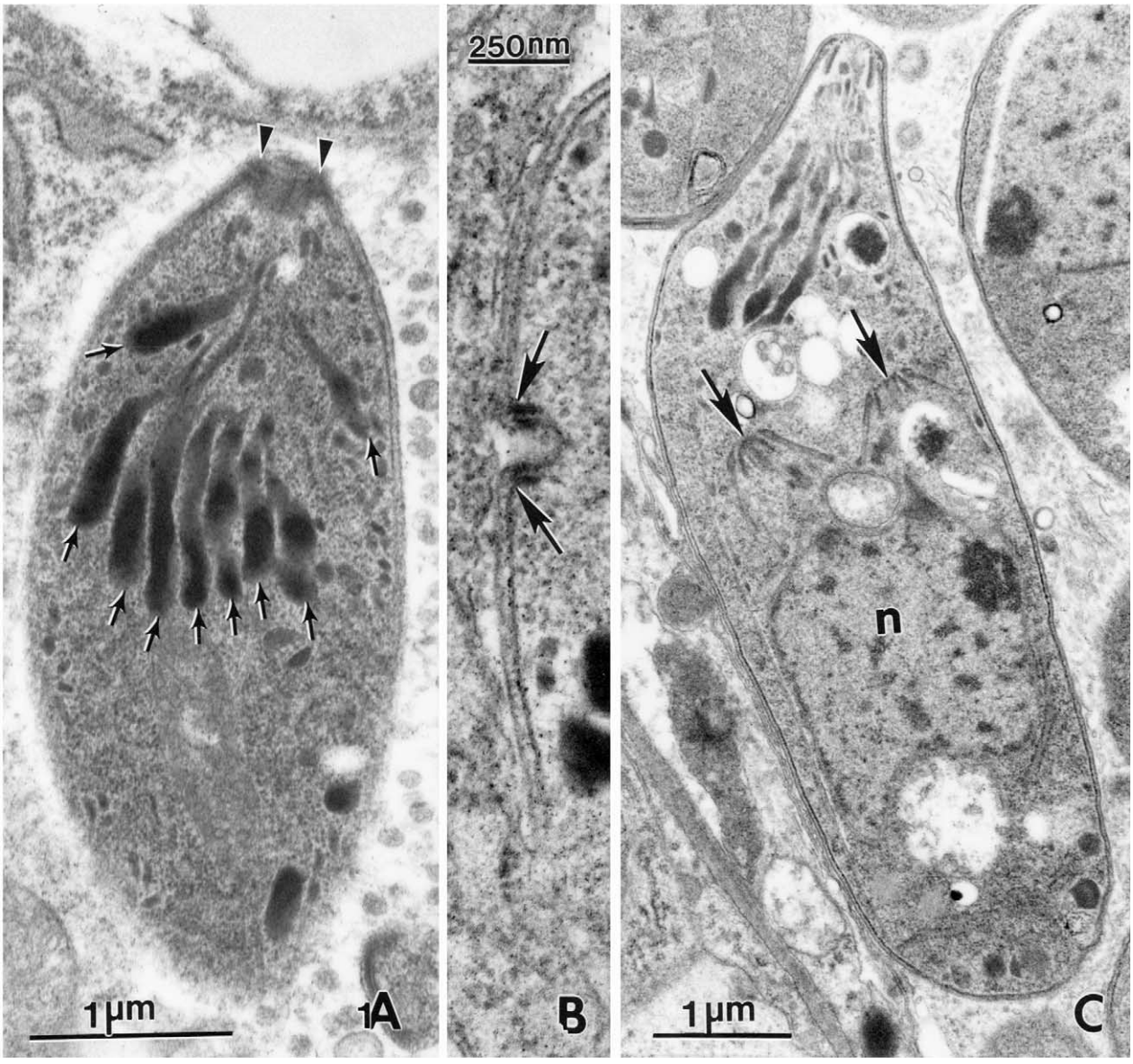

Fig. 4. TEM of tachyzoites of the NC-1 strain of *N. caninum* in the liver of interferon gamma gene knockout mouse perfused with glutarldehyde. (A) Apical end showing a conoid (arrowheads) and nine rhoptries in one plane of section. The rhoptry contents are electron-dense. The arrows point to blind ends of rhoptries, thus there is no uncertainty regarding their number in this illustration. (B) Section through a micropore (arrow) in the pellicle with two membranes. (C) Mother cell with apical ends (arrows) of two daughter zoites forming before the mother nucleus (n) has divided.

(Fig. 3A, C) (Lindsay and Dubey, 1989b), gerbils (Figs. 3B, 5C) (Basso et al., 2001; Gondim et al., 2001), and cats (Fig. 5A, B) (Dubey et al., 1990a). *Neospora caninum* thick-walled tissue cysts were also reported in the CNS of naturally infected cattle (Fig. 5E) (Dubey et al., 1989a), sheep (Fig. 5H) (Dubey et al., 1990b; Kobayashi et al., 2001), goats (Fig. 5F, G) (Barr et al., 1992; Dubey et al., 1992), deer (Fig. 5D) (Dubey et al., 1996), and possibly horses (Daft et al., 1996; Lindsay et al., 1996). Whether the thickness of the cyst wall varies with the duration of infection and tissues parasitised has also not been determined, and only a few tissue cysts have been studied ultrastructurally (Bjerkås and Presthus, 1988; Barr et al., 1991; Jardine, 1996; Speer et al., 1999). In two naturally infected dogs, the tissue cysts were found with walls 0.5–0.7 μm thick. These tissue cysts had a wavy outer contour, and their bradyzoites were of similar appearance to those of *N. caninum* (Dubey et al., 1998; Speer and Dubey, 1989a; Speer et al., 1999). In gerbils and mice, tissue cyst walls were up to 4 μm thick, 3 months p.i. (Fig. 3A, B).

4.2. Intramuscular tissue cysts

Recently, Peters et al. (2001) reported 0.3–1.0 μm thick-walled tissue cysts in muscles of cattle and dogs naturally infected with a *N. caninum*-like parasite. These intramus-

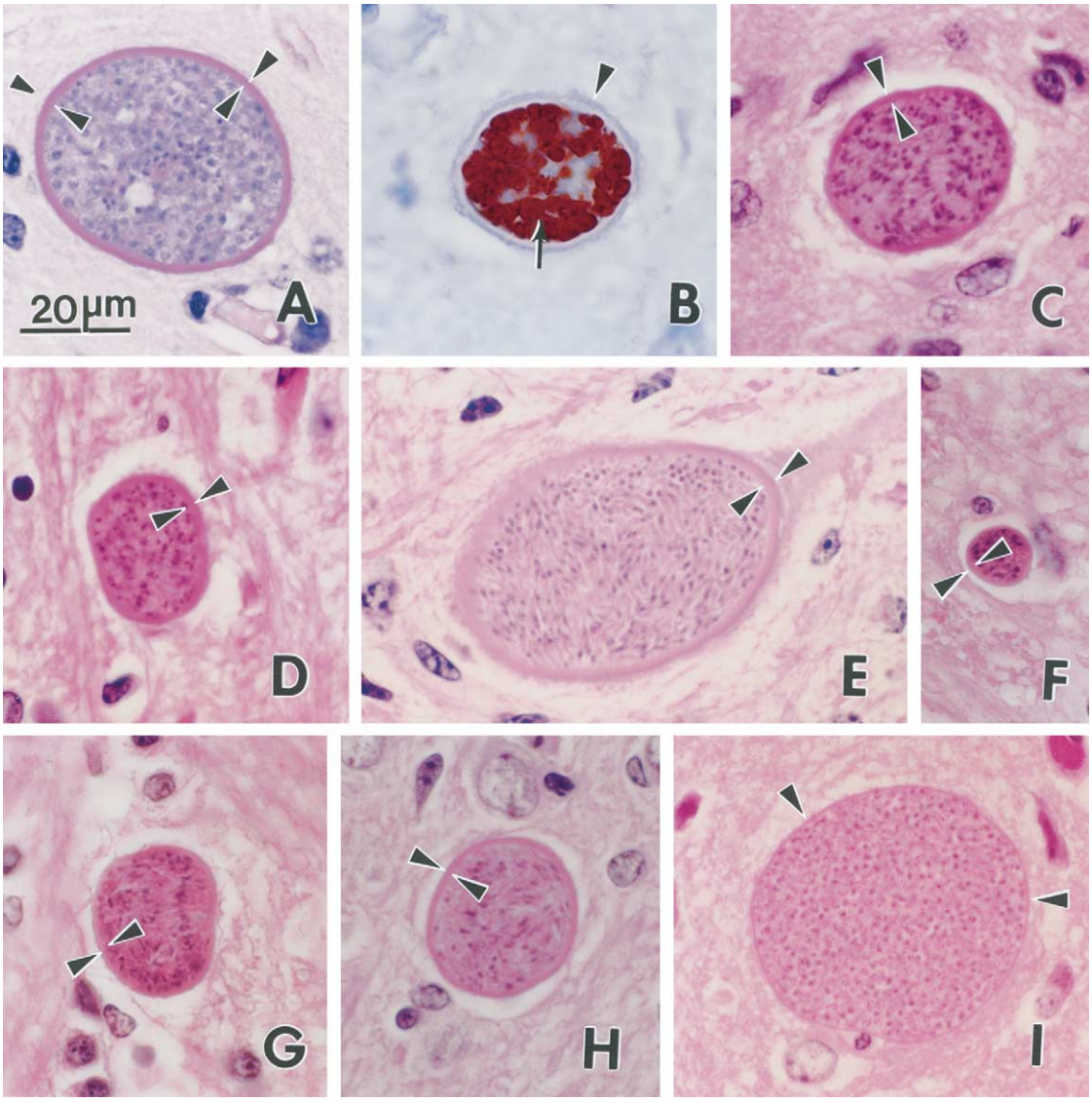

Fig. 5. Neural *Neospora caninum* (A–H) tissue cysts in histological sections from the brain or spinal cord of animals compared with a tissue cyst of *Toxoplasma gondii* (I). The thickness of the tissue cyst wall is indicated by arrowheads. Bar applies to all parts. Tissue cyst in the brain of an experimentally infected cat, 55 days p.i. (Dubey et al., 1990a). H&E. (B) Tissue cyst in the brain of the same cat as in Fig. 5A. Immunohistochemical stain with polyclonal *N. caninum* antibodies. The bradyzoites are stained red with antibodies whereas the cyst wall is unstained but the wavy contour (arrow) is visible. (C) Tissue cyst in the brain of a gerbil fed oocysts from the naturally infected dog from Basso et al. (2001), 75 days p.i. (D) Tissue cyst in the brain of the naturally infected deer from Dubey et al. (1996). H&E. (E) Intraneural tissue cysts from the spinal cord of a naturally infected newborn calf from Dubey et al. (1989a). H&E. (F,G) Tissue cysts in the brain of the naturally infected goat from Dubey et al. (1992). H&E. (H) Tissue cyst from the brain of the naturally infected sheep from Dubey et al. (1990b). H&E. (I) Tissue cyst in the brain of a mouse, 3 months p.i. This is one of the largest *T. gondii* tissue cysts in section. Note that the cyst wall is thin and encloses hundreds of bradyzoites.

cular tissue cysts were labelled with antibodies against *N. caninum* and were apparently situated in myofibers, although the precise location was not confirmed by ultrastructural analysis. The intramuscular tissue cysts each contained 14–50 bradyzoites, 5.2×1.6 µm in size, and morphologically they resemble *Neospora* more than *Toxoplasma* or *Sarcocystis*. Intramuscular tissue cysts have not been yet found in experimentally infected mice, gerbils, and cats (Dubey, unpublished). This may be due, in part, to the observation that it is difficult to induce tissue cyst formation with *N. caninum* in rodents (Dubey, unpublished).

4.3. The parasitophorous vacuole

In the original description of *N. caninum* (Dubey et al., 1988a), tachyzoites were not seen to be in parasitophorous vacuoles in samples of liver and dermal tissue from two naturally infected dogs. However, parasitophorous vacuoles around the parasite were demonstrated later when the para-

site was isolated in cell culture (Dubey et al., 1988b). This may be because the membrane of the parasitophorous vacuole is sometimes thin and not visible in all specimens (Fig. 2), or, the membrane may disintegrate in the early stages of host cell degeneration.

4.4. Oocysts

As already stated, the oocyst of *N. caninum* was not discovered until 1998 (McAllister et al., 1998), and many other aspects of the life cycle of *N. caninum* are currently not known, including whether oocyst production may be initiated in dogs after they ingest sporulated oocysts. Furthermore, the entero-epithelial stages of *N. caninum* preceeding oocyst formation in dogs are unknown. To date dogs have been shown to shed relatively small numbers of *N. caninum* oocysts (McAllister et al., 1998; Lindsay et al., 1999a, Lindsay et al., 2001), irrespective of their age or immune status, and it has not yet been possible to locate and describe schizonts and gamonts within the canine alimentary tract.

4.5. Immunohistochemical labelling of parasites from the original description of Bjerkås et al. (1984), and Dubey et al. (1988a)

In the original description of *N. caninum* by Dubey et al. (1988a) polyclonal rabbit antibodies to *T. gondii* were used in an attempt to immunohistochemically label protozoan organisms in tissues from affected dogs. At that time, immunohistochemical labelling with *N. caninum* organisms could not be performed because *N. caninum* specific antibodies had not been produced. Immunohistochemically, the *Toxoplasma*-like parasite described by Bjerkås et al. (1984) reacted with convalescent serum but not with polyclonal rabbit antibodies to *T. gondii* (Bjerkås and Presthus, 1988). In all 10 dogs from the USA infected with *N. caninum*, the *T. gondii* antibodies could not be shown to bind to the parasite (Dubey et al., 1988a). In 2002 (14 years later), cover slips from these sections were removed from the slides and the sections treated with *N. caninum* polyclonal antibodies (Lindsay and Dubey, 1989a) and bradyzoite-specific BAG-1 antibodies (McAllister et al., 1996). The latter (also called BAG-5) reacts with antigens found in the bradyzoites of *N. caninum* and *T. gondii* but not with tachyzoites of either parasite (McAllister et al., 1996). Using these procedures in the present study, it was demonstrated that the organisms in all 10 *N. caninum* infected dogs described by Dubey et al. (1988a) were labelled by the *N. caninum* antibodies. Large groups of tachyzoites were present in the muscles of the dogs reported by Bjerkås et al. (1984) and Dubey et al. (1988a); no BAG-1 positive characters were found. Thus, as of this time, tissue cysts of *N. caninum* were found only in the brain and spinal cords of dogs originally reported by Bjerkås et al. (1984) and Dubey et al. (1988a).

4.6. Characterisation of N. caninum isolates from different hosts

Neospora caninum has been isolated from tissues of bovine foetuses (Conrad et al., 1993; Stenlund et al., 1997; Davison et al., 1999; Kim et al., 2000), congenitally infected calves (Barr et al., 1993; Yamane et al., 1997; McAllister et al., 1998; Magnino et al., 1999; Fioretti et al., 2000; Kim et al., 2000), a cow (Sawada et al., 2000), tissues of naturally infected dogs (Dubey et al., 1988b; Hay et al., 1990; Cuddon et al., 1992; Barber et al., 1995; Marsh et al., 1998; Dubey et al., 1998; Peters et al., 2000; Gondim et al., 2001), and from the faeces of a naturally infected dog (Basso et al., 2001). No morphological, biological, or molecular differences have been found among *N. caninum* isolates obtained from cattle and dogs (Holmdahl et al., 1997; Marsh et al., 1998). The *N. caninum* isolate from an adult sheep (Koyama et al., 2001) has not yet been molecularly compared with the canine and bovine isolates.

5. Differentiation of *N. caninum* from *Neospora hughesi*

Marsh et al. (1998) proposed a new species of *N. hughesi* from the horse (*Equus caballus*), based on differences from *N. caninum* in proteins, ITS1 region profiles, and tissue cyst morphology. The parasite that was isolated had been obtained by inoculation of cell culture with neural tissue from a horse in California, USA (Marsh et al., 1998). Since then, *N. hughesi* has been isolated from one horse in Oregon, USA (Dubey et al., 2001) and one in Alabama, USA (Cheadle et al., 1999). There are several uncertainties about the differences between *N. caninum* and *N. hughesi*, and available information is summarised subsequently.

(1) The tachyzoites of *N. hughesi* appear morphologically similar to those of *N. caninum* although a critical comparison has not been made (Dubey et al., 2001). (2) Only a few tissue cysts were found in the brain of the California horse. The tissue cysts measured 6.9–16.0 × 10.7–19.3 μm ($n = 6$) and the thickness of the cyst wall was from 0.15 to 1.0 μm (Fig. 6A). The bradyzoites in sections measured 4.4–5.8 × 1.7–2.8 μm, and each contained 13–24 rhoptries with electron-dense contents. It would appear that *N. hughesi* bradyzoites are smaller in size than those of *N. caninum* bradyzoites. Tissue cysts were not found in the *N. hughesi*-infected horse from Oregon (Hamir et al., 1998; Dubey et al., 2001), and tissue cysts have not been seen in experimentally infected mice or gerbils (Walsh et al., 2000; Dubey et al., 2001). (3) The oocysts and the definitive host(s) of *N. hughesi* are currently unidentified. An attempt to infect two dogs by feeding them infected *N. hughesi* mouse brains was unsuccessful (Walsh et al., 2000). (4) Gerbils appear not to be clinically susceptible to *N. hughesi* but they are susceptible to *N. caninum* (Walsh et al., 2000; Dubey et al., 2001). (5) The surface antigens of *N. hughesi* (SAG1, SRS2) differ from the equivalent proteins in *N. caninum* (Marsh et al.,

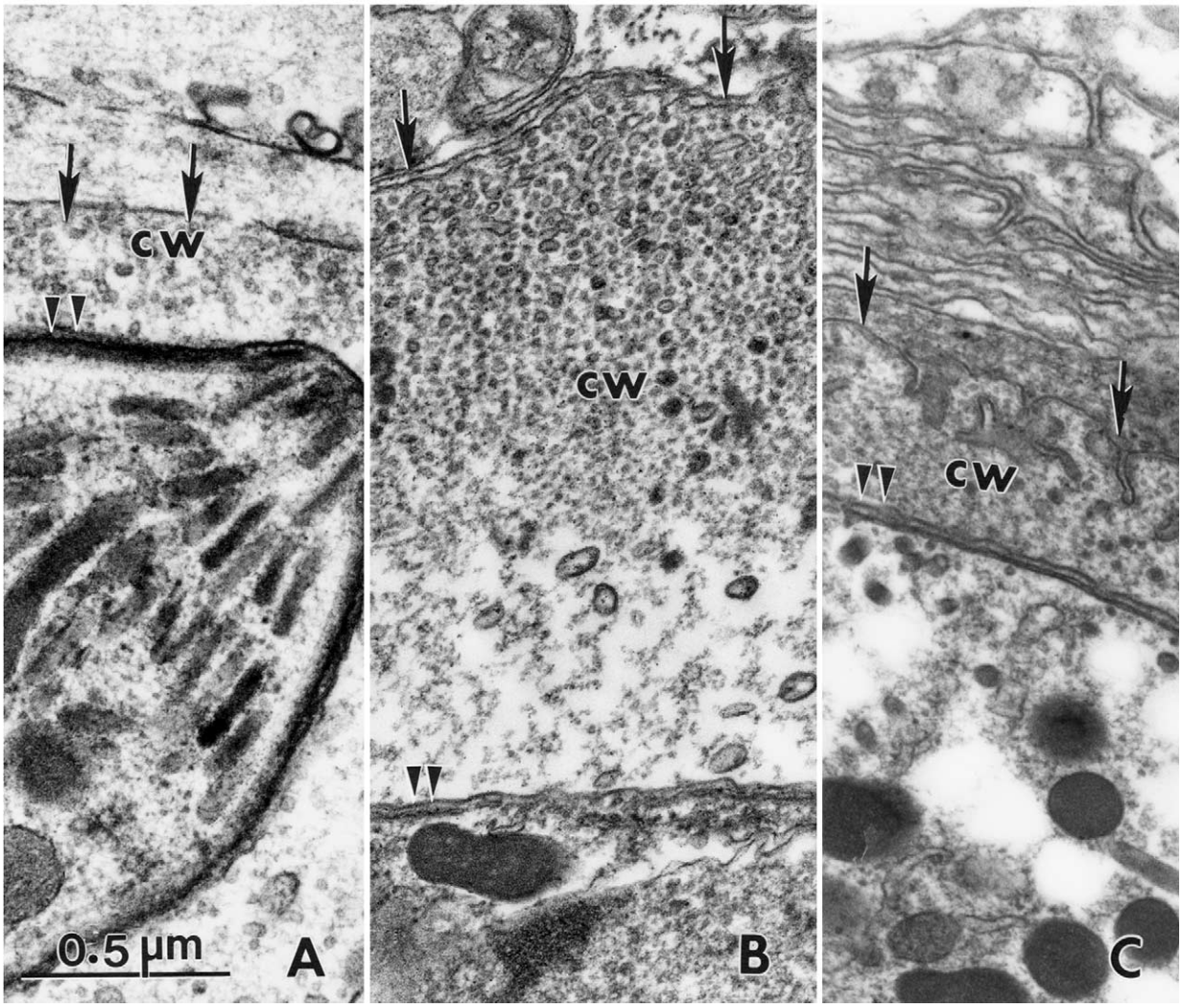

Fig. 6. TEM's of tissue cysts of *Neospora hughesi* (A) in the spinal cord of the horse from Marsh et al. (1998), *Neospora caninum* (B) in the spinal cord of a calf from Barr et al. (1991), and *Toxoplasma gondii* (C) from the brain of an experimentally infected mouse from Barr et al. (1991), 6 months p.i. Bar applies to all parts. The outer tissue cyst wall membrane is indicated by large arrows. In each illustration part of a bradyzoite is just beneath the inner margin of the tissue cyst wall; arrowheads point to outer membrane of the bradyzoite pellicle. Compare the thickness of the cyst walls (cw).

1999; Dubey et al., 2001). (6) The *N. hughesi* dense granule proteins GRA6 and GRA7 are different from corresponding proteins from *N. caninum* (Walsh et al., 2001). (7) The ITS1 sequences of *N. hughesi* are significantly different from those of isolates of *N. caninum* from dogs and cattle (Marsh et al., 1998; Cheadle et al., 1999; Dubey, 1999; Spencer et al., 2001).

Before the discovery of *N. hughesi*, there were reports of *Neospora* infection in an aborted foal (Dubey and Porterfield, 1990), a newborn foal (Lindsay et al., 1996), and in an aged mare (Daft et al., 1996). Thick-walled tissue cysts, 1.5–3.0 μm thick, were found in the foal (Lindsay et al., 1996), and cyst walls up to 2 μm thick were seen in tissue cysts in the aged mare (Daft et al., 1996; Dubey et al., 2001). It is uncertain whether the thick-walled tissue cysts in these horses were those of *N. hughesi* or *N. caninum*; antigenically *N. caninum* antibodies cross-react with *N. hughesi*.

6. Differentiation of *N. caninum* from *T. gondii*

In 1988, having established its distinctiveness from *T. gondii* and given the totally novel aspects of the host-parasite association and the lack of knowledge of its life cycle, a new genus (*Neospora*) and species (*N. caninum*) were erected. All *T. gondii* organisms derived from different warm-blooded hosts cannot be distinguished morphologically and can produce recombinant progeny during sexual reproduction in their feline definitive host (Howe and Sibley, 1995), they are considered to belong to one species, *T. gondii*. *Neospora caninum* is reproductively isolated from *T. gondii* because *N. caninum* uses a canine, not a feline, definitive host. Dubey and Lindsay (1996), Speer et al. (1999), and Mugridge et al. (1999) have summarised biological, morphological, molecular, and antigenic differences between *N. caninum* and *T. gondii*. In addition, major *N.*

caninum antigens are genetically, and immunologically distinct from those of *T. gondii* (Brindley et al., 1993; Dubey, 1999; Howe and Sibley, 1999; Ellis et al., 2000a; Atkinson et al., 2001). Furthermore, Naguleswaran et al. (2002) recently demonstrated that *T. gondii* and *N. caninum* also differ with regard to their affinities for host cell surface glycosaminoglycans which are used as receptors for adhesion and invasion of host cells. While *N. caninum* appears to preferentially bind to chondroitinsulphate proteoglycans, *T. gondii* has a clear preference for heparansulphate residues. In summary, *T. gondii* is morphologically, genetically, antigenically, and biologically distinct from *N. caninum*. Dogs are definitive hosts for *N. caninum* and neosporosis is a major disease of cattle, whereas cats are definitive hosts for *T. gondii* and toxoplasmosis is a major disease of sheep but not of cattle. Rhoptries in tachyzoites of *N. caninum* are electron-dense whereas rhoptries of *T. gondii* are electron-lucent (Dubey and Lindsay, 1996).

A preliminary way to distinguish *N. caninum* from *T. gondii* is by the thickness of the tissue cyst wall. *Toxoplasma gondii* tissue cysts have such a thin wall that it cannot be measured accurately under the light microscope, irrespective of the duration of infection (Figs. 1F, 3D, 5I). Antibodies to *T. gondii* and *N. caninum* do not cross-react in several serological assays, including the modified direct agglutination test (Romand et al., 1998), but may cross-react in some serological tests. Cross reaction may also be present in immunohistochemical tests, depending on whether tissues are pre-treated with pepsin or trypsin and on the specificity of the antibodies used (Dubey and Lindsay, 1996). However, cross-reactivity may be avoided by employing monoclonal antibodies specific to either species when attempting to label organisms in tissue sections (Cole et al., 1993) or parasite antigens in immunoblotting procedures (Harkins et al., 1998; Heckeroth et al., 2000; Schares et al., 2001b).

7. Differentiation of *N. caninum* from other related coccidia

The oocysts of *N. caninum* morphologically resemble oocysts of another group of coccidia in canine faeces previously called *I. bigemina*, *Isospora bahiensis*, and *H. heydorni*. Therefore, it is necessary to review their taxonomic history before attempting any comparison. For the present discussion, the small race of *I. bigemina*, *I. bahiensis*, and *H. heydorni* are considered one and the same parasite for the reasons stated subsequently.

Historically, Stiles (1891, 1892) found a coccidium in the lamina propria of a dog and named it *Coccidium bigeminum* and this was subsequently changed to *I. bigemina* (see Wenyon, 1923; Levine, 1973; Levine and Ivens, 1981). Nothing more was reported about the structure or life cycle of *I. bigemina* until Wenyon and Sheather (1925) described a parasite in the intestine of a dog with schizonts and gamonts in the surface epithelium, but not the lamina propria, of the intestine. They also recorded unsporulated oocysts, 11×10 μm in size. In the same article, the authors reported oocysts, 13.5×15.5 μm, to be present in the lamina propria of another dog. They concluded that in "chronic stages of *I. bigemina* infection in dogs and cats, mature oocysts occur in the sub-epithelial tissues and during acute infection they occur in the epithelium". This one page note by Wenyon and Sheather (1925) is critical to the following discussion and to the detailed article by Wenyon (1926). The size of oocysts in the surface epithelium of the dog reported by Wenyon and Sheather (1925) was re-measured by Wenyon (1926) and found to be 10–14 μm in length rather than 10–11 μm as originally recorded (Wenyon and Sheather, 1925). Additionally, the oocysts measuring 13.5×15.5 μm in the lamina propria of the second dog were redefined as sporocysts rather than oocysts (Wenyon, 1926). No mention was made in these articles that the parasite in the epithelium was anything other than *I. bigemina*, and the same parasite was also considered to occur in cats (Wenyon, 1926).

From 1926 until the discovery of the stages of *T. gondii* in the feline intestine (reviewed in Dubey et al., 1970) and the stages of *Sarcocystis* in the canine intestine (Heydorn and Rommel, 1972), nothing was known of the life cycle of *I. bigemina*. In 1973, Heydorn reported unsporulated oocysts, $10–14.6 \times 9.2–13.1$ μm, in the faeces of dogs fed cattle musculature and the asexual and sexual development of this parasite was shown to occur in the epithelium rather than in the lamina propria (Heydorn et al., 1975). Thus, it became apparent that the parasite in the lamina propria of dogs, initially termed *C. bigeminum* was a species of *Sarcocystis* while the parasite in the epithelium was an unnamed species that Heydorn called the small race of *I. bigemina* of the dog (Heydorn, 1973). Because dogs are definitive hosts for numerous species of *Sarcocystis*, the oocysts and sporocysts of which cannot be distinguished on morphological grounds alone (Dubey et al., 1989b), *I. bigemina* has been used for more than 1 parasites including several species of *Sarcocystis*. Heydorn (1973) also found that dogs fed sporulated '*I. bigemina*' oocysts did not shed oocysts and that the parasite was not infective to cats and mice (Table 1).

Levine and Ivens (1965) and Levine (1973) reviewed the worldwide reports of *I. bigemina*-like oocysts in the faeces of naturally infected dogs and gave their size as $12–14 \times 10–12$ μm. Levine (1978) named the small race of *I. bigemina* as *I. bahiensis* based on a report by Costa (1956) who had found oocysts measuring $12.3–13.6 \times 10.7–12.3$ μm in one dog and $11.4–13.6 \times 10.7–12.3$ μm in another dog from Bahia, Brazil; Costa had called the parasite he found *I. bigemina* var *bahiensis*.

Dubey and Fayer (1976) reported *I. bigemina*-like oocysts in the faeces of a naturally infected dog from Ohio, USA (Table 1). The oocysts were $10–14 \times 9–13$ μm in size and found to be infective to cattle. Dogs fed cattle musculature shed unsporulated *I. bigemina*-like oocysts in

Table 1
Evidence for obligatory two-host life cycle of *Hammondia heydorni*-like parasites from dogs

Lack of oocyst shedding by dogs fed oocysts				Strain designations and other observations[a]	References
No. of oocysts fed	Mean size of oocysts (μm)	No. of dogs	Observation period (weeks)		
4×10^4–1.3×10^6	11.9×11.1	21	6	Berlin 1971[b]	Heydorn (1973)
10^5–10^6	12.5×11	15	4	Ohio 1974[c]	Dubey and Fayer (1976)
5×10^4	12.4×11.2	2	8	Brazil[d]	Matsui et al. (1981)
10^7	12.6×11.9	3	3	Alabama1[e]	Blagburn et al. (1988)

[a] Strains were designated in the present article to facilitate discussion. Original articles did not have these designations.
[b] Oocysts were infective to cattle and dogs, but not to cats, mice or rabbits.
[c] Oocysts were not infective to mice and cats.
[d] Oocysts were not infective to mice, rats, rabbits, and hamsters, but were infective to guinea pigs.
[e] Oocysts were infective to goats but not to one calf.

their faeces, but dogs fed sporulated oocysts became infected but did not shed oocysts (Table 1). Schizonts and gamonts of this parasite were found in the intestinal epithelium of both experimental dogs and the naturally infected dog (Dubey and Fayer, 1976) and they were morphologically indistinguishable from the stages described by Wenyon and Sheather (1925), and by Heydorn et al. (1975). Dubey (1976) named this small race of *I. bigemina* as *Isospora wallacei* in an attempt to distinguish it from the name *I. bigemina*. That year, Tadros and Laarman (1976) named the same parasite *Isospora heydorni*. Because the latter article was published a month earlier than that by Dubey (1976), *I. heydorni* took precedence over *I. wallacei*. At the time of publication neither Dubey (1976) nor Tadros and Laarman (1976) were aware of the article by Costa (1956). There are no host sera or oocyst DNA available from these studies.

In 1975, a new genus of coccidia, *Hammondia* was proposed by Frenkel and Dubey (1975) to accomodate a feline coccidium *H. hammondi* with an obligatory two-host life cycle. Cats fed sporulated *H. hammondi* oocysts did not produce oocysts, a character shared by the small race of *I. bigemina* studied by Heydorn (1973) and Dubey and Fayer (1976) (Table 1). Therefore, Dubey (1977) proposed that *I. heydorni* be transferred to the genus *Hammondia*, and thus created the binomial *H. heydorni* (Tadros and Laarman, 1976) Dubey, 1977.

Since 1977, findings of unsporulated *H. heydorni*-like oocysts have been reported in the faeces of dogs, coyotes, and foxes fed muscles of naturally or experimentally infected cattle, sheep, goats, moose, reindeer, roe deer, mountain gazelle, camels, or water buffaloes (Tables 2 and 3). These parasites were assumed to be *H. heydorni*-like based solely on morphology of the oocysts. Levine and Ivens (1981) synonymised all *I. heydorni*, *H. heydorni* and *I. bigemina*-like organisms in dogs, foxes and other animals with *I. bahiensis* due to the size of oocysts in faeces.

Little is known about the life cycles of the *I. bigemina*, *I. bahiensis*, or *H. heydorni*-like parasites except that they have an obligatory two-host life cycle. This assumption is based on observations that the dogs fed sporulated oocysts did not shed oocysts in their faeces, whereas, sporulated oocysts were infective for cattle, sheep and goats (Table 1). However, dogs fed extra-intestinal tissues of animals, including dogs, shed unsporulated oocysts in their faeces (Matsui et al., 1981, 1986). Oocysts of these parasites were not infective for cats, mice, hamsters, or rabbits (Table 1) and their extra-intestinal stages have not been conclusively demonstrated histologically in tissues of ruminants or dogs. The intramuscular tissue cysts found by Heydorn (1973) in cattle infected with *I. bigemina* turned out to be tissue cysts (sarcocysts) of *Sarcocystis*.

The only report describing tissue cysts of *H. heydorni* is by Matsui (1991), who examined tissues of guinea pigs fed oocysts of this parasite. He found a tissue cyst in the brain of an animal killed 77 days p.i. and one tissue cyst in a brain smear of a guinea pig killed 189 days p.i. The tissue cysts measured 10.7×10.6 μm in the histological section and 13.0×12.1 μm in tissue smear. They had thin walls and were indistinguishable from *T. gondii* tissue cysts. To our knowledge, tissues or sera from these guinea pigs are not available for retrospective studies and therefore further verification of these tissue cysts is not possible. To our knowledge there is also no DNA or oocysts now available from this study.

Entero-epithelial stages of *I. bigemina*-like parasites were reported in histological sections of small intestines of naturally infected dogs (Wenyon and Sheather, 1925; Dubey and Fayer, 1976) and of experimentally infected dogs (Heydorn et al., 1975; Dubey and Fayer, 1976; Matsui et al., 1986). The schizonts were approximately 5–10 μm in diameter and each contained up to 16 merozoites. Female gamonts and unsporulated oocysts were approximately 10 μm in diameter. Similar stages were found in the small intestine of a naturally infected gray fox (*Urocyon cineroargenteus*) in the USA (Dubey and Lin, 1994). The schizonts, gamonts, and oocysts in these four reports appear to be morphologically similar. However, these stages cannot be compared

Table 2
Shedding of *Hammondia heydorni*-like oocysts by dogs fed muscles from naturally infected herbivores

Herbivore host	Country	Measurement (μm) of oocysts shed by dogs		Further studies done	References
		Average	Range (n = no. measured)		
Cattle (*Bos taurus*)	Germany	11.9–11.1	10–14.6 × 9.2–13.1 ($n = 150$)	Yes[a]	Heydorn (1973)
	USA	11.9 × 10.8	Not given ($n = 20$)	No	Fayer (1974)
	USA	13 × 11.5	10–14 × 9–13 ($n = 10$)	Yes[b]	Dubey and Fayer (1976)
Water buffalo (*Bubalus bubalus*)	Egypt	11.9 × 11.1	10–14.5 × 9.3–13.1	No	Nassar et al. (1983)
	Malaysia	Not given	10–12 × 9–10	No	Dissanaike and Kan (1977)
Camel (*Camelus dromedarius*)	Egypt	11.9 × 11.1	10–14.5 × 9.3–13.1	No	Nassar et al. (1983)
	Egypt	12 × 11	11.6–13.2 × 10.5–11.7	No	Hilali et al. (1992)
	Saudi Arabia	11.6 × 10.4	10.7–11.9 × 9.5–10.7	No	Hilali et al. (1995)
	Sudan	10.8 × 9.4	9.1–10.1 × 9.3–12.4	No	Warrag and Hussein (1983)
Sheep (*Ovis aries*)	Brazil	Not reported	Not reported	No	Pereira and Lopes (1990)
	USA	[c]	[c]	Yes[b]	Dubey and Williams (1980)
Moose (*Alces alces*)	USA	[c]	[c]	No	Dubey and Williams (1980)
Goat (*Capra hircus*)	USA	[c]	[c]	Yes[b]	Dubey and Williams (1980)
	India		11–14 × 10.5–13.0	No	Shankar et al. (1991)
	Brazil		11.24 ± 0.03 × 10.22 × 0.04	No	Pacheco et al. (1991)

[a] Heydorn (1973); Heydorn et al. (1975).
[b] See Dubey and Williams (1980).
[c] Considered to be same as seen in faeces of the naturally infected dog (Dubey and Fayer, 1976).

with *N. caninum* because schizonts and gamonts of *N. caninum* have not yet been demonstrated.

Before the discovery of *N. caninum* there was only one report of a cell culture of *H. heydorni* induced with sporozoites (Speer et al., 1988). The parasite grew in several cell lines, but died out after a few asexual cycles and could not be transferred to other cell lines by culture derived organisms inoculation. In this respect, the parasite shared more characteristics with parasites of the genus *Hammondia* than with *Toxoplasma* or *Neospora*.

After the discovery in 1998 of the oocysts of *N. caninum* in dog faeces and their resemblance to *I. bigemina* (*H. heydorni*) oocysts, there was a renewed interest in these parasites. Schares et al. (2001a, 2002) reported that *H. heydorni* (or *I. bigemina*-like) oocysts observed in faeces of foxes (Table 3) may belong to a different parasite than that in dogs. Of the dogs and foxes fed muscles from experimentally infected sheep and goats, only foxes shed *H. heydorni*-like oocysts which appeared to be slightly larger in size than those from dogs previously reported. They also pointed out that similar findings were reported by Ashford (1977), Entzeroth et al. (1978), and Gjerde (1983) (Table 3). Schares et al. (2001a) concluded that there is no way to determine whether studies conducted before 1998 with *I. bigemina*-like oocysts used *N. caninum*, *H. heydorni* or other protozoan parasites because host sera, parasite DNA and organisms from these studies are no longer available. Even the possibility that the initial life cycle description of *I.*

Table 3
Shedding of *Hammondia heydorni*-like oocysts by non-dog canids fed musculature of herbivores

Herbivore	Country	Carnivore	Oocyst measurements (μm)	Other experiments	References
Roe deer (*Capreolus capreolus*)	Germany	Fox[a]	13.1 × 11.6	No	Entzeroth et al. (1978)
Sheep (*Ovis aries*)	UK	Fox[a]	14 × 12 ($n = 10$)	No	Ashford (1977)
	Germany	Fox[a]	11.6–13.7 × 10.9–12.9, 12.7 × 11.7; $n = 50$	No	Schares et al. (2002)
Mountain gazelle (*Gazella gazella*)	Saudi Arabia	Fox		No	Ellis et al. (1999)
Reindeer (*Rangifer tarandus*)	Norway	Fox[a]	10.7–15.3 × 9.7–12.8 ($N = 95$)	No	Gjerde (1983)
Cattle (*Bos taurus*)	USA	Coyote (*Canis latrans*)	13 × 11	Yes[b]	Dubey and Williams (1980)

[a] Dogs did not shed oocysts.
[b] Oocysts from coyote faeces were infective to sheep and goats and the isolate was transmissible to dogs.

bigemina by Heydorn (1973) was conducted with a mixture of two or more species must be considered.

It was recently suggested that the *H. heydorni*-like isolates now designated Berlin 1971 (Table 1) and studied by Heydorn (1973) may be the same as *N. caninum*, and based on oocyst size different from the parasite studied by Blagburn et al. (1988) and Speer et al. (1988) (named Alabama 1 strain in this article). The mean oocyst size (12.6×11.9 μm) given by Blagburn et al. (1988) was about 1 μm bigger than the mean size of oocyst (11.9×11.1) stated by Heydorn in the 1973 article (Heydorn and Mehlhorn, 2002). However, Heydorn (1973) reported that the oocyst size for the parasite he studied ranged from 10–14.6×9.2 to 13.1 μm (Table 1). As stated earlier, based on oocyst size alone it is not possible to determine the identity of the *I. bigemina*-like parasite (Table 1). For example, oocysts initially identified as the small race of *I. bigemina* in cat faeces were found to be at least three distinct organisms, *T. gondii*, *H. hammondi*, and *Besnoitia darlingi*, all with oocysts approximately 10–14 μm in diameter (Dubey, 1993). Calculation techniques (for example, ocular micrometer versus digital imaging) can cause varying amounts of error in the case of these small objects, and the effects of sporulation on oocyst dimension has not been carefully examined for measurements of these small oocysts.

8. Comparison of *N. caninum* and *H. heydorni*

Neospora caninum differs biologically, immunologically, morphologically, and molecularly from *H. heydorni*. Although *H. heydorni* (*I. bahiensis*) may encompass more than one species, available differences are pointed out, and wherever applicable, strains or isolates of *H. heydorni* are identified. There is also the possibility that the parasites designated as strains may be separate species. With these limitations in mind, the differences between these two species are as follows: (1) *Hammondia heydorni* '(*I. bigemina*)' has a obligatory two-host life cycle demonstrated in all four isolates/strains listed in Table 1, whereas *N. caninum* can be maintained in cell culture, in rodents, and in cattle almost indefinitely. Transplacental transmission of *N. caninum* is very efficient in cattle, while *H. heydorni* has currently not been shown to be transplacentally transmitted. Additionally, the Alabama 1 strain of *H. heydorni* could not be maintained in cell culture after sub-passages (Speer et al., 1988; Speer and Dubey, 1989b).

(2) *Neospora caninum* is molecularly distinct from *H. heydorni* (Ellis et al., 1999; Mugridge et al., 1999; Basso et al., 2001; Dijkstra et al., 2001; Hill et al., 2001; Schares et al., 2001a) based on currently available analysis of genetic sequences summarised in Table 4. Increasing evidence shows that ITS1 remains a valuable species–specific marker for members of the Toxoplasmatinae (Ellis et al., 1999; Ellis et al., 2000b; Schares et al., 2002; Šlapeta et al., 2002; Tenter et al., 2002; Table 4). Mehlhorn and Heydorn (2000) and Heydorn and Melhorn (2002) interpretation of the phylogenetic relationships between *N. caninum* and *H. heydorni* may be due to the fact that different parts of rDNA evolve at different rates (Ellis et al., 2000b). Indeed, the high level of nucleotide similarity which exists among the small subunit and large subunit rDNAs of *T. gondii*, *N. caninum* and *H. heydorni* reflects their relatively slow tempo of evolution which results in a lack of informative characters for analysis. The ITS1 (and some protein encoding sequences), which have less biological constraints show a much higher level of genetic variation among these taxa. All isolates of *N. caninum* possess an ITS1 sequence characteristic of the species, whereas all isolates of *H. heydorni* analysed possess a completely different, yet species–specific sequence. (3) The thick-walled tissue cysts of *N. caninum* differ from the two thin-walled tissue cysts putatively termed *H. heydorni* by Matsui (1991). (4) At present, there is no evidence that *H. heydorni* oocysts from dogs are infective to gerbils, whereas *N. caninum* oocysts are (Dubey and Lindsay, 2000; Basso et al., 2001). However, not all isolates of *N. caninum* are pathogenic for gerbils (Basso et al., 2001; Dijkstra et al., 2001; Schares et al., 2001a). (5) *Neospora caninum* tachyzoites are morphologically distinct from culture-derived organisms of the Alabama 1 strain of *H. heydorni* (Speer and Dubey, 1989b; Dubey et al., 2002).

9. Uncertainties and conclusions

It has been reported recently that there were three isolates (Berlin 1971, Berlin 1974, Berlin 1996) of *I. bigemina* (*H. heydorni*-like) organisms in Heydorn's laboratory, all considered to be *H. heydorni* until the discovery of the oocyst stage of *N. caninum* in dog faeces by McAllister et al. (1998). The initial source or sources of the Berlin 1971 isolate used in reports of Heydorn (1973) and Heydorn et al. (1975) were tissues of naturally infected cattle, and thus it is uncertain whether there were one or more parasite species in this material. According to Schares et al. (2001a) there are no host sera or parasite DNA available from this material.

The Berlin 1974 isolate was obtained from the faeces of a naturally infected dog. Its oocysts were about 1 μm larger than the Berlin 1971 isolate and it was infective for cattle, sheep and goats. Nothing was published on this isolate until recently (see Heydorn and Mehlhorn, 2002) and, according to this report, Mugridge et al. (1999) used this isolate for their molecular characterisation of *H. heydorni* (see Table 4).

The Berlin 1996 isolate was obtained from the faeces of a naturally infected dog (Schares et al., 2001a). Sporulated oocysts from this dog were fed to an adult goat and an adult sheep. Subsequently, muscular tissue from the goat and sheep were fed to dogs to obtain oocysts for teaching purposes in Heydorn's laboratory. The dog (No. 1) fed goat

Table 4
Genetic analysis of *Hammondia heydorni*-like oocysts from naturally infected dogs or foxes

Country	Host	Oocyst measurement	GenBank Accession no.	Genes studied	Strain designation	References
Czech Republic	Dog	11.9 × 11.0 (11–13 × 10–12)	AF317282	ITS1	CZ-1	Šlapeta et al. (2002)
		11.9 × 10.3 (11–13 × 8–11.5)	AF317281	ITS1	CZ-2	
		12.2 × 11 (11.5–14 × 10–12)	AF317280	ITS1	CZ-3	
USA	Dog	12.6 × 11.9 (11.3–13.5 × 10.8–13.5)	AF096501	ITS1	Alabama1	Blagburn et al. (1988);
			AF096502	Partial large subunit rDNA		Speer et al. (1988); Speer and Dubey (1989b); Ellis et al. (1999)
Germany	Dog	Not reported	AF159240	Large	Berlin 1974	Mugridge et al. (1999); Heydorn and Mehlhorn (2002)
New Zealand	Dog	12.8 × 11.7 (12.6–14.1 × 11.5–11.9)	AF508020	ITS1		Ellis and Pomroy (unpublished)
			AF508029	Partial large subunit rDNA		
Germany	Fox	12.7 × 11.7 (11.6–13.7 × 10.9–12.9)	AF395866	Partial large subunit rDNA	Fox-2000/2 Fox-2000/1	Schares et al. (2002)
			AF395867	ITS1		
Saudi Arabia	Fox	10.9 × 10.1 (9.5–12.3 × 8.8−11.4)	AF076858	ITS1		Mohammed et al. (unpublished); Ellis et al. (1999)

tissues did not shed oocysts, while the dog (No. 2) fed sheep tissues shed *H. heydorni*-like oocysts. Dog No. 2 had been fed on the tissues of the sheep that had been given oocysts from the naturally infected dog, 641 days previously. After the discovery of the *N. caninum* oocysts in the faeces of dogs by McAllister et al. (1998), the oocysts from dog No. 2 were used for studies reported by Schares et al. (2001a). It was found that this isolate was infective but not pathogenic at the doses used for sheep, goats, guinea pigs, gerbils and multimammate rats. These oocyst inoculated animals developed antibodies to *N. caninum*, and *N. caninum* DNA was subsequently detected in their tissues by PCR (Müller et al., 2001; Schares et al., 2001a). However, the parasite was not isolated from tissues of any of the infected animals (Schares et al., 2001a). Thus, Schares et al. (2001a) provided evidence that the Berlin 1996 isolate was indistinguishable from *N. caninum* and that dogs can shed large numbers of oocysts after eating the infected musculature of sheep. There is, however, uncertainty about the original source of the infection because nearly 2 years had elapsed between the feeding of the dog faeces to the sheep and its euthanasia for bioassays. Besides the sheep used to further passage the Berlin 1996 isolate were found to be co-infected with *T. gondii*, *Hammondia* sp., and *Sarcocystis* sp. in addition to *N. caninum* (Schares et al., 2002).

Heydorn and Mehlhorn (2002) have proposed that the Berlin 1996 *I. bigemina* isolate is indistinguishable from *N. caninum*. Because the oocysts of the Berlin 1996 isolate are similar in size to oocysts of the Berlin 1971 *I. bigemina* isolate, those authors imply that Heydorn discovered *N. caninum* and its life cycle and suggest that *N. caninum* should be named *I. heydorni*. However, in light of the existence of two or more coccidian species of canids with morphologically similar oocysts, we do not believe that oocyst size by itself is sufficient information upon which to base such a taxonomic decision.

Heydorn and Mehlhorn (2002) also stated that the origin of the brain cysts of *N. caninum* first seen by Bjerkås et al. (1984) is not clear, since the young dogs had a natural infection. They cast doubt on the position that the parasite in Norwegian dogs is the same as that reported by Dubey et al. (1988a) in the USA from 10 unrelated dogs that died over a period of 40 years. The Norwegian dogs were up to 18 months old and came from the same bitch. Retrospectively, Dubey et al. (1990c) reported a similar parasite (*N. caninum*) from maternally related dogs that had died in 1957 in Ohio, USA; 29 of 39 dogs had developed the same clinical syndrome reported by Bjerkås et al. (1984) from Norway. The occurrence of the same parasite in littermates from Norway (Bjerkås et al., 1984) and the USA (Dubey et al., 1990c) suggest more than an incidental finding. Mehlhorn and Heydorn (2000) also make the assumption that intramuscular tissue cysts were present in the dogs reported by Bjerkås et al. (1984), although this is not stated in the original report and tissue cysts were not found in the retrospective study. Finally, they also argued that the parasite (NC-1) isolated by Dubey et al. (1988b) was not the same as described from the fixed material by Dubey et al. (1988a). The following arguments negate these conjectures: (1) The tissue cysts described by Bjerkås et al. (1984) and Bjerkås and Presthus (1988) and those observed by Dubey et al. (1988a) and Dubey et al. (1990c) were

demonstrated to be morphologically and antigenically indistinguishable (Bjerkås and Dubey, 1991). (2) The isolation of the NC-1 strain of *N. caninum* in cell culture was linked to the original description of the parasite based on the finding of thick-walled tissue cysts in the brains of mice inoculated with tissues of paralysed dogs and the reproduction of clinical disease in experimentally infected dogs, thus fulfilling Koch's postulates (Dubey et al., 1988b). Furthermore, the same thick-walled tissue cysts were produced experimentally in mice and cats inoculated with the NC-1 isolate of *N. caninum* (Lindsay and Dubey, 1989b; Dubey et al., 1990a) and in gerbils and mice inoculated with the NC-beef isolate of *N. caninum* obtained from cattle (present study). (3) Lindsay et al. (2001) induced production of *N. caninum* oocysts in the faeces of dogs fed thick-walled tissue cysts derived from cloned *N. caninum* tachyzoites, thus providing evidence that the donor mice had been infected with only one parasite species. (4) The parasite (NC-1) isolated from the tissues of dogs is morphologically, biologically, immunologically, and molecularly indistinguishable from the parasite strains isolated from the faeces of a naturally infected dog (Basso et al., 2001), and from tissues of cattle (Conrad et al., 1993; Barr et al., 1993; Dubey and Lindsay, 1996; Holmdahl et al., 1997, 1998).

In conclusion, *N. caninum* is a species distinct from *T. gondii* and *H. heydorni*-like parasites in dog faeces. However, whether *H. heydorni* or *I. bahiensis* consists of one or more species is not clear.

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