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The comparative pathogenesis of neosporosis

David Buxton, Milton M. McAllister and J.P. Dubey

Since its first description in dogs in 1984, the protozoan parasite *Neospora caninum* has been found to infect a wide range of animals, worldwide. In cattle, *N. caninum* has particular significance as a cause of abortion in which persistence of infection in the mother, recrudescence of the parasite during pregnancy, and the vulnerability of the placenta and foetus to invasion are important features. This article discusses how *Neospora* invades cells, how it infects and causes disease in several animal species, and particularly how it has evolved a special niche in cattle and dogs that ensures its survival.

Neosporosis is the name given to a disease caused by the apicomplexan parasites *Neospora caninum* and *Neospora hughesi* [1], which are obligate intracellular protozoa closely related to *Toxoplasma gondii*. This review is almost solely concerned with *N. caninum*, which was first described in dogs in 1984 [2], then in calves with myeloencephalitis, and was subsequently isolated and named in 1988 [3,4]. The parasite infects a wide range of animals and can cause illness in several species, most significantly in cattle and dogs, although it does not appear to cause significant infection or disease in humans [5]. The current incidence of neosporosis in dogs is not known, and seroprevalence rates range from 0% in Kenya to 29% in Italy [6], but it is noteworthy that higher levels of seropositivity in farm dogs correlate with the presence of infection in cattle on the same premises [7]. Neosporosis is a serious cause of abortion in cattle, although subclinical infection is very much more common [5].

Life cycle and cell invasion
Neospora undergoes a life cycle involving three principle stages. First, oocysts are produced in the faeces of dogs, the definitive host, following ingestion of bradyzoites [8]. Second, bradyzoites, which multiply slowly, are found in tissue cysts in the central nervous system (CNS), both in the canine definitive host and in a wide range of intermediate hosts. They represent a persistent, quiescent infection, held in check by host immunity [9]. Third, tachyzoites, the rapidly multiplying stage, trigger lesion development by multiplying in and rupturing cells. In the absence of a host immune response, tachyzoites would continue to multiply, causing progressively more cell death until the host dies. However, by extrapolation from *T. gondii*, it is assumed that, with the onset of the host immune response and the presence of other physiological factors, tachyzoites differentiate into bradyzoites and a persistent tissue cyst infection is established [10]. The occurrence of cell destruction, and therefore disease, depends upon a balance between tachyzoites being able to penetrate and multiply in host cells and the ability of the host to inhibit parasite multiplication.
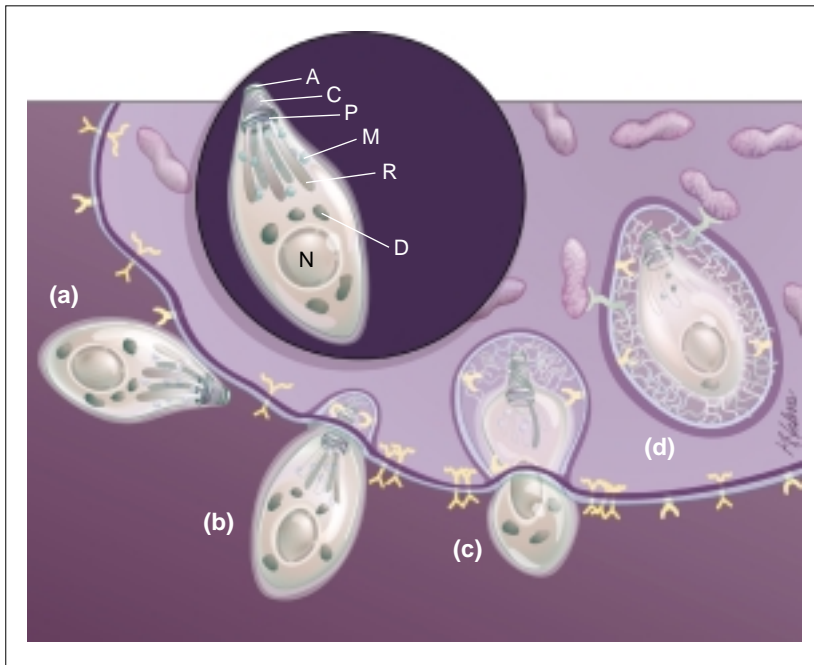
Fig. 1. Invasion of an apicomplexan tachyzoite such as *Neospora caninum* into a host cell. Inset figure: a depiction of the major ultrastructural features of the *N. caninum* tachyzoite. (a) Attachment: a tachyzoite is randomly orientated upon initial attachment to the host cell plasmalemma. (b) Initiation of invasion: the tachyzoite has reorientated itself to abut its apical end against the host. A tight attachment, called a moving junction, joins the parasite pellicle with the host plasmalemma. Organelles have begun to discharge their contents through the protruding conoid into the nascent parasitophorous vacuole. (c) Penetration: the tachyzoite pulls the moving junction posteriorly along its pellicle (against its cytoskeleton, which is not depicted). The parasitophorous vacuolar membrane (PVM) is created by inversion of the host plasmalemma as the parasite pushes in. Host surface proteins (yellow) having a cytoplasmic domain are excluded by the moving junction (process demonstrated using *Toxoplasma gondii* as an example). A tubulovesicular membrane network is forming within the parasitophorous vacuole. Non-transmembrane surface proteins of the host plasmalemma have passed the moving junction and protrude into the vacuole, but do not extend into the host cytoplasm. (d) An established parasitophorous vacuole: the PVM has separated from the host plasmalemma. Parasite proteins (green) span the PVM and create a PVM–organelle association. Abbreviations: A, apical ring; C, conoid; D, dense granules; M, microneme; N, nucleus; P, polar ring; R, rhoptries.

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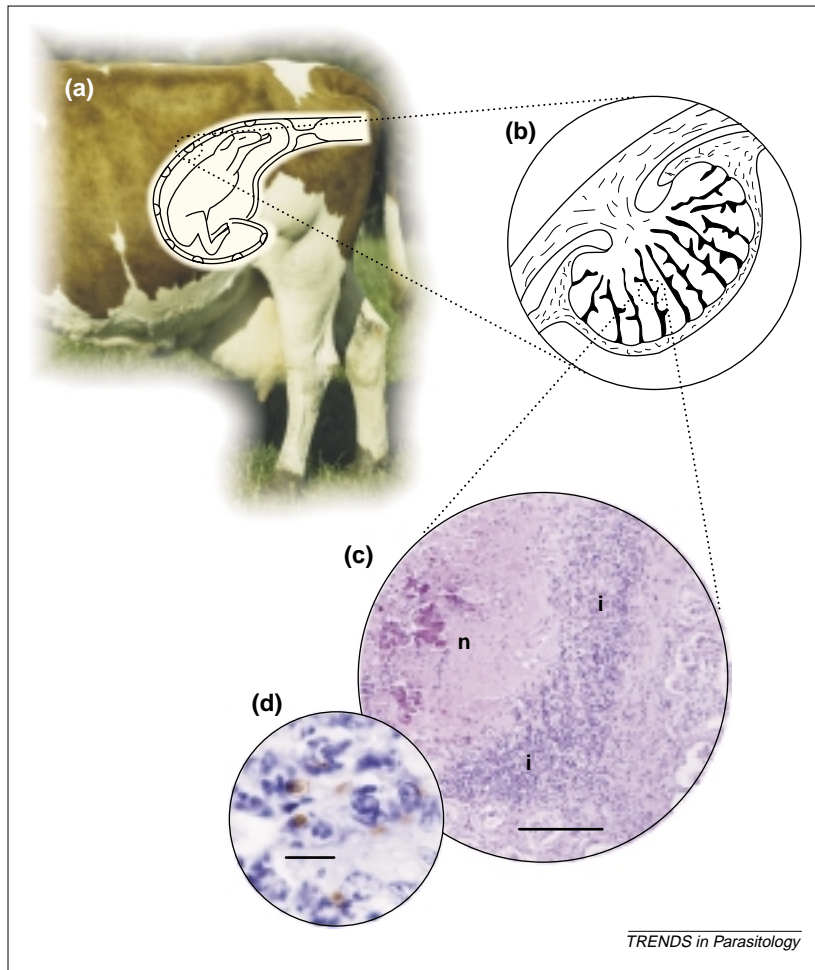
In the pathogenesis of neosporosis, cell invasion is a key element and the complex processes involved appear to be similar among apicomplexan species. These include surface receptors and a series of proteins that are released from micronemes, rhoptries and dense granules (Fig. 1). The initial attachment of the parasite to the host cell occurs without any consistent orientation [11] and involves immunodominant surface antigens such as *N. caninum* SAG-related sequence 2 (NcSRS2) [12]. After initial attachment, the parasite reorientates itself so that the anterior end abuts the host plasmalemma, the conoid is extruded, the host membrane becomes indented and various proteins are secreted into the nascent parasitophorous vacuole (PV) [13].

The outer membrane of the parasite forms a tight attachment zone with the host plasmalemma [11]; this is mediated by adhesin proteins secreted from micronemes [13]. This attachment zone forms a moving junction (MJ) that slides towards the posterior end of the parasite ('rearward capping') and pulls the parasite into the cell until it is entirely encased within the PV. The MJ is driven by the parasite cytoskeleton [14], without using energy from the host cell [11]. The host plasma membrane is used to form the parasitophorous vacuolar membrane (PVM), although host transmembrane proteins are excluded, which results in a vacuole that will not fuse with lysosomes [15].

In *T. gondii*, rhoptry proteins are discharged into the nascent PV and span the PVM to form a PVM–organelle association [16]. The PV of *N. caninum* also associates with host organelles, and a homologous rhoptry protein has been discovered [17], although its function has not been investigated. Mitochondria and endoplasmic reticulum of the host cell are thereby positioned adjacent to the PV. Dense granule proteins modify the PVM and contribute to the formation of an intravacuolar tubulo-vesicular membrane network.

Over a dozen secretory proteins and two major surface antigens have been identified from *N. caninum* as being associated with invasion and establishment of the PV. The functions of a few of these proteins have been investigated directly within *N. caninum*, but for many they have been inferred by analogy with homologous proteins that were previously described in *T. gondii* or other species. Research to test these assumptions is required.

A standardized system of naming proteins of *N. caninum* aids greatly in making interspecies comparisons [18]. Proteins located in micronemes are designated MIC or MAP (MIC-associated protein; e.g. M2AP); dense granules contain GRA proteins; rhoptries contain ROP proteins; and surface antigens are designated SAG or SRS. Microneme proteins of *T. gondii* are numbered TgMIC1, TgMIC2 and so on, and homologous proteins discovered in *N. caninum* are given an Nc prefix. Understanding the fine detail of cell invasion will explain why some cell types are more likely to be targeted than others, and why some species, such as rodents, appear to be more resistant to infection than other species, such as dogs and cattle.

Infection in cattle

Bovine infection can be established following ingestion of sporulated oocysts [19]. When this occurs during pregnancy, the parasite invades cells in the gravid uterus, providing an explanation for some outbreaks of abortion [20]. It is reasonable to assume that all foetal infections follow a maternal parasitaemia, but the majority also occurs in cattle that were already harbouring a persistent infection before pregnancy was established. Up to 95% of calves can be born clinically normal, yet seropositive, to cows that are themselves seropositive [21]. Thus, only in a minority does the foetus succumb to infection, to be expelled. In the UK, this represents 12.5% of diagnosed abortions [22], and while this figure could be somewhat higher or lower in different countries, losses are still of economic significance [23].

Why does a normally benign infection sometimes cause death? In part, the explanation lies in the stage

Fig. 2. (a) Pregnancy in cattle lasts ~280 days. In the gravid uterus, the maternofoetal interface can contain up to 100 placentomes. (b) A single placentome, consisting of a honeycomb of maternal caruncular septa, which interdigitate with a vast number of foetal placental villi. Here, the maternal immunomodulation that allows the foetus to develop without rejection by the dam does little to hinder the invasion of *Neospora* circulating in the maternal bloodstream. The intracellular multiplication of *Neospora* causes cell destruction (necrosis) and allows the parasite to spread to the foetal placental villi. Many foci of necrosis and inflammation, involving both maternal and foetal tissues, develop. (c) A focus of necrosis (n) and associated inflammation (i). (d) *Neospora* tachyzoites, coloured dark-brown, are characteristically infrequent. Scale bars: (c) = 200 µm; (d) = 10 µm.

of pregnancy at which the dam transmits infection to the foetus [24], which in most cases is dictated by the timing of recrudescence of a persistent maternal infection [25]. At the same time, the outcome will depend upon the age of the foetus and possibly also the magnitude of the parasitaemia and the characteristics of the particular strain of *N. caninum* [9].

In cattle, pregnancy lasts ~280 days, and the foetal immune system develops progressively throughout; hence, the calf is immunologically competent at birth. The foetus is particularly vulnerable during the first third of gestation, when the thymus, spleen and peripheral lymph nodes are first forming. During the middle third of pregnancy, these tissues start to recognize and respond to microorganisms [26].

Thus, before 100 days gestation (dg), the foetus is unable to recognize a pathogen such as bovine virus diarrhoea virus (BVDV) as being foreign [27] and those calves that do survive infection at this stage are

born immunotolerant to the virus, being both persistently infected with BVDV and seronegative for the virus. At ~100–150 dg, the foetus starts to be able to mount an immune response [26,27]; after 150 dg, it becomes progressively more competent at recognizing and responding in full to various pathogens [26]. Thus, in the first trimester, the foetus is exceptionally vulnerable to *N. caninum* infection, and is unlikely to survive. In the middle third of pregnancy, the foetus is able to launch a rudimentary immune response, which still might not be sufficient to save it because this is when the majority of abortions occur [24] and, in the third trimester, it is capable of an increasingly competent defence against the pathogen, leading to survival. As the majority of intrauterine transmissions result in the birth of clinically normal, infected calves, it is tempting to draw a correlation with human toxoplasmosis, where maternal to foetal transmission occurs more readily as pregnancy progresses, and this trend is inversely proportional to the foetal damage that ensues [28]. This suggestion is supported by the fact that the cell-mediated immune responses of pregnant cows to *N. caninum* are more effective in early pregnancy than mid-pregnancy [9]. With maternal parasitaemia and infection of the conceptus more likely to occur later in pregnancy, eliciting the factor(s) governing this timing is crucial to understanding why infection is sometimes triggered earlier in pregnancy to cause abortion.

In mammals, complex immunological mechanisms have evolved to allow the dam to nurture a foetus and not reject it as foreign [29]. While the immunology of pregnancy is subject to continuing debate, in ruminants (e.g. cattle, sheep, goats, deer) it is mediated through a maternofoetal interface consisting of up to 100 points of contact (placentomes). Each placentome comprises a foetal placental cotyledon intimately interdigitating with a maternal caruncle projecting from the inner surface of the uterus. Transfer of nutrients and oxygen from mother to foetus takes place within this structure, across the interface, between the maternal caruncular septa and the foetal placental villi (Fig. 2).

As a part of the very precise immunological balance that pertains in the placenta, beneficial maternal cytokines such as interleukin (IL)-10 and transforming growth factor (TGF)-β predominate, whereas potentially destructive cytokines such as IL-2, IL-12, interferon (IFN)-γ and tumour necrosis factor (TNF)-α are restricted [29]. Thus, the immunological balance that has evolved within this unique tissue to allow the mother to nurture an allograft might also favour certain microorganisms, including *N. caninum*. Any inflammatory response elicited by tachyzoite invasion and multiplication in maternal and foetal placental cells might then stimulate an inappropriate immune reaction [29] that could play a part in a subsequent abortion. This hypothesis remains to be proven, but it might be relevant that, in sheep, foetal production of TNF-α, a cytokine not normally present in the ovine placenta, is produced in the placenta in response to
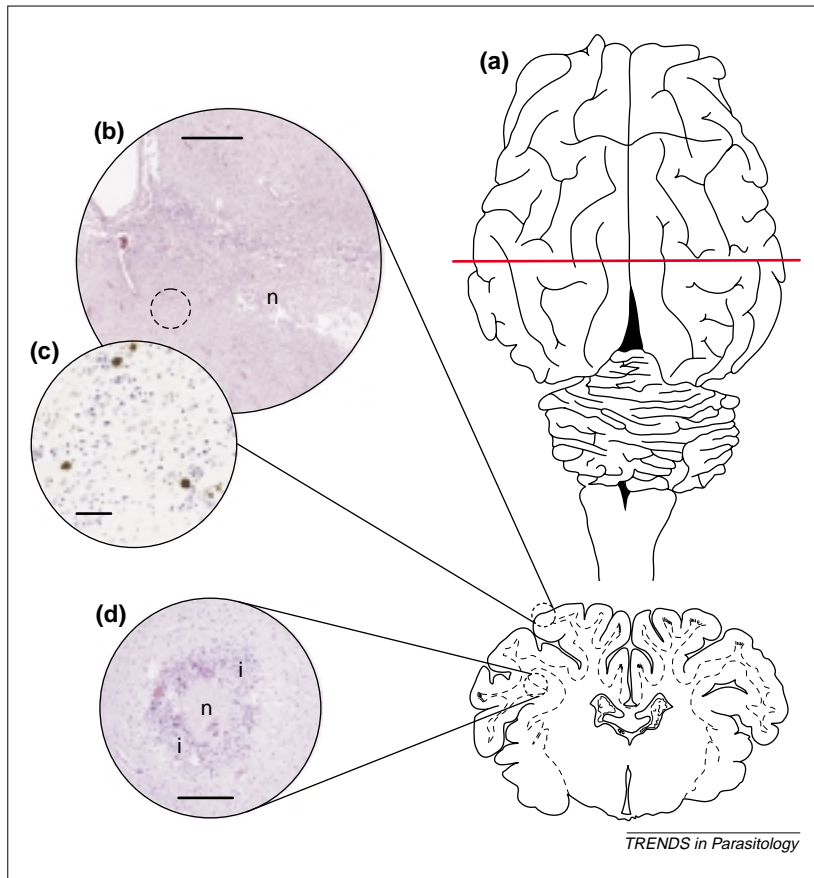
locates in and around blood vessels [25] and, in the younger foetus, its uncontrolled multiplication can cause lethal, widespread destruction of the neuropil, with relatively little inflammation (Fig. 3) [33]. In older foetuses, which are better able to respond to the parasite, multiplication is more restricted, and necrosis is confined to small foci of damage surrounded by a relatively intense foetal inflammatory response involving microglia, reactive astrocytes and cells of the monocyte and lymphoid series (Fig. 3) [31,32]. Associated mild meningitis might also be present. Destruction of foetal cells and associated lymphoid inflammation can also occur in the heart, skeletal muscle, lung and liver.

Other ruminants and pigs

Natural infection and disease in sheep and goats is uncommon [34,35], but their experimental inoculation with *N. caninum* during pregnancy causes pathology very similar to that observed in cattle [25]. Although persistent infection appears to establish in sheep [36], recrudescence of maternal infection is reported as being infrequent in experiments [37]. If this reflects better immunological control than that in cattle then this allows an interesting comparison with *T. gondii* because cattle are better able to control this parasite and so prevent significant disease [38], whereas, in sheep and goats, toxoplasmosis is an important cause of abortion and stillbirth in many countries [39]. However, ovine and caprine toxoplasmosis only follows a primary infection during pregnancy, subsequent immunity is protective and no significant incidence of recrudescence is recorded.

In pigs, transplacental neosporosis has been induced by the inoculation of tachyzoites during pregnancy [40], but natural disease has not been recorded. Foetal infection has also been recorded in Eld's deer (*Cervus eldi siamensis*) [41] and captive antelopes (*Tragelaphus imberbis*) [42], and fatal neosporosis has been described in a nonpregnant adult black-tailed deer (*Odocoileus hemionus*) [67]. While these appear to be rare occurrences of clinical disease, infection in white-tailed deer (*Odocoileus virginianus*) might be relatively common, suggesting that *Neospora* has a sylvatic cycle, at least in North America [43].

Dogs

Neosporosis was first reported in dogs in Norway [2], and it is now clear that canine infection occurs worldwide [6,7], although the proportion of dogs that develop clinical versus subclinical infection is very low. A subclinically infected bitch can transmit the parasite to her foetuses, and her successive litters can be born infected. Whether there is breed predisposition and differential sex susceptibility to neosporosis in dogs is not known, but most described cases have been in labrador and golden retrievers, boxers, greyhounds and basset hounds.

Dogs of any age can develop clinical disease, which can be either generalized, with virtually all organs

Fig. 3. (a) A foetal bovine brain viewed from above, with an outline of the cross-section made through the cerebrum at the level of the thalamus (red line). (b) Acute, fatal degeneration (liquifactive necrosis; n) in the cerebrum of a young bovine foetus naturally infected with *Neospora caninum*. (c) A magnified view of the broken circle in (b). This area of the cerebrum was immunohistochemically labelled to show individual tachyzoites and larger groups of parasites (probably bradyzoites in tissue cysts; coloured brown), indicating an active lesion. (d) A more-established lesion in an older foetus infected with *N. caninum*, showing a central focus of necrosis (n) encompassed by a thick band of foetal inflammatory cells (i). This lesion has been controlled by the foetus and is not life threatening. Scale bars: (b) = 500 μm; (c) = 20 μm; (d) = 200 μm.

infection with *Chlamydophila abortus*. It has been suggested that TNF-α could play a significant role in the subsequent collapse of pregnancy [30].

When *N. caninum* invades cells in the bovine uterus, it multiplies and causes focal destruction of both maternal and foetal tissue at the maternofoetal interface, as well as initiating an inflammatory response* (Fig. 2). From here, the damage extends out into the chorioallantois (the foetal placental membranes) between the cotyledons, both in naturally occurring disease [31] and in experimentally induced lesions [32]. How much of this damage is the direct result of parasite multiplication and how much is due to maternal (and foetal) immune responses to the parasite is not known.

At the same time as potentially lethal placental damage and associated maternal and foetal inflammation, the parasite enters the foetal bloodstream and invades further tissues, with a predilection for the CNS. Here, the parasite initially

*Maley, S.W. *et al.* (2001) Investigations into the early pathogenesis of neosporosis in experimentally infected pregnant cattle, p. 27, Proceedings of *Neospora* 2001, held 13–15 September 2001, in Edinburgh, UK.

involved (including the skin), or localized [44]. The most severe cases of localized disease occur in young, congenitally infected pups, which show an initial hind limb paresis that progresses to paralysis. Neurological signs are dependent on the site parasitized in the CNS and the hind limbs, which are usually more severely affected than the front limbs, are often in a rigid hyperextention. Dogs with hind limb paralysis can be alert and survive for months. Other dysfunctions that occur include difficulty in swallowing, paralysis of the jaw, muscle flaccidity, muscle atrophy and even heart failure.

Non-neurological clinical signs are also related to the cells parasitized, which include the vascular endothelium, myocytes and dermal cells [3]. It is perhaps significant that reported cases of canine cutaneous neosporosis have involved immunosuppressed or older animals [3,45] and the abundance of tachyzoites associated with the lesions also points to a lack of host immune control of parasite multiplication.

Congenital and postnatal neosporosis has been induced experimentally in dogs [46], but typical signs of congenital neosporosis, as observed in natural cases, have not been reproduced. Clinical disease has been produced in a blue fox (*Alopex lagopus*) inoculated with brain homogenate from a dog naturally infected with *N. caninum* [2], and inflammatory lesions and *N. caninum*-like tachyzoites were seen in the brain when it was examined ten weeks later. Currently, while there are no reports of clinical neosporosis in naturally infected canids other than dogs, antibodies to the parasite have been found in red foxes (*Vulpes vulpes*), grey foxes (*Urocyon cinereoargenteus*), American coyotes (*Canis latrans*) and Australian dingos (*Canis dingo*) [5].

Horses
Seroprevalence to *Neospora* in horses can exceed 10% [47], but only a few cases of clinical neosporosis have been reported and it is uncertain in these instances whether *N. caninum*, *N. hughesi,* or both were responsible. To date, clinical reports of neosporosis include two foetuses [47,48], a one-month-old foal, and five horses aged ten or more years and suffering with other concurrent diseases [47,49]. Except for the case of infection in an aborted foetus in France [48], all other reports were from horses infected in the USA, although in these countries antibodies to *Neospora* were recorded in 21% and 23% of clinically normal horses in France and USA, respectively [50,51]. Thus, whereas virtually nothing is known of the epidemiology of neosporosis in horses, it appears that they do encounter *Neospora* under natural conditions, but perhaps only succumb to disease when their immune system is compromised. It is of interest that the rhinoceros family is also in the order Perissodactyla (which includes horses), and neosporosis has been identified as the cause of death in a young white rhinoceros (*Ceratotherium simum*) calf in a game-breeding centre [52].

Experimental infections in other animals
Experimentally, *N. caninum* can infect other animals, including cats, rodents and birds. In cats, naturally occurring illness has not been reported, although clinical disease has been produced in pregnant animals following parenteral inoculation, with the dose of parasite, as well as the stage of pregnancy, influencing the outcome [53].

Although there are no reports of naturally occurring neosporosis in mice or other rodents, laboratory mice have been used in many studies of experimental neosporosis. It is difficult to induce clinical illness in outbred mice without the use of the immunosuppressor methylprednisolone because they are naturally resistant to the parasite [54], whereas inbred mice could be more susceptible [55]. Nude mice are especially sensitive to *N. caninum* and have been used for parasite isolation from cattle before transfer into cell culture [56]. Knockout mice that are IFN-γ–deficient or B-cell-deficient readily succumb to *N. caninum* [55].

When acute experimental neosporosis is induced in mice, lymphohistiocytic pneumonia is the predominant lesion [57] while, in mice developing subacute and chronic disease, the most significant lesions usually occur in the brain and spinal cord [54,57]. The lesions consist of lymphocytic meningitis, perivascular cuffing, multifocal gliosis and multifocal necrotizing encephalitis. Inflammatory lesions can also occur in other tissues such as the heart, skeletal muscles and liver. Tissue cysts of *N. caninum* have been found to develop in the brain of experimentally infected, immunosuppressed mice [58]. However, dogs produce fewer oocysts after consuming infected murine tissues, compared with dogs that consume infected bovine tissues [59], which suggests that mice are inefficient intermediate hosts.

Mouse models developed to study transplacental transmission of infection to foetal pups indicate that there is a higher rate of foetal infection and pathology if mothers are inoculated with tachyzoites in the first half of pregnancy compared with the second half of pregnancy [60]. Transplacental transmission can also occur in a second pregnancy, following experimental infection in the previous pregnancy, although the rate of foetal infection is much reduced [60].

Sprague Dawley rats (*Rattus norvegicus*) are naturally resistant to *N. caninum* infection [61], and treatment with 2 mg or 4 mg of methylprednisolone acetate per animal was required to induce clinical neosporosis. In those given 4 mg, fulminating disease was produced with 10^5 or more tachyzoites, with lesions in the liver, lungs and brain. However, tissue cysts were very rare.

Gerbils (*Meriones unguiculatus*) might have a role in neosporosis research [62,63] because they have been used successfully to test the infectivity of *Neospora* oocysts [62,64]. When gerbils were fed oocysts either of the NC-Liv or NC-2 isolates, they developed focal ulcerative enteritis in which parasites were readily found, and those that survived the enteric phase went on to develop pneumonia and encephalitis. Their relative susceptibility could make

them useful for detecting *Neospora* oocysts in canid faeces in epidemiological studies [64], although not all isolates of *N. caninum* are lethal for gerbils [64].

Rabbits are readily infected and have frequently been used to obtain polyclonal antibodies to *Neospora* but, even when inoculated parenterally with millions of tachyzoites, they remain clinically normal and parasites are not detected in their tissues [65].

Currently, there are no reports of natural *Neospora* infection in birds. Pigeons (*Columbia livia*) inoculated with 10^5 or more *N. caninum* tachyzoites developed patent infections, but zebra finches (*Peophila guttata*) inoculated with similar doses of tachyzoites were refractory [66], and antibodies and parasites were not found in four budgerigars (*Melopsittacus undulatus*) fed 10 000 sporulated oocysts of *N. caninum* (J.P. Dubey, unpublished).

Conclusions

Neospora caninum exists naturally throughout the world and can infect a wide range of animals, but consistently causes disease only in cattle and dogs. In both, *N. caninum* most commonly occurs as a symptomless persistent infection that might undergo recrudescence in the female host during pregnancy, resulting in a maternal parasitaemia and infection of the gravid uterus. Infection passes readily to the placenta and foetus, where the age, and hence the immunological maturity, of the foetus can significantly influence the outcome, at least in cattle.

In other pregnant ruminants, such as sheep, goats, deer and antelope, fatal foetal infections have been recorded. However, infection might be relatively uncommon under natural conditions, although experimental disease is readily achieved. Therefore, it would seem that maternal immunity is better at preventing recrudescence of the parasite in these animals when compared with cattle and dogs. Whether these other ruminants encounter *N. caninum* less frequently under natural conditions (possibly with some exceptions [43]) compared with cattle, and/or whether initial infection is less readily established in them as a result of innate resistance, is not clear.

Neospora caninum has evolved a strategy for survival and perpetuation by living in especially close harmony with its animal host. In cattle, it has reached a particularly fine balance whereby it is able to exploit the niche that the altered immune status of pregnancy provides in order to transmit itself to the next generation. If this equilibrium is altered, such as when foetal infection occurs earlier in pregnancy or when the immune system of other normally resistant animals is compromised, *N. caninum* is able to multiply unchecked to kill its host, to the obvious detriment of the host as well as the parasite in many cases. Why cattle are apparently more susceptible than other animals, particularly ruminants, such as sheep and goats, is only one of the many questions that neosporosis presents.

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