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NEOSPORA CANINUM AND NEOSPOROSIS IN ANIMALS – A REVIEW

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Summary

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The cyst-forming protozoan *Neospora caninum*, discovered in 1984 in dogs with encephalitis and myositis, is described as a separate species. The biological cycle of *N. caninum* involves two hosts: definitive and intermediate. Known definitive hosts are dogs and coyotes. As intermediate hosts, cows, sheep, goats, horses and deers are reported. The dog could be both intermediate and definitive host. The life cycle of *N. caninum* is characterized by 3 infection stages tachyzoites, tissue cysts and oocysts. The transmission routes of *N. caninum* are two: horizontal or oral and vertical or transplacental. The definitive hosts shed oocysts that are a potential source of horizontal transmission via infected food and water. At the same time, the vertical transmission from infected dams to their offspring, is also one of the natural routes of infections in cows. Transplacental infection could recur in the same animals. In cows, abortions are the main clinical signs. The cows at various ages could abort from the 3rd months of gestation onward, but abortions by the 5th – 6th month are predominant. Two categories of abortions are distinguishes: epidemic, when a higher percentage of cows abort within few weeks and endemic, when abortions occur within months or years. The serological analysis of sera from cows with abortions is indicator of a *N. caninum* infection. The final diagnosis is made through histological and immunohistological analysis of specimens from foetuses or by detecting *N. caninum* DNA by PCR. The epidemiology of infection and related abortions is still inadequately studied. The entity of risk factors, influencing the infection and related abortions at the farm level is still incompletely identified. The annual economical losses due to decreased production and losses from aborted foetuses amount to millions of dollars. By reducing the risk of faecal contamination of food and water and destroying aborted foetuses and placentas, the possibility for infection of potential hosts of *N. caninum* could be limited. In order to prevent the vertical transmission, serological investigations in animals and creation of reproductive programmes in farms according to the seroprevalence are recommended.

The present review attempts to summarize both the known information and our views about the life cycle of the parasite, the pathogenesis and clinical signs of the disease, the mechanisms of transmission, the diagnostic methods and the means of infection control.

Key words: abortions, cattle, dogs, goats, horses, life cycle, *Neospora caninum*, parasitic diseases, wild animals

INTRODUCTION

Neospora caninum is a recently discovered protozoan that was wrongly identified as *Toxoplasma gondii* up to 1988. A disease resembling neosporosis was reported for the first time by Bjerkas *et al.* (1984). The authors identified a cyst-forming protozoan in newborn puppies with nervous signs in Norway. In sera

from these dogs, no antibodies against *T. gondii* have been detected. Dubey *et al.* (1988a) found similar parasites in 10 dogs in the USA. Having differentiated these parasites from *T. gondii,* the authors referred them to a new genus – *Neospora*, while the species was determined as *Neospora caninum.*

N. caninum is highly pathogenic for cows and dogs and could also induce a disease in sheep, goats, horses and deers. The infection with *N. caninum* is one of the main causes of abortions in cows in many countries over the world. The economical losses caused by abortions, reduced productivity and reproductive pathology are estimated to millions of dollars.

The literature reviews of Dubey & Lindsay (1996), Dubey (1999, 2003a, 2003b) and Toolan (2003) provide information about the biology, epidemiology, pathogenesis, clinical manifestation, diagnosis and prophylaxis of neosporosis in animals, updated with the newest scientific facts. So far, not all hosts of the protozoan as well as the routes of transmission, the risk factors for the infection, the methods of prevention and control are determined.

The incomplete information that is available so far and the necessity of complex knowledge on the problem provoked the performance of a detailed assessment and analysis of existing literature data and the composition of the present literature review.

GENERAL FEATURES OF *N. CANINUM* INFECTION

Biological cycle

N. caninum is an Apicomplexa coccidian protozoan. Its biological cycle involves the participation of two hosts. Dogs (McAllister *et al.*, 1998) and recently coyotes (Gondim *et al.*, 2004) are described as definitive hosts. Apart definitive, the dog could be an intermediate host too (McAllister *et al.*, 1998; Lindsay *et al.*, 1999a, 1999b; Basso *et al.*, 2001a; Dubey *et al.*, 2002). As intermediate hosts of *N. caninum* are shown also cows (Dubey *et al.*, 1990), goats (Dubey *et al.*, 1992; Barr *et al.*, 1992), horses (Daft *et al.*, 1997) and deers (Dubey *et al.*, 1996b). Antibodies against *N. caninum* are also detected in sera from naturally infected buffaloes, coyotes, red foxes and camels and it is suggested that these species are also natural intermediate hosts (Lindsay *et al.*, 1996; Buxton *et al.*, 1997; Simpson *et al.*, 1997; Dubey *et al.*, 1998). According to Bartles *et al.* (1999) and Ould-Amrouche *et al.* (1999) birds could also be reservoir hosts, but this hypothesis needs further confirmation.

The life cycle of *N. caninum* comprises three infection stages: tachyzoites, tissue cysts containing bradyzoites and oocysts. Tachyzoites and tissue cysts are stages, detected in intermediate hosts. Both forms are intracellular and are localized in host cell' cytoplasm, with or without parasitoform vacuole. The size of tachyzoites is about 6 × 2 μm. In infected intermediate hosts they are found in nervous cells, macrophages, fibroblasts, vascular endothelial cells, myocytes, renal tubular epithelial cells and hepatocytes (Dubey *et al.*, 1988a; Dubey & Lindsay, 1989; Dubey *et al.*, 2002).

Tissue cysts are with an oval or round shape and size up to 107 μm. They are detected in the brain, the spinal cord and the retina of intermediate hosts. The bradyzoites enclosed into the cyst are 7–8 × 2 μm in size (Dubey *et al.*, 1988a; Dubey & Lindsay, 1996). Oocysts with dimen-

sions 11.7 × 11.3 μm are shed with the faeces of definitive hosts. They sporulate in the environment and become infective for intermediate hosts (Lindsay *et al.*, 1999a; Lindsay *et al.*, 2001; Slapeta *et al.*, 2002).

Dogs, as definitive hosts, are infected through eating of foetal membranes, placenta, organs of aborted foetuses containing tachyzoites or tissue cysts (McAllister *et al.*, 1998; Lindsay *et al.*, 1999a). A sexual reproduction takes places in canine intestines and consequently, non-sporulated oocysts are shed for 5 to 17 days after the infection with tissue cysts. The oocysts' size is 11.7 × 11.3 μm and they sporulate in the environment for 3 days. Sporulated, infective oocysts contain 2 sporocysts with 4 sporozoites in each (Lindsay *et al.*, 1999a; Lindsay *et al.*, 2001; Slapeta *et al.*, 2002). Despite the opinion of cited authors that dogs are shedding a small number of oocysts, Gondim *et al.* (2002) reported recently that dogs, fed tissues from experimentally infected cows, shed more than 100 000 *N. caninum* oocysts. Yet, little is known about the frequency of oocyst shedding, the time of their survival in the environment or whether other canides are their definitive hosts.

Intermediate hosts could be infected via food and water contaminated with sporulated *N. caninum* oocysts. Sporozoites are freed into the intestines, penetrate the host cells and then, became transformed into tachyzoites. The latter are rapidly replicated by endodyogeny in host cells and after destroying them, penetrate into new, intact cells (Dubey, 1999; Innes *et al.*, 2001). In nervous cells, tachyzoites could be transformed into bradyzoites (slowly replicating via endodyogeny), when a strong immune response against the protozoan is present. Around the bradyzoites, tissue cysts are formed and within, they remain in a latent state. In immunosuppressive states of the intermediate host (McAllister *et al.*, 1998; Dubey, 1999) bradyzoites could be reactivated.

Tachyzoites could be transmitted vertically from the mother to the foetus via the placenta in case of primary infection with oocysts or during reactivation of tissue cysts. The infection of the embryo could results in abortion, neurological affection of offspring or congenital infection without clinical manifestation. A repeated transplacental infection in the same animals is possible (Dubey & Lindsay, 1996; Davison *et al.*, 1999).

A transplacental infection was experimentally reproduced in cows, sheep, mice, dogs, cats and swine (Dubey *et al.*, 1992; Barr *et al.*, 1993; McAllister *et al.*, 1996; Buxton *et al.*, 1997, 1998; Jensen *et al.*, 1998).

Clinical signs

The commonest clinical signs in dogs younger than 6 months are paresis and paralysis of hindlimbs. Infected dogs could also exhibit difficulties in swallowing, myocarditis, dermatitis and pneumonia (Hay *et al.*, 1990; Odin & Dubey, 1993; Dubey *et al.*, 1995; Greig *et al.*, 1995; Ruehlmann *et al.*, 1995).

Abortions are the first clinical sign in intermediate hosts. The liveborn congenitally infected animals often do not manifest clinical signs of disease. In other cases they could be with a lower body weight at birth, no weight gain, with contracted or strongly extended limbs and neurological signs including ataxia, decreased patellar reflex and lack of orientation (Dubey, 1999).

Pathoanatomical changes

N. caninum causes cellular death accompanied by visible necrotic lesions in several days due to active replication of tachyzoites. This could result in sever nervous and muscular disease because of nervous cell destruction, including brain and spinal cord cells and due to the effect upon the conductivity of infected cells (Mayhew *et al.*, 1991; Dubey & Lahunta, 1993).

Tissue cysts are often surrounded with a cellular reaction zone. Although the duration of tissue cysts' persistence is not known, Lindsay *et al.* (1992) observed that they remain vital in the tissues of experimentally infected mice for an year. The formation of granulomas around degenerated tissue cysts and bradyzoites was probably due to formation of inflammation foci consequently to host response to their destruction (Dubey *et al.*, 1992, 1996b).

In 85% of cases, the lesions of aborted foetuses are localized in the central nervous system, although the heart, skeletal muscles, liver and kidneys could also be affected (Barr *et al.*, 1990; Anderson *et al.*, 1991; Wouda *et al.*, 1997).

Epidemiology

After detecting that the definitive hosts of *N. caninum* shed oocysts with faeces, the horizontal route of transmission was discovered by McAllister *et al.* (1998). Regardless of the successful experimental infections of intermediate hosts with oocysts, the transmission mode of infection in natural conditions is still not clear.

In intermediate hosts, the vertical route of transmission from infected cows to offspring is the best studied (Pare *et al.*, 1996; Anderson *et al.*, 1997). Experimentally, the colostrum has also been shown as carrier of infection (Uggla *et al.*, 1998).

In dogs the efficacy of vertical transmission is very variable although in some publications, a transmission from a bitch to her puppies is reported (Dubey *et al.*, 1988b, Dubey & Lindsay, 1989).

By now, a comparative evaluation about the role of horizontal and vertical routes of transmission was not performed.

Diagnosis

The oocysts of *N. caninum* in definitive hosts are detected by investigations of faeces using flotation methods (McAllister *et al.*, 1998; Lindsay *et al.*, 1999a). Morphologically, they are identical to oocysts of *Toxoplasma gondii* and *Hammondia hamondii* in feline faeces and *Hammondia heydorni* in canine faeces (Schares *et al.*, 2001a; Dubey *et al.*, 2002). As the microscopy is not enough for differentiation between *N. caninum* from *H. heydorni* oocysts , Hill *et al.* (2001) and Slapeta *et al.* (2002) have developed genetic methods for differentiation of species.

Serologically, it could not be said whether the dog sheds oocysts because the presence of titres does not correlate with oocyst shedding (McAllister *et al.*, 1998).

The dogs as intermediate hosts in whose tissues unsexual replication occurs, manifest clinical signs such as hindlimb hyperextension resulting in progressive spastic paralysis, only in rare cases. Regardless of manifested signs, such dogs do not shed oocysts (Dubey *et al.*, 1988a; Scheahan *et al.*, 1993; Barber & Trees, 1996).

The detection of antibodies against *N. caninum* in intermediate hosts is performed by several serological tests: indirect fluorescence assay (IFA), immunosorbent analysis (ELISA), direct agglutination (DA) and Western blot analysis

(WB) (Pare *et al.*, 1995a, 1995b; Dubey & Lindsay, 1996; Baszler *et al.*, 1996; Bjorkman & Uggla, 1999). The positive findings of applied serological tests indicate only that the animal has been infected with *N. caninum*, but do not provide a definitive diagnosis in case of abortions.

The analysis of tissues from aborted foetuses for presence of specific lesions, tissue cysts and tachyzoites is necessary for confirmation of diagnosis. Specimens of choice are those from the brain although most often, tissue cysts and tachyzoites could be detected in lungs, kidneys and skeletal muscles too. Specific lesions are determined by histological examination of tissues but the identification of the causative agent is performed via immunohistochemical analysis and polymerase chain reaction (PCR) (Lindsay & Dubey, 1989; Dubey & Lindsay, 1996; Jenkins *et al.,* 1997; Cole *et al.*, 1993; Müller *et al.*, 1996; Gottstein *et al.*, 1998).

Prevention and control

Both the prevention and control are directed towards reduction of postnatal and congenital infection with *N. caninum*. The restriction of postnatal infection is focused on decreasing the risk of infection with oocysts, shed with the faeces of definitive hosts. For this purpose, reduction of populations of farmers' dogs and limitation of their access to food and water sources of intermediate hosts, to carcasses of aborted foetuses or dead animal are recommended (McAllister *et al.*, 1998; Toolan, 2003).

As the congenital transmission is most commonly encountered in cows, the prevention measures are related to determination of seroprevalence in the respective farm and histopathological study of aborted foetuses (Pare *et al.*, 1996; Davison *et al.*, 1999). In highly infected farms, the results of serological studies are used in the elaboration of reproductive schedules.

At present, there are no effective means for treatment of neosporosis. There is a vaccine produced by Intervet and licensed in the USA but the results of its application in practice are still under observation.

NEOSPOROSIS IN DOGS

Biological cycle

The experimental infection in dogs and subsequent oocyst shedding is reproduced after feeding with brain (McAllister *et al.*, 1998; Lindsay *et al.*, 1999a; Gondim *et al.*, 2004; Rodrigues *et al.*, 2004), various tissues or carcasses, including brain or spinal cord (Schares *et al.*, 2001a; Gondim *et al.*, 2002), heart and skeletal muscles (Schares *et al.*, 2001a) of intermediate hosts or placenta of infected cows (Dijkstra *et al.*, 2001). Bergeron *et al.* (2001) and Dijkstra *et al.* (2001) have found no oocyst shedding in a dog that have consumed naturally infected bovine foetuses and milk containing *N. caninum* tachyzoites. Little is known about sources producing infection in dogs shedding *N. caninum* oocysts in natural conditions (Basso *et al.*, 2001a, 2001b; Slapeta *et al.*, 2002; McGarry *et al.*, 2003). As the oocysts of the two species *N. caninum* and *H. heydorni* whose definitive host are dogs could not be morphologically distinguished, Dubey *et al.* (2002) suggested their identification in isolates to be performed by a biological test and molecular methods. Through the performance of a screening study in Germany on 24 089 canine faecal samples, Schares *et al.* (2005) observed oocysts similar to those of *Neospora* or *Hammondia* in 47 out of them. After performance of a biological

test and PCR, *N. caninum* oocysts were detected in only 7 isolates.

A congenital neosporosis was experimentally reproduced in bitches via subcutaneous and intramuscular inoculation of tachyzoites from tissue cultures (Dubey & Lindsay, 1989; Cole *et al.*, 1995). The bitches remained clinically healthy, but the *N. caninum* infection could cause an early embryonic death, mummification, absorption or birth of weak puppies with neurological signs. Barber & Trees (1998) described for the first time cases of naturally acquired vertical transmission of *N. caninum* in dogs.

Clinical signs

Diarrhoea in dogs, accompanied by *N. caninum* oocysts shedding, was described by Blagburn *et al.* (1988), Basso *et al.* (2001a) and Gondim *et al.* (2002).

Clinical cases of neosporosis were observed by Lindsay & Dubey (2000), Boydell & Brogan (2000) and Cantile & Arispici (2002). The heaviest form of neosporosis is encountered in young, congenitally infected dogs. Young dogs develop hindlimb paresis that advance to progressive paralysis. Other observed dysfunctions are: difficult swallowing, paralysis of jaws, muscle weakness, muscular atrophy and even cardiac troubles. The dogs with hindlimb paralysis could move and even survive for several months. The disease could be localized or generalized, with affection of all organs, including the skin. The dermatitis could be severe, caused by a huge number of *N. caninum* parasites. Four cases of skin form of neosporosis, due to mixed infection with *Leishmania* sp. (Tarantino *et al.*, 2001; La Perle *et al.*, 2001; Ordeix *et al.*, 2002) are reported.

Dogs at various age could be affected. Cases of nodular dermatitis, polymyositis and multifocal encephalitis are described in dogs aged between 18 months to 6 years (Knowler & Wheeler, 1995; Patitucci *et al.*, 2001).

Subclinically infected bitches could transmit the parasite to their foetuses and the offspring of such bitches could be born infected. It is not known whether there is a breed or gender predisposition to neosporosis in dogs. Most reported cases are in Labrador retrievers, Boxers, Greyhounds, Golden retrievers and Bassets.

Diagnosis

The clinical signs shown by dogs infected with *N. caninum*, could aid to the in life diagnosis.

The dogs infected with *N. caninum* are shedding unsporulated oocysts whose size and morphology are similar to those of *Hammondia heydorni*. That is why, the use of molecular methods for differential diagnosis at this stage is mandatory (Dubey *et al.*, 2002, Gondim *et al.*, 2004).

The blood serological analysis is also used for in life diagnosis. IFAT is the most commonly used diagnostic test. However, the detection of antibodies only by IFAT is not indicating clinical neosporosis (Lindsay *et al.*, 1990). A small number of cross reactions between *N. caninum* and *T. gondii* are reported by Dubey *et al.* (1995); Trees *et al.* (1993) in naturally infected dogs. Significant part of dogs, shedding *N. caninum* oocysts following experimental infection, showed no seroconversion when studied for *N. caninum* by IFAT (McAllister *et al.*, 1998; Lindsay *et al.*, 1999a; Dijkstra *et al.*, 2001; Schares *et al.*, 2001a; Gondim *et al.*, 2002). The immunoblot test against the 152 kDa antigen of *N. caninum* could however serve as an useful indicator for a preceding infection (Schares *et al.*,

2001b). Antibodies against *N. caninum* were also determined by iscom- ELISA from Bjorkman *et al.* (1994).

The post mortem diagnosis is based on the detection of parasites in lesions of affected dogs. Big lesions, characteristic for neosporosis, are CNS and liver necroses (Dubey *et al.*, 1988a), granulomas (up to 1 cm diameter) in visceral tissues (Dubey *et al.*, 1988a), yellowish-white strips in muscles, especially in the diaphragm (Dubey *et al.*, 1988a; Dubey & Lindsay, 1990), cerebral atrophy (Bjerkas *et al.*, 1984; Jackson *et al.*, 1995) and ulcerative dermatitis (Dubey *et al.*, 1988a; Dubey *et al.*, 1995). For confirmation of the diagnosis, immunohistochemistry tests using specific antigens are applied.

N. caninum was isolated mainly from dogs showing nervous and muscle signs (Peters *et al.*, 2000; Basso *et al.*, 2001a).

Prevalence

Antibodies against *N. caninum* are reported in 121 out of 320 (37.8 %) examined dogs in Argentina (Basso *et al.*, 2001b); in 22 % of 200 dogs in New Zealand (Reichel *et al.*, 1998); in 10 % of 150 dogs in Turkey (Coşkun *et al.*, 2000); in 6.7 % of 163 dogs in Brazil (Mineo *et al.*, 2001); in 10 % of 500 domestic dogs and 25 % of 611 stray dogs in Brazil (Gennazi *et al.*, 2002); in 6.4 % of 1058 dogs in Italy (Cringoli *et al.*, 2002), in 12 % of 120 urban and 26 % of 81 rural dogs in Chile (Patitucci *et al.*, 2001). Klein and Müller (2001) detected antibodies in 4 % of 50 dogs in Germany without clinical signs and in 13 % of 200 dogs with clinical manifestation. Antibodies against *N . caninum* were evidenced in 21% of 134 dogs in cattle farms in Brazil too (de Souza *et al.*, 2002).

Comparative studies about the incidence of neosporosis among urban and rural dogs were performed. Sawada *et al.* (1998) detected antibodies in 31 % of 48 dogs from dairy farms and in 7 % of 198 dogs from urban areas in Japan. Wouda *et al.* (1998) reported a more extensive distribution in farm dogs (23.6 % of 152), than in urban dogs (5.5 % of 344) in Holland. Basso *et al.* (2001b) observed a higher seroprevalence in dogs from dairy farms (48 % of 125) beef-cattle farms (54.2 % of 35) than in dogs from urban regions (22.2 % of 160) in Argentina.

NEOSPOROSIS IN COWS

Biological cycle

Thilsted & Dubey (1989) were the first to discover *N. caninum*-like organism in the brain of bovine foetuses originating from a farm in New Mexico with persisting abortions. The diagnosis was definitively confirmed after obtaining a specific serum against *N. caninum* by Lindsay & Dubey (1989) that allowed to be shown that parasites in bovine foetal tissues react with antibodies against *N. caninum*.

The cows could be infected either postnatally (via horizontal transmission) or congenitally (via vertical transmission). The postnatal infection is realized via food and water, contaminated with faeces of definitive hosts that are shedding oocysts. If the cow is pregnant, the infection could be transmitted transplacentally and an abortion could follow. The vertical transmission is encountered more frequently than the horizontal one and the infection of foetus ends commonly not with abortion but with giving birth to an infected calf. The heifers originating from transplacentally infected calves could transmit the infection to the next generation when pregnant (Anderson *et al.*,

2000).

The infection of cows with *N. caninum*, either natural or experimental, could result in reproductive losses. The foetuses could die intrauterinely, be absorbed, mummified, autolyzed. A preliminary delivery of living, but ill or clinically normally born but chronically infected calves could also occur (Dubey, 1999). The gestation period, during which the cow is infected, determined the outcome of pregnancy. The early infection results in absorption of the foetus, while the infection after the 6 month of gestation could end with stillbirth or congenital infection (Anderson *et al.*, 2000).

Clinical signs

Clinical signs are described only in congenitally infected calves, younger than 2 months (Barr *et al.*, 1990; Dubey & Lahunta, 1993). According to Barr *et al.* (1990), the clinical signs are observed most commonly 3 to 5 days after birth but could also appear later – 2 to 3 weeks after birth. The calves infected with *N. caninum* are born with a lower body weight, unviable or without clinical signs of the disease. The hindlimbs or/and the forelimbs could be bent or extremely extended. The neurological examination shows ataxia, decreased patellar reflex and lack of orientation. Sometimes, an exophtalmus, asymmetry of the eyes, hydrocephaly or narrowing of the vertebral column are observed (Dubey & Lahunta, 1993; Bryan *et al.*, 1994).

Abortions are the principal clinical sign in cows (Dubey & Lindsay, 1996). *N. caninum* causes abortions in both dairy and meat cows, at various ages, from the 3rd month after fertilization onward. Most abortions caused by *N. caninum* occur by the 5th–6th month of gestation (Dubey & Lindsay, 1996; Anderson *et al.*, 2000). Although the pathophysiology of abortions caused by *N. caninum* is not well studied, the foetus is most commonly autolyzed, with placentitis and placental oedema as accompanying factors. The *N. caninum* induced abortions, rarely result in retention of the placenta or development of metritis (Dubey *et al.*, 1996a; Dubey, 2003a).

Seropositive cows (with proved antibodies against *N. caninum*) abort more frequently than seronegative ones (Thurmond & Hietala, 1997b; Moen *et al.*, 1998). *N. caninum* could cause repeated abortions in a small percentage (about 5%) of cows (Obendorf *et al.*, 1995; Wouda *et al.*, 1995; Anderson *et al.*, 1995; Dannatt *et al.*, 1995; Moen & Wound, 1995).

Immunity

Initially, the fact that the abortion could be repeated in seropositive cows, cast suspicion on the development of the protective immunity against neosporosis in natural conditions (Barr *et al.*, 1993; Thurmond & Hietala, 1997a). Further studies showed that natural immunity against *Neospora*-induced abortion develops for a long period of time. The prevalence of abortions in acute infections (seronegative cows) is higher than those in chronic infections (seropositive dams) (McAllister *et al.*, 2000). In the latter, the cases of abortions decrease with the number of successive pregnancies (Anderson *et al.*, 1995; Wouda *et al.*, 1998). These results show that some levels of immunity develop in chronically infected animals and that it is reinforced during the succeeding pregnancies.

As *Neospora* is an intracellular parasite, it could be expected that cell mediated immunity (CMI) plays an essential role. In cows, infected with *N. caninum*

(naturally or experimentally), specific antibodies and a CMI response are evidenced (Innes *et al.*, 2002). In cows, experimentally inoculated with tachyzoites, the production of specific for the parasite CD4 + cytotoxic T-lymphocytes (CTL), able to kill cells autoinfected with *N. caninum* is shown (Staska *et al.*, 2003). This fact also substantiates the hypothesis that the CMI response plays an important role in protective immunity. If the main protective response in naturally infected cows is cellular, the status of pregnancy could change the host-parasite balance and thus, to increase the number of parasites. In chronically infected cows, bradyzoites are activated and differentiated into infective tachyzoites thus manifesting the altered equilibrium between the host protective response and the parasite balance. Data for reactivation of *N. caninum* infection in pregnant cows are obtained by serological studies too. It is proved that in chronically infected cows, abortions are preceded by increased titres of specific anti-*N. caninum* antibodies (Dubey, 2003a). The increased antibody titres does not necessarily result in abortions. This depends on the period of gestation. The early reactivation produced an absorption, that prior to the 20^{th} week of gestation – to abortions and after the 20^{th} week – to premature births or birth of congenitally infected calves (Anderson *et al.*, 1997; Williams *et al.*, 2000). There is a substantial difference between the type of immunity against *N. caninum* in cows that are infected with the parasite during the foetal development and these that become infected after the parturition. When heifers are experimentally infected with *Neospora* prior the pregnancy and are challenged with live tachyzoites in mid-gestation, they give birth to living, non-infected calves (Innes *et al.*, 2001) unlike cows, that become infected for the first time during the pregnancy. Apparently, the immunization of heifers with live tachyzoites vaccine is possible, similarly to the circumstances with toxoplasmosis. Congenitally infected cows could also be preserved from abortions by challenge with live tachyzoites by the 10^{th} week of gestation (Williams, 2001) or by vaccination with killed tachyzoites, processed with adjuvant during the first third of gestation.

Pathogenesis

Little is known about the pathogenesis of *N. caninum* lesions and the neonatal death rate in cows. Tachyzoites and tissue cysts are detected in considerable amounts in both early and later stages of gestation (Anderson *et al.*, 1991; Barr *et al.*, 1991). Tissue cysts prevailed in stillborn calves or calves necropsied at an age of < 7 days (Dubey & Lindsay, 1990, Barr *et al.*, 1991). Degenerative to inflammatory lesions could be found out in foetal tissues. Most commonly they are localized in the central nervous system (CNS), the heart, skeletal muscles and liver (Barr *et al.*, 1990; Barr *et al.*, 1991; Wouda *et al.*, 1998). The characteristic CNS lesions are organized as a central necrotic zone, infiltrated by inflammatory cells (glial or mononuclear). Most commonly, foetuses are autolyzed or mummified (Thornton *et al.*, 1991; Nietfeld *et al.*, 1992).

Epidemiology

The *N. caninum* infection in cows is most efficiently realized via vertical transmission that could be manifested in some consecutive generations (Bjorkman *et al.*, 1996; Anderson *et al.*, 1997; Schares *et al.*, 1998). According to Toolan (2003) this is a very efficient means for preserving the infection within the population. The horizontal transmission is necessary

for introduction of a new infection in the farm (Pare *et al.*, 1996, 1997; Thurmond & Hietala, 1997a; Wouda *et al.*, 1998; French *et al.*, 1998; Schares *et al.*, 1998).

A horizontal transmission from cow to cow is not proved (Dubey, 1999). In an experiment carried out by Anderson *et al.* (1997), 25 seronegative heifers were reared together 25 seropositive heifers. Their progenies were examined for *N. caninum* infection. Seronegative heifers remained seronegative and gave birth to non-infected calves. Seropositive heifers remained clinically healthy but gave birth to congenitally infected calves. The necropsy of 7 congenitally infected calves revealed histological changes, characteristic for *N. caninum* infection. Guy *et al.* (2001) housed 3 non-infected cows together with seropositive pregnant ones, but no seroconversion was observed.

Davison *et al.* (2001) did not succeed to accomplish transmission of *N. caninum* in susceptible cows by feeding infected placentas.

A lactogenic transmission is experimentally evidenced in newborn calves fed with tachyzoites-infected colostrum, but there are no data for a natural infection of this kind (Davison *et al.*, 2001).

Baillargeon *et al.* (2001) proved experimentally that *N. caninum* could not be transmitted venereally or via embryo transfer. The authors recommend the practical performance of embryo transfer as a means of prevention of vertical transmission.

The fact that once the cow is infected either via horizontal or vertical transmission, the protozoan persisted in its organism as a chronic infection that could be transmitted to the foetus during the gestation, is very important from epidemiological point of view (Anderson *et al.*, 2000).

Abortions due to *N. caninum* infection are encountered all year round (Anderson *et al.*, 1991; Thurmond *et al.*, 1995; Moen & Wouda, 1995). In California, more cases are detected in winter than during summer or early autumn (Anderson *et al.*, 1991). According to Moen & Wouda (1995) the abortions in Holland due to *N. caninum* infection are more common during summer than in autumn.

N. caninum infections in cows are evidenced in the USA (Anderson *et al.*, 1991;1997), New Zealand (Thornton *et al.*, 1991), Holland (Wouda, 1998), Argentina (Campero *et al.*, 1998; Basso *et al.*, 2001b), Belgium (de Kruif *et al.*, 1997), Canada (Duivenvoorden & Luis, 1995), Denmark (Agerholm *et al.*, 1997), Germany (Conraths *et al.*, 1996), Hungary (Hornok *et al.*, 1998), Italy (Magnino *et al.*, 1998), Spain (Fondevila *et al.*, 1998), Sweden (Stenlund *et al.*,1997), the United Kingdom (Graham *et al.*, 1996; Trees & Williams, 2000), Austria (Edelhofer *et al.*, 2003), Switzerland (Gottstein *et al.*, 1998), the Czech Republic (Vaclavek *et al.*, 2003). The reports for the respective studies showed that 12–42 % of aborted foetuses were infected with *N. caninum* (it is known that this percentage could vary broadly – from 2.5 to 77 %). Analyzing these results, Wouda *et al.* (1998) stated that abortions could be either epidemic or endemic. According to Wouda *et al.* (1999) and Schares *et al.* (2002), the abortions are epidemic when more than 10 % of the risk contingent of cows abort within 6 to 8 weeks. Outbreaks of abortions with more than 30% foetal losses are communicated by McAllister *et al.* (1996), Neitfeld *et al.* (1992), Cox *et al.* (1998). The authors assume the possibility of a horizontal transmission of infection through concentrating of the infective agent in food and water.

Diagnosis

Several serological reactions are used for detection of antibodies against *N. caninum*: the indirect fluorescent antibody test (IFAT), the direct agglutination (DA) test and various variants of ELISA (Conraths *et al.*, 1996; Dubey & Lindsay, 1996, Dubey *et al.,* 1997; Jenkins *et al.*, 1997; Wouda, 1998). The ELISA with diagnostic sensitivity of 96.4 % and specificity of 96.8 % is an easy and rapid method for precise detection of the *N. caninum* infection status in cows. By means of the ELISA immunoblot test, specific antibodies against *N. caninum* are successfully evidenced. Avidity ELISA, that differentiates the recent from chronic infection, could be effectively used for distinguishing endemic from epidemic abortions (Bjorkman & Uggla, 1999; Bjorkman *et al.*, 1999). Though *N. caninum* is closely related to *T. gondii*, *Sarcocystis spp*. and other Apicomplexa protozoa, no cross-reactions are observed in experimental *N. caninum* infections (Dubey *et al.*, 1996a; Wouda *et al.*, 1998).

Some serological tests are permitted for commercial distribution: Herd Chek Idexx (Intervet); Civest Bovis Neospora Hipra (Cypress Diagnostic c.v.); Mastazyme^{TM} (Mast Diagnostics); P38 – ELISA (Animal Welfare and Food Safety GmbH).

Von Blumroder *et al.* (2004) compared the serological tests in use in Europe with a standard sera pack. Most of these tests studied in various laboratories, showed a high level of compliance and very good to excellent similarity of the protocols and of data from the statistical analysis. The results show that mentioned tests could be used when performing parallel epidemiological studies. This would contribute to standardization of data interpretation.

The detection of anti-*N. caninum* antibodies in the serum of aborted cows is only indicative for contact with these protozoa and does not conform that the cause of abortion is neosporosis. Very often, sera of aborted cows show positive titres against 3 other agents: *Salmonella Dublin*, *Leptospira hardjo* and the bovine viral diarrhoea virus (BVDV). The negative maternal serology however is an almost certain indicator that *N. caninum* was not involved in the abortion (Wouda *et al.*, 1998; Williams *et al.*, 2000; Toolan, 2003).

The detection of antibodies against *N. caninum* in foetal sera and precolostrum sera from calves is indicative of infection. The negative result could not however exclude that the abortion was caused by *N. caninum* as the antibody synthesis of the foetus depends on the stage of gestation, the degree of infection and the time between the infection and the abortion (Barr *et al.*, 1995; Wouda *et al.*, 1997).

The ELISA is approved as a method for detection of antibodies against *N. caninum* in cow's milk (Schares *et al.*, 2004). It was shown that a higher number of diagnozed *N. caninum* abortions in cows is revealed by analysis of milk samples via ELISA than by the results of blood sera analysis. Andrianarivo *et al.* (2001) established that when infected with *N. caninum*, cows produce IgG_1 and IgG_2 antibodies in a amount, depending on the time after the infection. Soon after infection, IgG_1 antibodies are produced at higher rates than IgG_2 do. As IgG_1 is the IgG subclass present at a higher amount in cow's milk, it could be understood why in aborted cows the ELISA, based on milk analysis was more sensitive than the tests based on blood serum analysis.

The analysis of milk samples had also better advantages than the analysis of sera,

because of the easier and cheaper collection of specimens. The milk samples are not infective. Thus, the hazard of transmission of the infection by the needle and the production losses due to stress would be eliminated.

Apart the serological analysis, the detection of the agent in tissues from aborted foetuses is needed for the definitive diagnosis of neosporosis (Dubey & Lindsay, 1996; Wouda *et al.*, 1997). The histological investigation could be performed on brain, heart, liver, placenta or tissue fluids, but the diagnosis value is more confident when more tissues are studied. Despite that the lesions due to neosporosis are present in various organs, the foetal brain is most commonly affected. As most foetuses are autolyzed, even the semiliquid brain tissue should be fixed in 10% buffered neutral formalin for histological examination of haematoxylin-eosin stained sections. *N. caninum* tachyzoites are immunohistochemically identified in 85 % of brains, 14 % of hearts and 26 % of livers of 80 foetuses with confirmed neosporosis (Wouda *et al.*, 1997). Detection of DNA of *N. caninum* via PCR could be performed in formalin-fixed, paraffin-embedded brain tissues of aborted foetuses. PCR is more sensitive for detection of *N. caninum* infection in foetuses than immunohistochemistry (Baszler *et al.*, 1999).

Prevention

The prevention of horizontal transmission could be directed towards preservation of food and water for animals as well as their stores, from contamination with faeces of dogs and wild canidae. Dogs should have no access to calving premises and recently calved cows. Aborted foetuses and placentas should be collected and made harmless in a way such that the access of dogs to them is impossible (Toolan, 2003).

As in 81 to 95% of liveborn calves from seropositive dams, a vertical transmission of *N. caninum* was proved (Pare *et al.*, 1998; Davison *et al.*, 1999) programmes for prevention and control of this route of transmission are developed. If the prevalence in a farm is low, the culling of seropositive cows and their seropositive progeny could be feasible. In severely affected farms however, the seropositivity against *N. caninum* could be used as an additional criterion when deciding which animals should be culled and which – included in reproductive programmes. According to Wouda *et al.* (1998), in heavily infected farms it would be advisable to use for reproduction only seronegative heifers instead of culling numerous seropositive lactating cows. Some seropositive animals that are kept in farms could be fertilized by a beef bull and their offspring – fattened and slaughtered so that congenitally infected offspring could not be used for breeding.

For limiting the vertical transmission, Williams *et al.* (2000) recommended the maintenance of a closed cycle in farms. If an infected animal is introduced, the infection would not spread from it to the other animals. The purchased cow and its progeny however, would be at higher risk of abortions and congenital infections. Moreover, the infected animals in the farm are a potential source of infection for farm dogs and this could result in epidemic abortions.

That is why, the programmes for prevention during the purchase of animals include the serological test for antibodies against *N. caninum*. The use of this test would help to perform a screening of neosporosis-free animals rather than to diag-

nose abortions (Wouda *et al.*, 1998; Williams *et al.*, 2000).

On the basis of laboratory experiments with mice (Innes *et al.*, 2001; Trees & Williams, 2003) the Bovilis Neogard vaccine was created (Intervet), that is licensed for production and marketing in the USA and Canada in 2001. The vaccine contains killed *Neospora* tachyzoites and a SPUR adjuvant. It is applied twice subcutaneously, at a 3-4 week interval, the first vaccination being done in the first third of gestation. A similar course of two injections is necessary for each following pregnancy. It is anticipated that the application of this vaccine would reduce the incidence of abortions and prevent giving birth of congenitally infected calves. The vaccine was experimentally studied in field conditions in Costa Rica (Romero *et al.*, 2002; Frankena *et al.*, 2004) and New Zealand (Heuer *et al.*, 2004). A reduction of abortions with 11.2% and 24.6% respectively was reported in vaccinated cows vs non-vaccinated.

Economical losses

There are no exact data about the economical losses due to neosporosis, but they are assessed to be millions of dollars (Hoar *et al.*, 1996). The principal economical losses in *N. caninum*-infected cows are related to abortions. From 20 to 43% of all abortions in cows in California (Anderson *et al.*, 1991, 1995) and 15%–20% in Holland (Wouda *et al.*, 1997) are due to neosporosis. For California, the annual economical losses directly associated with abortions are calculated to about $ 35 millions.

Indirect economical losses include: expenses for professional help, diagnostics, repeated fertilization, prolonged lactation period, reduced milk and meat productivity, replacement of aborted cows if they are culled etc. (Thurmond & Hietala, 1996, 1997a, 1997b; Barling *et al.*, 2000; Hernandez *et al.*, 2001). In a study in California, Thurmond & Hietala (1997b) observed that seropositive cows produced daily about 1 litre milk less than seronegative. In Australia, the neosporosis impact is estimated to $ 85 millions for dairy and $ 25 millions for meat industry annually (Ellis, 1997).

These data confirm the strong need of a scientific study upon the economical importance of bovine neosporosis in other regions of the world as well.

NEOSPOROSIS IN SHEEP

Neosporosis in sheep that are intermediate hosts of *N. caninum* is inadequately studied. Sheep could be infected per os with N. caninum oocysts (O'Handley *et al.*, 2002). Pregnant sheep are very susceptible to experimental infection with *N. caninum* tachyzoites (Dubey & Lindsay, 1990; McAllister *et al.*, 1996; Buxton *et al.*, 1997, 1998, 2002; Jolley *et al.*, 1999; Innes *et al.*, 2001). They abort dead lambs 25–30 days after the infection. Lesions in the brain, the spinal cord, the muscles and placenta are observed. An encephalitis characterized by multiple foci, hemorrhages and necroses is found out. In later stages of gestation (65, 90 and 130 days) infected sheep abort, give birth to weak or clinically normal lambs. Lesions are detected in the brain, placenta, heart, liver, lungs, even in autolyzed and mummified foetuses. Tissue cysts are observed only in the brain. The easy induction of abortions in sheep, the delivery of weak but infected lambs with or without clinical signs make sheep, on the opinion of Innes *et al.* (2001), an alternative model for studying bovine neosporosis. However, after post natal infection of 3-week old lambs inocu-

lated IV, SC or IM with tachyzoites, the animals remained clinically healthy. Therefore, that proved that such a route of infection did not result in the development of clinical neosporosis (Dubey *et al.*, 1996a).

For the first time, Dubey & Lindsay (1990) detected *N. caninum* in a congenitally infected lamb in England. The lamb was born weak, with partial ataxia and died at the age of 1 week. Only tissue cysts were found out, without tachyzoites. Similar incidences of clinical neosporosis in sheep are reported in Japan. Kobayashi *et al.* (2001) discovered a natural neosporosis in a pregnant sheep and its twin foetuses. A focal encephalitis and thick wall tissue cysts of *N. caninum* were present in all three animals. *N. caninum* was also isolated by Koyama *et al.* (2001) from another lambed sheep.

Experimentally infected sheep produce antibodies against *N. caninum.* By the day of infection, a titre ≤ 1:50 was detected by IFAT and all sheep showed seroconversion up to ≥ 1:400 3 weeks after the infection. Little is known about seropositive infections with *N. caninum* in naturally infected sheep and yet, there are no data about the role of *N. caninum in* ovine abortions (Otter *et al.*, 1997). The studies performed by Helmick *et al.* (2002) in the United Kingdom has revealed antibodies against *Neospora caninum* only in 3 out of 660 ewes with abortions.

NEOSPOROSIS IN GOATS

Pregnant goats are susceptible to experimental infections. *N. caninum*-infected pregnant goats abort infected foetuses (Barr *et al.*, 1992; Lindsay *et al.*, 1995).

Abortions and stillbirths are described in goats in the USA (Dubey *et al.*, 1992), in dairy goat farms in Costa Rica (Dubey *et al.*, 1996a) and Brazil (Corbellini *et al.*, 2001). Antibodies against *N. caninum* are detected in 5 out of 77 dairy goats with abortions in Costa Rica. The percentage of seropositive goats is not known. Ooi *et al.* (2000) have detected no antibodies against *N. caninum* in 24 goats in Taiwan.

NEOSPOROSIS IN HORSES

A parasite resembling *N. caninum* was detected in tissues of 2 aborted equine foetuses (Dubey & Porterfield, 1990; Pronost *et al.*, 1999), in congenitally infected foals (Lindsay *et al.*, 1996) and 5 adult horses (Daft *et al.*, 1997; Gray *et al.*, 1996; Marsh *et al.*, 1996; Cheadle *et al.*, 1999b). Marsh *et al.* (1998) proposed a new name – *Neospora hughesi* for the parasite discovered in horses in 1996. Three *N. caninum* isolates from adult horses were reported by Marsh *et al.* (1998); Cheadle *et al.* (1999b); Dubey *et al.* (2001). Molecular and biological characterization of these 3 isolates was performed by Cheadle *et al.* (1999a) and Dubey *et al.* (2001).

The tissue cysts of *N. hugehesi* are smaller than those of *N. caninum*, with a thin cyst wall (less than 1.0 μm) and with bradyzoites, smaller than *N. caninum* ones (Marsh *et al.*, 1998). Thin-walled cysts characteristic for *N. caninum* are reported in horses from California (Daft *et al.*, 1997) and congenitally infected foals in Wisconsin (Lindsay *et al.*, 1996). Until now however, it is not clear whether *N. hughesi* is the only *Neospora* species that is able to infect horses. The highest prevalence of *Neospora caninum* in horses is observed in France 23 % (Pitel *et al.*, 2001) and the USA: 21 %, 3 %, 17.0 % and 11.5 % in four different studies (Cheadle *et al.*, 1999b; Dubey *et al.*, 1999b; Vardeleon *et al.*, 2001).

NEOSPOROSIS IN WILD ANIMALS

Wild animals could be involved in the life cycle of *N. caninum* as both intermediate and definitive hosts.

Neosporosis was diagnosed during the necropsy of 2 black tailed deers found dead in California (Woods *et al.*, 1994), in deers from a zoo in France (Dubey *et al.*, 1996b) and in antilopes in Germany (Peters *et al.*, 2001). Tissue cysts of *N. caninum* are detected in the brain of a full-term stillborn deer in the Paris zoo (Dubey *et al.*, 1996b). Peters *et al.* (2001) found out antibodies against *N. caninum* in amniotic fluids and DNA of *N. caninum* via PCR in brain, heart, lungs, liver and spleen or full-term antilope twins in the Hannover zoo (Germany).

The presence of antibodies in 40% of wild deers shows that the *N. caninum* cycle was probably a natural one. In a great number of these deers, the titres were high (>1:1600). The antibody titre and the prevalence percentage do not increase with age thus showing that the transmission was congenital (Dubey *et al.*, 1999a).

Anti-*Neospora caninum* antibodies are detected in coyotes (Lindsay, *et al.*, 1996), dingoes (Barber *et al.*, 1997), silver and red foxes (Barber *et al.*, 1997; Buxton *et al.*, 1997; Lindsay *et al.*, 2001) and wolves (Vitaliano *et al.*, 2004). In Belgium, Shares *et al.* (2001b) observed a wide distribution of *N. caninum* in red foxes and proved a congenital infection. The serological results showed that wild canidae most probably play an important role in the epidemiology of neosporosis and in the life cycle of *N. caninum*.

CONCLUSION

N. caninum is one of the principal causes of bovine abortions in many countries all over the world. During the last decade, a great progress in the identification of the pathogen and in the knowledge about the life cycle of the parasite, the epidemiology and pathogenesis of neosporosis was achieved. So far, all possible definitive and intermediate hosts, the risk factors for the transmission, the pathogenesis of abortions caused and the measures for control and prevention of infection are not fully recognized. It is proved that the vertical transmission is the main route of infection but the elimination of vertical transmission is not sufficient for its eradication in farms as the horizontal transmission of infection is also possible. The control of *N. caninum* infections includes prevention of both vertical and horizontal transmission, including a serological study for determination of seroprevalence and excluding the seropositive animals from reproduction. The economical losses related to reduced productivity, abortions, birth of congenitally infected offspring with or without neurological signs that are a reservoir for horizontal transmission in the farm, are fairly motivating the extension of studies and control of this parasitosis.

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