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VERSITA

INVITED REVIEW

Diagnosis of bovine neosporosis: Recent advances and perspectives

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Abstract

Neospora caninum is considered a major cause of abortion in cattle. Appropriate techniques for diagnosis of bovine neosporosis, both *in vivo* and in aborted foetuses, have been developed in the last ten years and some of them are commercially available. For diagnosis in live animals, detection of antibodies in serum or milk has been shown to be the best option both at the herd and the individual level. These techniques are excellent tools to examine *N. caninum*-associated abortion problems and to adopt some basic herd-control measures. Concerning foetal diagnosis, detection of compatible lesions by histological examination and parasites by PCR in brain (as well as heart and liver) are the best choices. Diagnostic criteria to distinguish foetal infection and *Neospora*-associated abortion are based not only on the demonstration of the parasite in the foetus but also on the extent and severity of the lesions in the foetus, foetal age and the assessment of neosporosis at the herd level. In the near future, new tools to diagnose infection should help to detect animals with parasite reactivation by testing the immune response to stage-specific antigens and lead to the development of molecular typing methods to characterise different parasite isolates. Finally, uniform diagnostic procedures need to be established between laboratories and countries in order to standardise result interpretation. The role of National or Regional Reference Laboratories is essential in countries or regions where control programmes for the disease are being developed.

Key words

Neosporosis, cattle, diagnosis

Introduction

Neospora caninum is a heteroxenous cyst-forming apicomplexan protozoan closely related to *Toxoplasma gondii*, which was recently redescribed (Dubey *et al*. 2002). *N. caninum* is now a matter of international concern as it has been recognised as a major cause of infectious abortion in the main cattle-producing countries (Dubey 2003a). Cattle and other ungulates such as sheep, goats, horses, white-tailed deer, rhinoceros, South American camelids and water buffaloes may act as natural intermediate hosts (Dubey 2003b, Chávez-Velásquez *et al*. 2004, Rodrigues *et al*. 2004). *N. caninum* infection has been detected in other animal species such as cats, opossums, foxes and other wild canids (Dubey 2003b, Moore 2005). Canids such as dogs (McAllister *et al*. 1998) and coyote (Gondim *et al*. 2004a) are the definitive hosts, although they may act as intermediate hosts as well.

The *N. caninum* life cycle involves three distinct stages (Dubey and Lindsay 1996, Basso *et al*. 2001). The sporozoite is a latent stage within oocysts by which intermediate hosts could be infected after ingestion. In the intermediate host, two different intracellular stages may be observed, the fast-replicating tachyzoite, which can be located in several organs during the acute phase of the disease, and the slowly-dividing bradyzoite, which remains latent in tissue cysts located principally in the central nervous system until reactivation.

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Transmission and clinical signs

Routes of *N. caninum* transmission in cattle include transplacental infection through tachyzoites (vertical transmission, from the dam to the foetus during gestation) and infection by ingestion of sporozoite-containing oocysts shed by a definitive host (horizontal transmission). Recently, the terms endogenous and exogenous transplacental infection (TPI) have been suggested to describe two different situations in *N. caninum* transmission to the foetus (Trees and Williams 2005). The "exogenous TPI" takes place when the dam is infected during the pregnancy from an exogenous source. Conversely, "endogenous TPI" refers to a congenital infection caused by parasite reactivation in a chronically infected pregnant cow, probably congenitally infected. The TPI is a very efficient route of *N. caninum* transmission and plays a major role in the maintenance and spread of the infection (Anderson *et al*. 2000). The efficiency of this mode of transmission has been reported to be from 44% (Bergeron *et al*. 2000) to over 95% (Davison *et al*. 1999) based on precolostral serology in calves born to seropositive dams. Most of these calves are clinically normal and play a very important role in maintaining the infection in the herd (Anderson *et al*. 2000). Repeated TPI in infected dams is possible during subsequent gestations (Barr *et al*. 1993). However, mathematical models indicate that endogenous TPI alone is not sufficient to maintain the infection in cattle herds (French *et al*. 1999). Thus, data from epidemiological (Wouda *et al*. 1999; Dijkstra *et al*. 2001, 2002) and experimental studies (De Marez *et al*. 1999, Trees *et al*. 2002, Gondim *et al*. 2004b) have confirmed the existence of horizontal transmission.

Other sources of postnatal transmission, such as the ingestion of contaminated colostrum or milk, could be possible and have been demonstrated experimentally (Uggla *et al*. 1998, Davison *et al*. 2001), but there is no evidence that this occurs in nature. Venereal transmission could be feasible, as evidenced recently in heifers experimentally infected by intrauterine inoculation of semen contaminated with tachyzoites (Serrano *et al*. 2006). The detection of *N. caninum* DNA in fresh and frozen semen of naturally infected bulls has been reported, although it was sporadic and had a low parasite load, suggesting that the risk of sexual transmission of *N. caninum* infection is also probably low (Ortega-Mora *et al*. 2003, Caetano-da-Silva *et al*. 2004, Ferre *et al*. 2005).

Neospora caninum infection is generally latent and asymptomatic in non-pregnant cattle, although bovine neosporosis in a pregnant cow is associated with repeated abortion and birth of clinically healthy but persistently infected calves (Buxton *et al*. 2002). Infected cows of any age may abort from 3 months gestation to term, with most abortions occurring between 5 to 7 months of gestation (Pereira-Bueno *et al*. 2003, Dubey 2005), and a cow may abort in successive gestations. Foetuses may die *in utero* and be reabsorbed, mummified, and/or autolysed (Dubey 2005). The infection has been associated with sporadic, endemic and epidemic abortions (Thurmond *et al*. 1997, Wouda *et al*. 1997b, Schares *et al*. 1999b, Anderson *et al*. 2000). A limited number of cases of new born congenitally infected calves with neuromuscular disorders have been detected (De Meerschman *et al*. 2005). In these cases, calves were underweight, unable to rise and had neurologic signs including ataxia, decreased patellar reflexes and loss of conscious proprioception (Barr *et al*. 1993). Exophthalmia or an asymmetrical appearance of the eyes may also be observed (Dubey and Lindsay 1996). However, it must be emphasised that these cases are rare, and most congenitally infected calves are clinically normal but persistently infected.

Diagnosis

To diagnose bovine neosporosis, clinical history and epidemiological data are very important. Information about the abortion pattern, foetal age and foetal status should be considered. Two patterns of abortion, endemic and epidemic, have been mainly described in association with neosporosis in the field (Thurmond *et al*. 1997). Herds with a persistent rate of abortion greater than 5% per year have an endemic pattern of abortion. In the epidemic pattern, abortion storms take place during which a high proportion of pregnant cattle (dams at risk) abort over a relatively brief period of time (within a few weeks) (Anderson *et al*. 2000). Recently, the abortion pattern was considered to be epidemic when more than 10% of the dams at risk aborted within a period of 42 days. Cows and heifers were considered at risk if they had been pregnant for at least 58 days to 260 days when the abortion storm started (Schares *et al*. 2002).

In addition, there are some risk factors which are related to *N. caninum* abortions that should be considered. It has been demonstrated that there is a strong association between the occurrence of abortion outbreaks, seroprevalence to *N. caninum*, the presence and the number of dogs with access to cattle or fodder (Paré *et al*. 1998, Bartels *at al*. 1999, Schares *et al*. 2004a, Hobson *et al*. 2005). Other risk factors such as the presence of other species of possible definitive hosts, causes of immunosuppression and others such as: Age, breed, climatic factors and herd location have been described (reviewed in Hemphill and Gottstein 2000; Schares *et al*. 2003, 2004a; Haddad *et al*. 2005; Thurmond *et al*. 2005).

Data described above can be suggestive of a *N. caninum* infection but for a final diagnosis the assistance of a veterinary diagnostic laboratory is needed (Anderson *et al*. 2000), and the examination of both aborted foetus and maternal serology is recommended. In this sense, there are two main problems that are related with this diagnosis: First, the diagnosis of infection and/or disease in the live animal, due to the absence of clinical signs in chronically infected cattle or in new born calves congenitally infected; and second, the diagnostic criteria and techniques used in the aborted ruminant foetus.

In vivo

The most useful techniques for diagnosing *N. caninum* infection *in vivo* are those aimed at detecting specific antibod-

ies. Several assays including: Indirect fluorescent antibody test (IFAT), various enzyme-linked immunosorbent assays (ELISAs), immunoblotting (IB) and direct agglutination tests (DATs) have been developed, and some of them are commercially available. Each test system has both advantages and drawbacks and these should be carefully considered when choosing tests for different applications. Other techniques of *in vivo* diagnosis of *N. caninum* include the detection of parasite DNA using PCR methodologies in blood or semen and the detection of the pro-inflammatory cytokine interferon-gamma, which is known to be important in host protection. Nevertheless, up to date these techniques have not been validated for diagnosis and have only been used as important research markers.

Detection of specific antibodies

At present, culture-derived tachyzoites of either bovine or canine *N. caninum* isolates are the source of antigens used in techniques directed to detect specific antibodies. These tachyzoites can be used formalin-fixed as in IFAT (Conrad *et al*. 1993), DAT (Packham *et al*. 1998, Romand *et al*. 1998) and in an indirect ELISA (Williams *et al*. 1997) or as a total or soluble extract in indirect ELISAs (Table I) and IB (Schares *et al*. 1998). Tachyzoite antigens included within immune stimulating complexes (ISCOMs) (Björkman *et al*. 1997, Frössling *et al*. 2003) as well as tachyzoite antigens purified by immunoaffinity (Schares *et al*. 2000) or recombinant antigens have also been used in ELISA (Table II) and, recently, in an immunochromatographic test (ICT) (Liao *et al*. 2005). These diagnostic tests are developed to detect specific IgG antibodies, and some of them, like ELISAs based on the use of ISCOMs, specifically detected IgG1. In addition, several avidity serological tests, such as an indirect avidity ELISA (Björkman *et al*. 1999) and an avidity immunoblot (Aguado-Martínez *et al*. 2005) have been developed to detect the avidity value of the specific IgG antibodies and permit differentiation between a recent and a chronic *N. caninum* infection.

Regarding the samples, serum or milk (Björkman *et al*. 1997, Chanlun *et al*. 2002) from cattle are used for diagnosis of *N. caninum* infection based on specific antibodies detection. Other body fluids of cattle, such as vaginal secretion and saliva can be used, but the frequency of antibodies detection is lower than in sera or milk (Ooi *et al*. 2000).

Techniques

The IFAT was the first serological test applied to neosporosis (Dubey *et al*. 1988) and was the serological technique most widely used to diagnose *N. caninum* infection in the recent past (Conrad *et al*. 1993, Otter *et al*. 1997, Atkinson *et al*. 2000). IFAT has been used as a reference test for other techniques (reviewed in Björkman and Uggla 1999). IFAT is based on the principle of affixing intact tachyzoites to microscopic slides. An IFAT result is considered positive when unbroken

Table I. Characteristics of ELISAs developed for serological diagnosis of *N. caninum* infection in cattle

Antigen	Target group	Se[a]/Sp[b]	Reference techniques	Reference
Soluble antigen	adults	88.6/96.5	IFAT	Paré *et al.* 1995a
Soluble antigen	foetuses adults	89/100 97/100	IFAT	Osawa *et al.* 1998
Soluble antigen	aborting cattle normal herds	98/100 98/92	IHQ	Wouda *et al.* 1998a
Soluble antigen	non aborting cattle aborting cattle foetuses	>90/>90	IFAT & IB	Álvarez-García *et al.* 2003
Soluble antigen (competition-inhibition)	adults	na[c]	IB	Baszler *et al.* 1996
Soluble antigen (competition-inhibition)	adults	97.6/98.6 96.4/96.8	IFAT & foetal IHC IFAT	Baszler *et al.* 2001
"Iscoms"	adults	100/96	IFAT	Björkman *et al.* 1997
"Iscoms"	adults	98/96	IFAT	Frössling *et al.* 2003
Fixed tachyzoites	adults	95/96	IFAT	Williams *et al.* 1997
Fixed tachyzoites	normal cattle aborting cattle endemic herds	62/97 97/95 85/90	IFAT	Williams *et al.* 1999
p38 (NcSRS2) (affinity purified antigen)	adults epidemic abortion endemic abortion	83/83 78/78 85/85	IFAT & IB	Schares *et al.* 2000

[a]Sensibility/[b]specificity, [c]data not available.

tachyzoite membrane fluorescence is seen (Paré *et al*. 1995b) (Fig. 1). The cut-off titre in IFAT differs between laboratories from 1:100 to over 1:640 for adult bovines and from 1:16 to 1:80 for foetal serology (Björkman and Uggla 1999, Álvarez-García *et al*. 2003). A recommended cut-off value in IFAT to detect infection is a 1:200 dilution in adult cattle (von Blumröder *et al*. 2004) and 1:16–1:25 in foetal fluids (Álvarez-García *et al*. 2003). Performance of this test requires training and experience and the result always depends on the subjectivity of the reader.

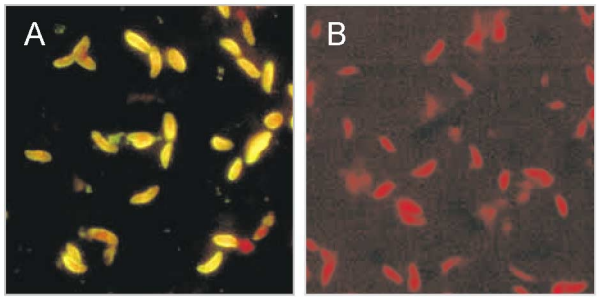

Fig. 1. Detection of specific antibodies using IFAT. **A.** IFAT is considered positive when unbroken tachyzoite membrane fluorescence is seen (× 1,000). **B.** Negative IFAT result (× 1,000)

ELISA has the advantage that the reaction is registered objectively and the assay can be easily automated. It is therefore a suitable technique for processing of large number of samples. Different ELISA formats such as indirect ELISA and competitive-inhibition (CI)-ELISA have been developed (Tables I and II). Different antigen preparations have also been used, but the most commonly used is an indirect ELISA based on soluble tachyzoite antigens. Both serum and milk can be tested using this technique. Results of an indirect ELISA can be expressed as optical density (OD) value (Osawa *et al*. 1998), percent positivity (PP) value (Williams *et al*. 1999), relative index percent (IRCP) (Álvarez-García *et al*. 2003) or sample/positive control (S/P) ratio (Wouda *et al*. 1998a). In the CI-ELISA, in which specific antibodies against *N. caninum* compete for an epitope on the captured p65 antigen with a conjugated monoclonal antibody, the result is presented as a percentage inhibition by the test serum (Baszler *et al*. 1996).

In serological assays, results are dependent on a range of factors such as: Antigen composition, conjugated characteristics and other reagents (Björkman and Uggla 1999). Age and purpose of testing (detection of infection or abortion) also have a great influence on the cut-off selection (Atkinson *et al*. 2000, Álvarez-García *et al*. 2003). From a practical standpoint and in absence of a perfect cut-off value for infected breeding cattle, the use of a cut-off value for maximum sensitivity would be useful to investigate the status of individual cattle prior to purchase or entry into a *Neospora*-free herd. On the other hand, a cut-off for maximum specificity could be of use when evaluating culling. In some cases, the use of IB could be recommended as a confirmatory or *a posteriori* test (Bartels *et al*. 2006).

Recently, several ELISAs and IFAT have been compared in a multi-centred study carried out in several European laboratories (von Blumröder *et al*. 2004). Most techniques showed a high level of agreement in the interpretation of the test results (positive and negative). Furthermore, a distinct increase in agreement between tests was obtained after the

Table II. Recombinant ELISAs developed for serological diagnosis of *N. caninum* infection in cattle

Antigen		Target group	Se[a]/Sp[b]	Reference techniques	Reference
NcDG1(NcGRA7) NcDG2 (NcGRA6)	&	adults	na[c]	IFAT	Lally *et al.* 1996 Jenkins *et al.* 1997
N54 N57		adults	95/96 82/93	IB	Louie *et al.* 1997
NcDG1 (NcGRA7) NcDG2 (NcGRA6)	&	adults foetuses	na[c]	IFAT and NAT	Venturini *et al.* 1999
SRS2		adults	na[c]	IFAT	Nishikawa *et al.* 2001
Nc-p29 (NcSAG1)		adults	na[c]	IFAT	Howe *et al.* 2002
Truncated NcSAG1		adults	na[c]	IB	Chahan *et al.* 2003
Ncp43P		adults	na[c]	ELISA and IB	Ahn *et al.* 2003
Truncated NcSRS2		adults	na[c]	IB	Gaturaga *et al.* 2005
NcGRA6 (sELISA)		adults	78.4/79.3[d]	IFAT-25, IFAT-100	
HPLC-NcGRA6 (dELISA)		adults	83.7/83.0[d]	IB ISCOM-ELISA	Jenkins *et al.* 2005

[a]Sensibility/[b]specificity; [c]data not available; [d]sensibility and specificity relative to the reference standard, which in the study were defined as sera that were positive or negative in at least three of the reference techniques.

application of standardised cut-offs offered by a two-graph receiver operating characteristic analysis. This procedure allows a standardised interpretation of results obtained with different tests used in independent, parallel seroepidemiological studies.

Indirect *N. caninum* ELISA has been modified to enable analysis of IgG avidity to distinguish between acute and chronic neosporosis (Björkman *et al*. 1999, Maley *et al*. 2001, Schares *et al*. 2002, Sager *et al*. 2003, Aguado-Martínez *et al*. 2005). Avidity assays are based on the fact that the first antibodies synthesised after primary infection have a lower affinity than those produced later on. By adding an incubation step with urea (6M or 8M), low affinity antibodies are eluted. The antibody titres obtained with and without urea are then used to calculate the IgG avidity index (titre with urea multiplied by 100 divided by titre without urea). Low avidity values are related with recent infection and high avidity values are observed in animals with chronic infection (Jenkins *et al*. 2000; McAllister *et al*. 2000; Schares *et al*. 2002; Björkman *et al*. 2003, 2005; Aguado-Martínez *et al*. 2005; Frössling *et al*. 2005). However, up to date this technique does not allow to discriminate a recrudescence or a re-infection from a chronic infection (Aguado-Martínez *et al*. 2005). Recently, several IgG avidity ELISA tests used in four European laboratories have been compared (Björkman *et al*. submitted). The results showed a moderate agreement between the different assays used to estimate the IgG avidity.

Detection of antibodies can also be done using individual milk samples or with bulk milk samples by ELISA. This source for testing antibodies is inexpensive and samples can be easily collected. In addition, milk sampling is non-invasive and harmless to the animal. Several studies have been done in individual milk samples using different ELISA tests with good agreement between sera and milk results (Björkman *et al*. 1997, Moskwa *et al*. 2003, Schares *et al*. 2004b), but unfortunately no comparable results exist among different labs. Recently, an ELISA based on the p38 (NcSRS2) affinity purified antigen (Schares *et al*. 2000) has been adapted for the detection of antibodies against *N. caninum* in bovine milk (Schares *et al*. 2005). Bulk milk testing can also be done by ELISA (Chanlun *et al*. 2002, 2006; Schares *et al*. 2003; Frössling *et al*. 2006; Varcasia *et al*. 2006) whenever intra-herd prevalence is higher than 10–15%. Moreover, this technique can be used to monitor control programmes. Recently, several ELISAs have been compared to detect specific antibodies to *N. caninum* in bulk milk (Bartels *et al*. 2005). Results of the comparison showed that two commercial ELISAs could adequately detect a 15% or higher intra-herd seroprevalence of *N. caninum* in lactating cows.

The IB combines the resolution of gel electrophoresis with the specificity of immunochemical detection. However, it would be very time-consuming as a routine tool for screening cattle sera. Instead, IB has been used for identification of immunodominant tachyzoite antigens (IDAs) by host sera (Bjerkås *et al*. 1994, Baszler *et al*. 1996, Schares *et al*. 1999a, Álvarez-García *et al*. 2002) and as an *a posteriori* test for other serological assays (Schares *et al*. 1998, 1999b; Atkinson *et al*. 2000; Álvarez-García *et al*. 2003; Bartels *et al*. 2006). One proposed cut-off for a positive result was the identification of one or more of four tachyzoite IDAs (Schares *et al*. 1999a, Álvarez-García *et al*. 2002). Recently, an avidity IB has been developed to detect the pattern of IgG avidity maturation against different specific antigens of *N. caninum* tachyzoites (Aguado-Martínez *et al.* 2005).

The DATs are based on the principle that intact tachyzoites agglutinate in the presence of specific IgG antibodies (Packham *et al*. 1998, Romand *et al*. 1998, Canada *et al*. 2004, Dubey and Thulliez 2005). The DAT has been carried out in 96 round-bottom-well microplates, using formalin-fixed *N. caninum* tachyzoites of the canine NC-1 isolate (Romand *et al*. 1998) or the bovine BPA-1 isolate (Packham *et al*. 1998). Diffuse opacity across the entire diameter of the well on the next day was regarded as a positive result. A cut-off titer of 1:80 gave the greatest sensitivity (100%) and specificity values (97%) (Packham *et al*. 1998). The advantages of DAT are its simplicity and non-requisite of species-specific conjugates.

At present, several IFAT, ELISA and DAT kits for *N. caninum* diagnosis are commercially available (Björkman and Uggla 1999, Williams *et al*. 1999, Atkinson *et al*. 2000, Baszler *et al*. 2001, Reichel and Pfeiffer 2002). Finally, an immunochromatographic test (ICT) with recombinant NcSAG1 has recently been developed for the rapid detection of antibodies to *N. caninum* in cattle (Liao *et al*. 2005).

Practical approaches

Infection-abortion relationship. At the individual level, post-abortion serology is useful because dams aborting due to an infection with *N. caninum* often have high levels of antibodies shortly after abortion (Quintanilla-Gozalo *et al*. 2000). Levels may decrease below the cut-off value after abortion (Jenkins *et al*. 2002). However, the presence of antibodies does not prove that the infection caused the abortion, as many chronically infected cows are serologically positive. Once *N. caninum* infection has been demonstrated, a seroepidemiological approach can be proposed to estimate the implication of *N. caninum* in those abortions (Thurmond and Hietala 1995, Thurmond *et al*. 1997, Sager *et al*. 2001, Hall *et al*. 2005). Although intra-herd seroprevalence provides information about the infection status, it is the seropositivity rate in aborting cows which is essential to establish the relationship between *N. caninum* infection and abortions. This rate should be significantly higher in aborting cows than in non-aborting cows. In addition, to know the pattern of abortion produced by *N. caninum* in the herd, it is necessary to estimate the odds ratio, which is a parameter indicative of the abortion risk. An endemic pattern of abortion is related with an odds ratio of around 2, whereas a higher odds ratio is indicative of an epidemic pattern (Thurmond and Hietala 1995).

Investigation of the predominant route of transmission. The analysis of the paired samples from dams and their daughters, samples from precolostral calves and the age-distribution of seropositive animals contribute to determine if the

vertical or horizontal route of transmission is predominant in the herd (Dijkstra *et al*. 2003). If the transmission is predominantly vertical, dams and their daughters are seropositive, as are precolostral calves, and there is a uniform distribution of seropositive animals across the age-groups. In the horizontal transmission of the infection, seropositive animals are in clusters and there is a lack of association between the serological status of dams and their daughters. In addition, the abortion pattern as well as avidity values in aborting dams are essential data (Jenkins *et al*. 2000, McAllister *et al*. 2000, Björkman *et al*. 2003). To determine the avidity value of antibodies, samples obtained immediately after the abortion from a representative number of seropositive aborted cows (8–10 sera) should be used. In herds with an endemic pattern of abortion and high avidity antibodies in aborting dams the vertical should be considered as the principal route of transmission. On the contrary, the presence of low avidity antibodies with an epidemic abortion pattern must be indicative of a recent exposure to *N. caninum* by the horizontal route (Dijkstra *et al*. 2002, Schares *et al*. 2002, Aguado-Martínez *et al*. 2005).

Adoption of basic herd-control measures. In addition to the identification of the main route of transmission of *N. caninum* infection in a herd, serological techniques may also help to adopt some basic measures concerning replacement. In some cases, as with purchase or sale, a study of *N. caninum* infection in non-aborting cows is needed. It should be taken into account that, in cattle, antibodies may fluctuate substantially and may even drop below the cut-off value of the serological test used (Quintanilla-Gozalo *et al*. 2000, Jenkins *et al*. 2002). In some cases, sampling after a period of 4–6 weeks is recommended and in doubtful samples the use of an *a posteriori* method such as IB is also useful (Álvarez-García *et al*. 2003). Antibody detection could also be used to determine whether a new born calf is congenitally infected (Wouda *et al*. 1998b). In such cases, a serum sample should be taken before suckling as colostral antibodies may cause false positive results and maternal antibodies may persist for several months. In precolostral calves, a positive result would confirm transplacental transmission, and it would permit the adoption of control measures related with vertical transmission in the farm.

Other methodologies for in vivo *diagnosis*

At present, other diagnostic tools can be used for *in vivo* diagnosis, although they are mainly used for research purposes and need to be validated with current diagnostic techniques. Thus, a nested PCR can be used for parasite DNA detection in blood (Okeoma *et al*. 2004, Ferre *et al*. 2005) and semen (Ortega-Mora *et al*. 2003, Caetano-da-Silva *et al*. 2004, Ferre *et al*. 2005) and it can be quantified by a real-time PCR in blood (Okeoma *et al*. 2005) as well as in semen (Ortega-Mora *et al*. 2003, Caetano-da-Silva *et al*. 2004, Ferre *et al*. 2005). Parasite DNA was sporadically detected in white blood cells in naturally and experimentally infected bulls and cows. In experimentally and naturally infected bulls, parasite DNA was also sporadically detected in semen, where it was demonstrated in the cellular fraction with a low parasite load.

On the other hand, determination of specific IFN-gamma production allows an indirect quantification of cell-mediated responses. Lymphocytes from peripheral blood from *N. caninum* infected cattle will proliferate *in vitro* when stimulated with specific antigens, and supernatants from these cultures have been shown to contain IFN-gamma. In the near future, this procedure could be used to diagnose the disease although, to date, this method represents only a valuable research marker (Lunden *et al*. 1998, Marks *et al*. 1998, Andrianarivo *et al*. 2001, Almería *et al*. 2003, Ferre *et al*. 2005, Moore *et al*. 2005, Serrano *et al*. 2006).

In the aborted foetus

The ideal diagnostic samples include the aborted foetus submitted with placenta and sera from the dam. If this is not possible, samples from brain, heart, and liver should be submitted. The brain is the target organ although the probability of diagnosing the infection increases when other tissues such as the heart and liver are analysed. A higher number of positive-PCR tissue samples have been observed in foetuses corresponding to the first and second trimesters compared with those aborted in the last trimester (Collantes-Fernández *et al*. 2006). Tissues should be collected as rapidly as possible after expulsion in order to avoid autolysis. Samples for serological analysis may be obtained from serosanguinous fluid accumulated in foetal body cavities (Anderson *et al*. 2000).

Gross lesions are rare in *Neospora* abortions, but white linear foci may be seen in skeletal muscles and the heart, and minute pale to dark foci may be present in the brain (Dubey and Lindsay 1996, Anderson *et al*. 2000). Histological examination of brain, heart and liver of the foetus, parasite detection by PCR techniques in target organs (alternatively immunohistochemistry) and detection of specific antibodies in dam and in foetal fluids in foetuses over five to six months of age may contribute to a successful diagnosis of bovine *Neospora* abortion.

Histological examination

Preparation of tissues for examination under the microscope should be carried out according to universally accepted protocols. Tissue sections of approximately 4 µm should be cut with a microtome, mounted and stained with haematoxylin and eosin. Inflammatory and degenerative lesions may be found histologically throughout foetal tissues but are most common in the central nervous system (CNS), heart, skeletal muscle and liver (reviewed in Dubey and Lindsay 1996). The histological lesions caused by *N. caninum* in these tissues are often distinctive, particularly the brain lesions. Diagnosis of the most significant lesions in the brain consist of non-suppurative encephalomyelitis characterised by multifocal non-suppurative infiltration (Fig. 2A), with or without multifocal necrosis (Fig. 2B). The lesions in the brain are often accom-

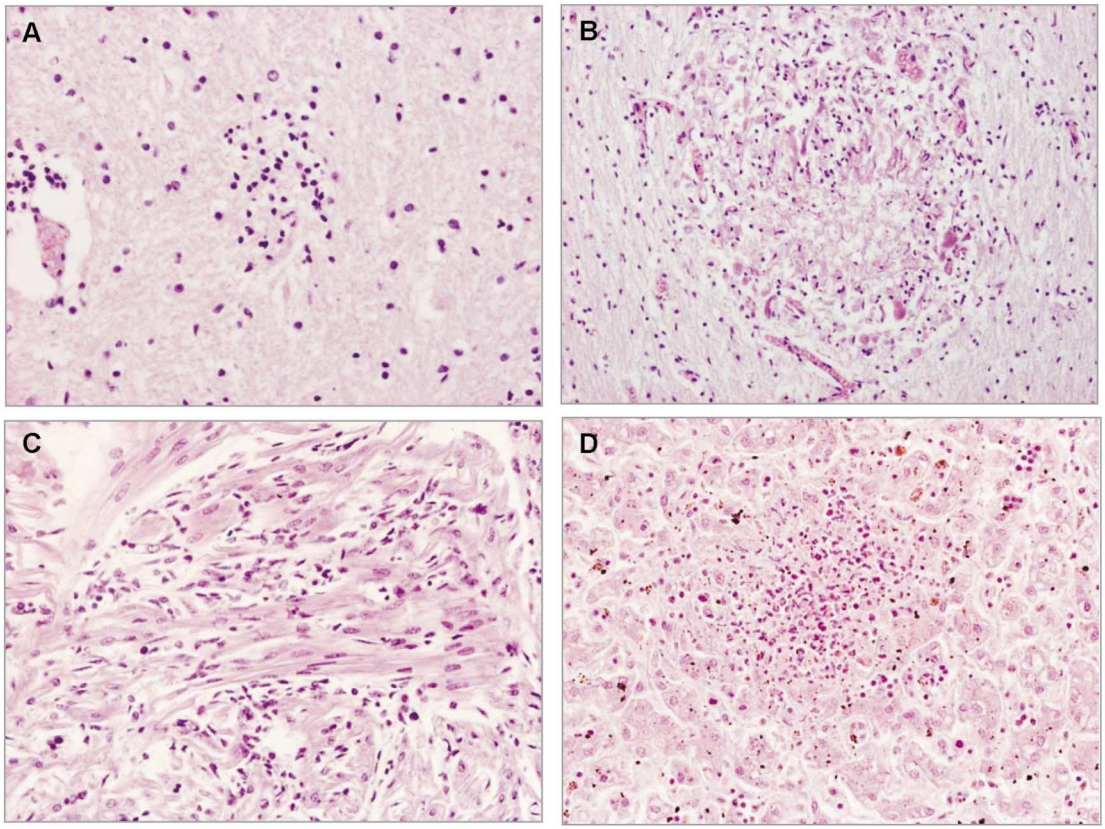

Fig. 2. Histological lesions observed in bovine tissues caused by *N. caninum* infection. **A.** Glial focus in foetal brain. H and E, × 200. **B.** Focal necrosis surrounded by non-suppurative infiltration in foetal brain. H and E, × 100. **C.** Lymphocytic myocarditis. H and E, × 200. **D.** Non-suppurative periportal hepatitis. H and E, × 100

panied by some vascular endothelial hyperplasia and associated mild infiltration by mononuclear cells. Although not specific to neosporosis, leukomalacia may be seen in some cases in the cerebral white matter. Other histologic lesions that are consistently found include: Non-suppurative myocarditis (Fig. 2C), focal non-suppurative myositis and non-suppurative periportal hepatitis (Fig. 2D) – associated in some cases with focal hepatic necrosis – and focal non-suppurative interstitial pneumonia (Wouda *et al*. 1997b). Although the placenta is not always available, the presence of a non-suppurative placentitis and necrosis has been described in abortion in early gestation. Characteristic histological foetal lesions such as multifocal non-suppurative encephalitis and myocarditis, although not pathognomonic, allow a presumptive diagnosis (Barr *et al*. 1991, Wouda *et al*. 1997b). Moreover, histology has been used as a reference procedure for the comparisons of other techniques (Baszler *et al*. 1999). Confirmation of the infection is necessary since other protozoa such as *Sarcocystis* spp. may cause similar lesions (Jenkins *et al*. 2002).

Parasite detection

The most commonly applied method for detection of *N. caninum* in the past has been immunohistochemistry (IHC) of foetal brain and other tissues such as lung, liver and heart (Lindsay and Dubey 1989). However, IHC is a relatively insensitive technique for detecting the parasites in severely autolysed foetuses. One of the advantages is its high specificity, although some cross-reactivity with *T. gondii* has been reported (van Maanen *et al*. 2004). The demonstration of *Neospora* antigens by IHC depends to a large extent on the number of sections made and the time spent on microscopic examination (Wouda *et al*. 1997b). Parasite tachyzoites and antigens are usually found associated with lesioned areas in the brain and other organs such as heart and liver (González *et al*. 1999). The number of tissue cysts in the brain is low and generally not associated with cellular responses (Fig. 3).

PCR techniques have been useful as diagnostic tools for detection of the parasite in aborted bovine foetuses (Gottstein *et al.* 1998, Baszler *et al*. 1999, Sager *et al.* 2001, Pereira-Bueno *et al.* 2003, van Maanen *et al.* 2004, Medina *et al*. 2006). PCR methods generally have a higher sensitivity than IHC methods and also a high specificity (van Maanen *et al.* 2004). Fair agreement has been observed between both probes (Pereira-Bueno *et al*. 2003, Medina *et al*. 2006). Several PCR-based methods have been developed in the last few years targeting the parasite ITS1 region (Holmdahl and Mattsson 1996) and the repeated *Neospora*-specific Nc5 sequence (Muller *et al*. 1996) with different modifications, such as nested or seminested PCR test, in an attempt to increase the sen-

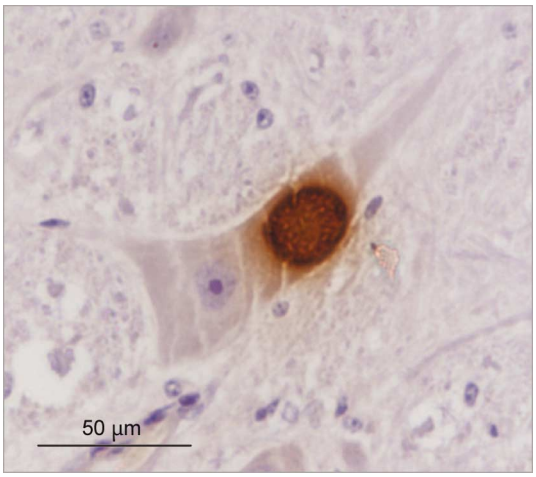

Fig. 3. *Neospora caninum* tissue cyst evidenced by IHC. A tissue cyst of *N. caninum* located in a neurone which is recognised by an anti-*N. caninum* polyclonal rabbit serum using IHC and counterstained with H and E (× 400)

sitivity and specificity of the technique. There appeared to be no clear relationship between the PCR format (i.e. single or nested) and diagnostic sensitivity (van Maanen *et al*. 2004). The advantages of these PCR procedures include high specificity and sensitivity, and the ability to amplify small amounts of *Neospora* DNA in a larger quantity of tissue. In addition, PCR works well when foetuses are autolysed, which is often the case with *Neospora* abortions. At present, two quantitative PCR techniques for the detection of *Neospora* in host tissues have been reported. A quantitative-competitive PCR (QC-PCR) technique based on the inclusion of a known competitor to the target *Neospora*-specific Nc5 genomic sequences was described, but it is labour-intensive and requires post-PCR analysis (Liddell *et al*. 1999). Recently, a specific quantitative PCR based on the Nc5 sequence of *N. caninum* that monitors the reaction in real time, employing the double-stranded DNA-binding dye by SYBR Green I, was standardised and successfully tested in aborted bovine foetuses (Collantes-Fernández *et al*. 2002).

A new method to detect *N. caninum* using indirect *in situ* PCR has been described (Loschenberger *et al*. 2004). *In situ* PCR combines the advantages of the extraordinarily high sensitivity and specificity of PCR and the *in situ* representation of immunohistochemical methods. The indirect *in situ* PCR is able to detect the amplified products using a primed *in situ* (PRINS) reaction with hapten-labelled nucleotides and visualising them using fluorochrome-labelled antibodies. This technique has been carried out in both infected cell cultures and formalin-fixed paraffin-embedded tissues. Clear signals were obtained in the *N. caninum* positive samples using *in situ* PCR, whereas control slides with *Toxoplasma gondii* infected tissues always yielded negative results (Loschenberger *et al*. 2004).

Finally, isolation of *Neospora* in cell culture or bioassay by inoculation in highly receptive mouse strains such as IFN-gamma knock-out and Balb/c nu/nu mice are not suitable techniques for routine foetal diagnosis since the success of isolation depends on the number of organisms present and most parasites in bovine foetuses die during autolysis of host cells (Dubey 1999). However, these techniques may be employed to obtain new *Neospora* isolates to pursue further work on molecular epidemiology, pathogenicity studies and vaccine development.

Foetal serology

The demonstration of *N. caninum* antibodies in sera or foetal fluids by IFAT, ELISA or IB indicates foetal infection (Conrad *et al*. 1993, Barr *et al*. 1995, Paré *et al*. 1995b, Otter *et al*. 1997, Slotved *et al*. 1999, Söndgen *et al*. 2001) since there is no transplacental transfer of antibodies from dam to foetus in cattle. The bovine foetus develops the ability to produce antibodies during the fifth month of gestation. Therefore, foetal serology can be applied to aid in diagnosing *N. caninum* infection in foetuses aged five months or older (Pereira-Bueno *et al*. 2003). However, several reports showed the low sensitivity of foetal serology even after improvement with IB, a negative result does not rule out the presence of a *N. caninum* infection (Barr *et al*. 1995, Wouda *et al*. 1997a, Gottstein *et al*. 1998, Slotved *et al*. 1999, Söndgen *et al*. 2001). Lack of foetal immunocompetence (Wouda *et al*. 1997a), a short interval between infection and foetal death (Söndgen *et al*. 2001), and autolysis, which may cause degradation of foetal immunoglobulins and thus lead to low levels of specific antibodies (Wouda *et al*. 1997a), are thought to be possible causes for low sensitivity of foetal serology. On the other hand, the detection of *Neospora* antibodies in foetal fluids does not necessarily prove that the infection caused foetal death, since many clinically normal calves have congenital antibodies. Therefore, foetal serology cannot be used as the sole diagnostic test to confirm *N. caninum* abortion in individual foetuses.

Conclusions and perspectives

Appropriate techniques for diagnosis of bovine neosporosis, both *in vivo* and in aborted foetuses, have been developed in the last ten years and some of them are commercially available. For diagnosis in live animals, detection of antibodies in serum or milk has been shown to be the best option both at the herd and individual level. These techniques are excellent tools to examine *N. caninum*-associated abortion problems and to adopt some basic herd-control measures such as culling of seropositive aborting dams, the use of the progeny of seropositive dams for beef and the exclusion of the progeny of seropositive dams from breeding (Conraths and Ortega-Mora 2005). Serological tests may also be applied to control neosporosis in the international animal trade, as has been suggested (Moore 2005), since infected animals can introduce the parasite in naive herds or areas where the disease does not exist or prevalence is very low. A combination of different techniques (IFAT+IB or ELISA+IB) is recommended in cer-

tain cases to classify an individual animal as seropositive or seronegative since there is no serological technique that can be considered as a gold standard.

Concerning foetal diagnosis, detection of compatible lesions by histology and parasites by PCR in brain (as well as heart and liver) are the best choices for foetal diagnosis. Diagnostic criteria to distinguish foetal infection and *Neospora*-associated abortion are based not only on the demonstration of foetal infection by PCR (or alternatively by IHC or foetal serology), but also, as has been suggested previously (Jenkins *et al*. 2002), on the extent and severity of the lesions in the foetus, foetal age and the assessment of neosporosis at the herd level. The poor agreement observed when histology, PCR and serology are compared underlines the need to use different and complementary techniques if we want to increase the probability of detecting infection in aborted foetuses (Pereira-Bueno *et al*. 2003, Medina *et al*. 2006). IHC techniques have a relatively low sensitivity in the detection of parasites in host tissues, due to either low parasite numbers or the poor quality of autolysed, mummified or macerated foetal samples. In this respect, PCR sensibility and specificity are higher compared with IHC. Nonetheless, some diagnostic laboratories can experience problems with contamination in the PCR and measures to avoid cross-contamination and carry-over contamination are highly recommended (van Maanen *et al*. 2004). At present, qualitative PCR is widely used in the laboratory diagnosis of foetal neosporosis, but some quantitative PCR techniques have also been applied to bovine foetuses (Collantes-Fernández *et al*. 2002). Recently, quantitative PCR has been employed to record parasite infection intensity and to obtain an accurate estimation of parasite load in different foetal tissues and to make statistical comparisons (Collantes-Fernández *et al*. 2006). This method represents a powerful tool to elucidate host-parasite interaction in foetal tissues and to evaluate the protective effect of vaccines and drugs.

Future research approaches to improve the diagnosis of bovine neosporosis and the use of diagnostic tools in the investigation of host-parasite interactions and neosporosis epidemiology and control may include the description of *Neospora*-stage specific antigens. These markers may be useful not only to discriminate between recent and past infection, as avidity ELISAs allow at present, but also to diagnose parasite-reactivation in persistently infected cattle. In this sense, the first reported gene to be expressed specifically during the *N. caninum* bradyzoite stage (the Nc*SAG4* gene) has been recently identified, cloned and expressed as a recombinant antigen (Fernández-García *et al*. 2006) and its utility as a diagnostic marker is being evaluated.

Another field of interest for foetal diagnosis is the combination of PCR with other molecular tools to investigate the molecular epidemiology of this disease and clarify aspects related with strain/isolate variability. In this sense, several molecular approaches have been used for the genetic characterisation of *N. caninum* isolates. Recently, microsatellite markers have been developed which have proven very useful for the molecular characterisation and tracking of individual laboratory isolates of *N. caninum* (Regidor-Cerrillo *et al*. 2006). These markers are now being evaluated in clinical samples both from natural and experimental infections with the aim of studying the population structure of this parasite and the molecular epidemiology of neosporosis.

Finally, it should be taken into account that, in some countries and regions, control schemes for the disease are being developed. This is the case in Spain where, although a nationwide programme to control the disease does not exist, several regional governments have already placed neosporosis on the list of diseases included in bovine herd-health programmes. For instance, in the Autonomous Region of Galicia, the Spanish Autonomous Community with the highest cattle census, approximately 90,000 animals have been tested in 2005. Massive screening of cattle challenges the diagnostic robustness of serological techniques and makes the role of the veterinary diagnostic laboratories essential. Thus, all veterinary diagnostic laboratories should validate their diagnostic techniques for *N. caninum* and a comparison of these techniques among laboratories from different geographical regions should be undertaken in order to obtain a standardised interpretation of results (von Blumröder *et al*. 2004, van Maanen *et al*. 2004). In countries with control programmes under way, National or Regional Reference Laboratories should be promoted. This idea is particularly important since the OIE (World Organisation for Animal Health) does not have standard protocols for bovine neosporosis. The role of these reference laboratories should be of particular importance for many reasons: To evaluate and define reference diagnostic methods, to prepare and supply antigens and reference serum and tissue controls, to organise inter-laboratory comparison for veterinary diagnostic laboratories, to test some samples to confirm unusual results, to give assessment, to provide training and to allow for the establishment of quality assurance programmes for other veterinary diagnostic laboratories for the purpose of accreditation. In this sense, as a part of an EU initiative, a guidelines manual is being prepared by several European laboratories for the diagnosis of protozoal abortifacients in farm ruminants. These guidelines will contain recommendations about the diagnostic procedures to be followed by official and private institutions across Europe when dealing with neosporosis cases and could be of use to several other countries.

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