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Research Advances in the Diagnosis of Cattle Neosporosis

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Abstract: *Neospora caninum* is an apicomplexan protozoan parasite of animals. Since its first recognition in 1984 in dogs in Norway by Bjerkås and the description of a new genus and species in 1988 by Dubey it has become the focus of significant research attention worldwide. Therefore, numerous diagnostic assays have been developed for use in dogs, cattle and a variety of other potential host species. This study reviews some methods that diagnose neosporosis in cattle including clinical signs, histopathology and Immunohistochemistry (IHC), Agglutination Test (AT), Indirect Fluorescent Antibody Test (IFAT), various Enzyme-Linked Immunosorbent Assays (ELISAs) and Polymerase Chain Reaction (PCR). Advantages and disadvantages of these methods for diagnosing neosporosis in cattle are discussed, with emphasis on IFAT and ELISA which are mainly used for detecting serum antibody to *N. caninum* in cattle. It is clear that further studies are necessary in improving the protocols.

Key words: *Neospora caninum*, neosporosis, diagnosis, cattle

INTRODUCTION

Neospora caninum is an apicomplexan protozoan parasite of animals. Since its first recognition in 1984 in dogs in Norway by Bjerkås and the description of a new genus and species by Dubey *et al.* (1988) it has become the focus of significant research attention worldwide. Neosporosis has a worldwide distribution. Evidence of exposure to *N. caninum* has been described in cattle, goats, sheep, dogs, felids, deer, horses, water buffaloes, red and gray foxes, alpacas, llamas, South American opossums, coyotes, wolves, mastiffs and other wild canids (Moore, 2005; Wapenaar *et al.*, 2007b). In addition, cats, mice, rats, gerbils and monkeys are experimental intermediate hosts of *N. caninum* (Moore, 2005; Jakubek *et al.*, 2007). Seropositivity of *Neospora*-infection has been reported in China (Liu *et al.*, 2003; Liao *et al.*, 2005) but no pathogen of this parasite has been isolated.

Similar to *Toxoplasma gondii*, *N. caninum* has a two-host herbivore-carnivore life cycle. Recent advances in the studies of Neospora life cycle have identified domestic dogs and coyotes as both the intermediate and definitive host for *N. caninum* (Lindsay *et al.*, 2001a; Dubey *et al.*, 2002; Gondim *et al.*, 2004) and both of them are capable of shedding infective oocysts (Lindsay *et al.*, 1999; Gondim *et al.*, 2004). The life cycle is typified by 3 infectious stages: Tachyzoites, tissue cysts and oocysts. Transplacental transmission is considered the major route of transmission of *N. caninum* in cattle in many countries. *Neospora* parasites cause abortion and neonatal morbidity in cattle, sheep, goats and horses and *N. caninum* infection is reported as a significant cause of economic loss in dairy and beef cattle herds worldwide due primarily to abortions and effects on productivity including reduced reproductive efficiency, poor milk production, increased culling, reduced weight gain and poor feed efficiency.

Since the discovery of *N. caninum*, many diagnostic tests have been developed to help in identifying this parasitic infection in cattle, which include Immunohistochemistry (IHC), Agglutination Test (AT), Indirect Fluorescent Antibody Test (IFAT), Enzyme-Linked Immunosorbent Assay (ELISA) and Polymerase Chain Reaction (PCR). This study reviews some of these methods, with emphasis on IFAT and ELISA, which are mainly used for detecting serum antibody to *N. caninum* in cattle.

DIAGNOSIS OF CATTLE NEOSPOROSIS

The diagnosis of *N. caninum* in individual cattle is assisted through the clinical signs and based upon examination of fetal tissues for histological lesions, for

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tachyzoites by immunohistochemistry, or for evidence of *N. caninum*-infection by serologic tests and molecular detection assays.

Clinical signs: Neosporosis induced abortion can occur all year around, no matter cow or beef cattle would be infected by *N. caninum*. *N. caninum*-associated abortions are always sporadic or endemic, epidemic in cattle herds. Abortion of infected cows may be repeated from generation to generation and it is the only clinical sign observed in adult cows. *Neospora*-infected cows of any age may abort from 3 months of gestation to term, with most *Neospora*-induced abortions occurring at 5-6 months of gestation (Dubey and Thulliez, 2005). When a naïve cow is infected with *N. caninum*, there are 4 manifestations, including early embryonic death, abortion, stillbirth, or birth of a feeble abnormal calf and birth of a normal calf with no obvious effect of *N. caninum* infection (Innes *et al*., 2002). *N. caninum* infected calves may have neurologic signs, be underweight, unable to rise, limbs may be flexed or hyperextended. Neurologic examination may reveal ataxia, decreased patellar reflexes and loss of conscious proprioception. Calves may have exophthalmia or asymmetrical appearance in the eyes. *N. caninum* occasionally causes birth defects including hydrocephalus and narrowing of the spinal cord (Dubey *et al*., 1990; 1992), or calves born without clinical signs of disease may exhibit neurologic symptoms 1-2 weeks later. When the congenitally infected calves would become adult cows, the neosporosis may cease or deteriorate, or become subclinical infection. When clinical signs associated with neosporosis are observed in cows or calves, especially in one or more herds, *N. caninum*-infection may be suspected.

Histopathological and immunohistochemical examinations: The brain, heart, liver, lung, kidney, placenta, thymus, adrenal gland, thyroid gland, spleen and skeletal muscle of aborted fetuses can be collected and submitted to histopathological examination. *N. caninum*-positive aborted fetuses have histopathological changes consistent with *N. caninum* infection and non-suppurative inflammation of the brain and/or myocardium and placental cotyledons can be identified by light microscopy (Otter *et al*., 1995). Typical fetal lesions, which are not pathognomonic, include multifocal non-suppurative necrotizing encephalitis and non-suppurative myocarditis with or without focal necrosis in the liver (Barr *et al*., 1991; Sadrebazzaz *et al*., 2007).

By histological examination, widely disseminated changes are present in many organs, however, the most diagnostically significant lesions are found in the brain, including scattered foci of non-suppurative cellular infiltrates, followed by occasional foci of necrosis. Other histological lesions that are consistently found include non-suppurative epicarditis and/or myocarditis, focal non-suppurative myositis and non-suppurative portal hepatitis, frequently with focal hepatic necrosis and focal non-suppurative interstitial pneumonia (Barr *et al*., 1991). However, tachyzoites within affected tissues can be detectable by *N. caninum*-specific immunohistochemistry.

Immunohistochemistry (IHC), in which an avidin-biotin-peroxidase complex was used, was the first test established to identify the parasite and demonstrate that there was no cross-reaction with the closely related *T. gondii* or other extra-intestinal coccidian (Lindsay and Dubey, 1989). This method was developed to detect *N. caninum* in formalin-fixed, paraffin-embedded tissue sections. Specificity of the test was evaluated by testing anti-*Neospora* rabbit sera against known parasites from experimentally induced infections, by using pre-immune rabbit serum (1:500 dilutions) instead of parasite-specific antiserum, by absorting antisera with homologous tachyzoites and by omitting parasite-specific antiserum in the test. Samples were considered positive when the entire organism surface was stained (Lindsay and Dubey, 1989).

The test also can detect tachyzoites and bradyzoites of *N. caninum*. From fetal material, brain provides the tissue of first choice for the diagnosis of neosporosis, although frequently tissue cysts or tachyzoites can also be found in lung, kidney and skeletal muscle. In addition, this test is still used to confirming *N. caninum* parasites in tissue where characteristic inflammatory lesions are observed on histological examination. For example, 5 of the 12 positive cases (41.7%) were identified as a result of IHC staining (Boger and Hattel, 2003). The results of this test revealed no additional *N. caninum* positive cases in the group of 56 fetuses that had previously exhibited histological lesions but were IHC negative as standard diagnostic procedures. Therefore, the standard IHC procedures appear to be quite specific and further evaluation of cases that have been diagnosed as *N. caninum* negative with standard procedures is not necessary.

Serological tests: A definitive diagnosis of neosporosis in cattle requires the examination of the aborted fetus. However, in many instances fetal material is not available. Therefore, most methods are based on serological tests. Agglutination Test (AT), Indirect Fluorescent Antibody Test (IFAT) and various Enzyme-Linked Immunosorbent Assays (ELISAs) are usually utilized. AT, IFAT and ELISA are the main methods for detecting serum antibody to *N. caninum* infection in cattle.

Agglutination Test (AT): Agglutination Test (AT), which has been used to diagnose American trypanosomiasis by Muniz and Dos Santos in 1950 (Muniz and Dos Santos, 1950), is one of the earliest serological methods for the diagnosis of parasitosis. To date, it has been used to diagnose a great quantity of parasitosis include *N. caninum*. AT that diagnoses neosporosis in cattle (Packham *et al*., 1998; Canada *et al*., 2002), raccoon dogs (Kim *et al*., 2003), camels (Hilali *et al*., 1998), wild animals (Lindsay *et al*., 2001b; Dubey and Thulliez, 2005) marine mammals (Dubey *et al*., 2003) and other animals (Packham *et al*., 1998; Ferroglio *et al*., 2005) has been reported. AT is a serologic test to demonstrate the presence of antibodies in the blood. It is dependent on the clumping of cells, microorganisms, or particles when mixed with specific antiserum. The basic principle of an AT is the formation of clumps of small particles coated with antigen when antigen exposed to specific antibody.

A cut-off titer of 1: 80 gives the greatest sensitivity and specificity values for the AT (Thulliez, 1995). However, a recent study of the optimal cut-off value for the *Neospora* AT for serodiagnosis in cattle shows that 1:40 is an optimal AT cut-off titer (Canada *et al*., 2004).

In order to examine a wide variety of animal species that may be infected with *N. caninum*, a Modified direct Agglutination Test (N-MAT) was developed by Packham *et al*. (1998). The N-MAT gave a higher sensitivity (100%) and specificity (97%) than ELISA (74 and 94%, respectively) and had a higher sensitivity but a lower specificity than the IFAT (98 and 99%, respectively) for the examination of cattle serum samples (Packham *et al*., 1998). In summary, AT is rapid, highly sensitive and easy to use and would be ideal for screening large numbers of wildlife or domestic animal samples for the presence of *N. caninum* antibodies.

Indirect Fluorescent Antibody Test (IFAT): The Indirect Fluorescent Antibody Test (IFAT) is the first serological test used for the demonstration of antibodies to *N. caninum* (Dubey *et al*., 1988). Since then, it has been widely used for diagnosing *Neospora*-infection in dogs and cattle and often as a reference test for *N. caninum* antibodies with which other assays are compared. The test is based on the principle of affixing intact *N. caninum* organisms (tachyzoites) to microscopic slides that are incubated with the diluted test serum and in a second step with fluorescence-labelled antibodies directed against immunoglobulins of the animal species under investigation. The test mainly detects antibodies directed to antigens present on the cell surface of the parasite. With apicomplexan species, such antigens are considered more specific than intracellular components (Packham *et al*., 1998).

The reaction is evaluated under a fluorescence microscope. For a positive result, bright, unbroken peripheral fluorescence of the organisms is required, which occurs when moderate or high-titre sera are tested. When low-titre sera are tested, apical or reduced fluorescence occurs and this might also occur as a result of cross-reactivity with *T. gondii*. Furthermore, the cut-off values employed in IFAT is dependent on a range of factors, such as conjugate characteristics and microscope properties and can be arbitrarily selected by varying the dilution of the conjugate. The divergence in test performance between laboratories makes direct comparison of IFAT titres difficult.

With the purpose of probing the sensitivity and specificity of IFAT, prevalence of antibodies to *N. caninum* was determined in a cross-sectional consensus survey of 1,029 bovines in a dairy herd with endemic *Neospora*-induced abortion (Dyer *et al*., 2000). The prevalence of *N. caninum* antibody in the IFAT was 17.9% in 107 neonates, 26.2% in 233 yearling heifers and steers, 39.07% in 218 mature heifers and 26.9% in 465 milking cows (Dyer *et al*., 2000). The result showed that the IFAT was highly sensitive and specific for detecting serum antibody to *N. caninum* in cattle.

Reagents for *N. caninum* IFAT (VMRD Inc. and in-house USDA) are commercially available (Wapenaar *et al*., 2007a). The *N. caninum* IFAT has been used to detect antibodies from a large number of animal species, including dog, fox, cat, cattle, sheep, goat, water buffalo, horse, rodent and primate.

Enzyme-Linked Immunosorbent Assays (ELISAs): To date, various Enzyme-linked Immunosorbent Assays (ELISAs) have been reported to diagnose neosporosis in cattle, which are based upon the principles of indirect ELISA and competitive ELISA. In indirect ELISA (I-ELISA), the immune stimulating complexes (iscoms) ELISA and recombinant antigen ELISA are widely used for detecting serum antibody to *N. caninum* in cattle. In competitive ELISA (C-ELISA), a Monoclonal Antibody (MAb) is used as a competitor with field serum against *N. caninum* tachyzoites. Furthermore, *N. caninum* ELISA kits are commercially available and widely used for screening *N. caninum*-infection in cattle in recent years.

Indirect ELISA: The indirect ELISA (I-ELISA) is widely applied for the demonstration of antibodies directed to a variety of infectious agents, including *N. caninum*. The principle is as follows: The particular antigen is coated onto the plastic surface of multi-well microtitre plates. After incubation with the diluted sera to be analysed, an enzyme-labelled, species-specific anti-immunoglobulin antibody (conjugate) is applied. In a final step, a substrate

is added which, in the presence of the conjugate, is transformed to a coloured product. After a fixed time the enzyme-substrate reaction can be stopped before the absorbance or Optical Density (O.D.) is measured by a spectrophotometer.

Traditional I-ELISA uses tachyzoite lysate as antigen. A number of antigens have been used, such as membrane antigen, intracellular antigen and cytoplasimic origin. The immune stimulating complexes are used to avoid the problems with exposure of intracellular antigens and cytoplasimic origin, which are experienced with a conventional ELISA employing a crude antigen preparation.

Iscoms are cage-like structures of about 40 nm composed of Quil A, cholesterol, phospholipids and antigen. Their main area of use has been as adjuvants and carriers of immunogens in vaccines (Björkman and Lunden, 1998). Iscoms can also be used for selection of surface membrane proteins of micro-organisms (tachyzoites) for use in ELISA, thus decreasing the number of internal proteins that might cause problems with non-specific antibody and cross-reactivity. The first ELISA for demonstration of *N. caninum* antibodies was an iscom ELISA developed for analysis of canine sera (Björkman *et al*., 1994). Soon after, the preparation of *N. caninum* iscoms is described and ELISA based on iscom antigen preparations that have been used for diagnosis of protozoan infections are reviewed (Björkman and Lunden, 1998). It has also been modified to enable analysis of sera from cattle (Björkman *et al*., 1997), water buffaloes (Huong *et al*., 1998) mice (Pinitkiatisaku *et al*., 2005). Moreover, no cross-reactivity with *T. gondii* or other closely related protozoa was observed with the iscom ELISA (Lally *et al*., 1996a). This ELISA also showed promising results as a method for screening specific antibodies against *N. caninum* in bovine foetal fluid (Slotved *et al*., 1999).

To validate iscom ELISA used to detect antibodies to *N. caninum*, sera from 244 cattle in five Swedish dairy herds infected with *N. caninum* were analysed. The iscom ELISA produced fewest incorrect test results over all at a cut-off value of 0.200. The sensitivity and specificity at this cut-off were 99 and 96%, respectively (Frossling *et al*., 2003). Therefore, the iscom ELISA has been proved very reliable, with high sensitivity and specificity.

To provide a sensitive and specific assay for detecting antibodies to *N. caninum* in the sera of infected bovines, recombinant form of the antigens which conduct as the major surface protein of the parasite are utilized.

Various ELISAs based upon recombinant *N. caninum* antigens such as Nc4.1 and Nc14.1 (Lally *et al*., 1996a), N54 and N57 (Louie *et al*., 1997) have been established for the diagnosis of neosporosis. The data showed that all the ELISAs using recombinant proteins were able to differentiate between naturally infected and uninfected control bovine sera. In addition, ELISAs using rNcp29 (Howe *et al*., 2002), Nc-p43 (Ahn *et al*., 2003), GST-NcSAG1t (Chahan *et al*., 2003) and the Reverse Phase-High Performance Liquid Chromatography (RP-HPLC) purified NcGRA6 (dELISA) (Jenkins *et al*., 2005) have been confirmed to diagnose neosporosis in cattle. Recently, the surface antigen 1-related sequence 2 of *N. caninum* (NcSRS2) was tested in an ELISA for the detection of antibodies to *N. caninum* in cattle. In the 197 samples analyzed, 64 (32.5%) samples were positive for antibodies to *N. caninum*. In the 64 ELISA-positive samples, 58 (90.6%) were confirmed as positive by Western blot analysis with whole-parasite antigens (Gaturaga *et al*., 2005). In addition, it is significant that bovine milk can be tested for antibodies against *N. caninum* by the ELISA based on a surface or recombinant antigen of *N. caninum* tachyzoites. Excellent agreement has been observed between milk and serum results (Chanlun *et al*., 2006; Frössling *et al*., 2006; Varcasia *et al*., 2006). These results suggest that ELISA with recombinant antigen is a specific and sensitive assay for diagnosis of *N. caninum* infection in cattle.

Competitive ELISA: Competitive ELISA (C-ELISA) is an indirect test in which a Monoclonal Antibody (MAb) is included and allowed to compete with antibodies in the test sera for available epitopes on the assay antigen. The secondary antibody is directed to mouse immunoglobulins, i.e. the MAb. If the test serum contains antibodies directed to the same epitope as the MAb, the absorbance will be lower in wells containing a mixture of MAb and test serum than in wells with only MAb. The result is most often presented as percentage inhibition by the test serum.

To investigate specific methods of antemortem diagnosis of neosporosis, the antibody responses of infected cows were evaluated by C-ELISA by using a MAb (MAb4A4-2), against *N. caninum* tachyzoites (a 65kDa carbohydrate epitope on a single *N. caninum* tachyzoites surface antigen) (Baszler *et al*., 1996). The binding of MAb4A4-2 to *N. caninum* tachyzoite antigen was consistently inhibited by sera from *Neospora*-infected cows in a C-ELISA format and was not inhibited by sera from *Neospora* antibody-negative cows. Furthermore, sera from cattle experimentally infected with *T. gondii*, *S. cruzi*, *S. hominis*, or *S. hirsuta*, which did not inhibit binding of MAb 4A4-2 in the C-ELISA. Thus, MAb 4A4-2 binds a carbohydrate epitope on a single *N. caninum* tachyzoite surface antigen that is recognized consistently and specifically by *Neospora*-infected cattle (Baszler *et al*., 1996).

Moreover, the MAb-based C-ELISA was modified and the assay was validated in various defined cattle populations for detection of serum antibody to *N. caninum*. Modifications to the C-ELISA included capturing native *N. caninum* antigen with a parasite-specific MAb (MAb5B6-25) and directly conjugating the competitor MAb (MAb4A4-2), with both MAbs binding different epitopes of a conserved, immunodominant 65-kDa tachyzoite surface antigen. For example, an independent assay to validate the test used a "gold standard" set of 184 cow sera (42 positives and 142 negatives) defined by fetal histopathologic examination and *N. caninum* IHC staining and by maternal *N. caninum* IFAT at a 1: 200 serum dilution. The sensitivity was 97.6% and specificity was 98.6% (Baszler *et al*., 2001). It has been shown to be un-reactive to antigens of 2 closely related apicomplexan protozoa, T.gondii and S. cruzi (Baszler *et al*., 2001). In summary, the modified *N. caninum* C-ELISA provides a simple, rapid and versatile method to identify *N. caninum* infection status in cattle using a single cut-off value. Currently, the C-ELISA is widely used for detecting antibody against *N. caninum*.

The application of the ELISA kits for the detection of *N. caninum* in cattle: Two ELISA test kits are produced which based upon I-ELISA and are extensively used for detecting serum antibody to *N. caninum* in USA, Canada and other countries, with the names of IDEXX (IDEXX Laboratories, Westbrook, Maine, USA) and Biovet (BIOVET Laboratories, St. Hyacinthe, Quebec), respectively. Recent studies showed that both of them produced results that correlated very well with the C-ELISA results. When compared with immunoblotting (the gold standard) by using 150 field sera from an infected beef herd, the two ELISAs worked equally well (Wu *et al*., 2002). Sensitivities for the Biovet and IDEXX ELISAs on the field samples were 95.1 and 97.6%, respectively, while specificities were 100 and 98.5%, respectively (Wu *et al*., 2002). When compared to C-ELISA results on bovine sera from an infected herd, the identity scores of the 2 ELISAs were 98% (IDEXX) and 97.33% (Biovet) (Wu *et al*., 2002). For the IDEXX test, the sensitivity and specificity were 97.56 and 98.53%, whereas for the Biovet assay 95.12 and 100% were recorded, respectively (Wu *et al*., 2002). Currently, both Biovet and IDEXX ELISAs are commercially available.

Recently, a study of the 2 commercial milk ELISAs from IDEXX and LSI were also developed. A cut-off value 0.6 for the IDEXX and 0.2 for the LSI were defined and specificity at these cut-off values was 92% (95% CI: 87-98%) for the IDEXX and 94% (95% CI: 90-99%) for the LSI ELISA (Bartels *et al*., 2005).

N. caninum Antibody Test Kits currently are commercially available and widely used for screening *N. caninum*-infection in cattle.

Molecular detection Assay-Polymerase Chain Reaction (PCR): In the last few years, diagnosis of neosporosis was much improved by the development of Polymerase Chain Reaction (PCR) tests, which allow highly sensitive detection of the parasite through the amplification and subsequent demonstration of parasite-specific DNA sequences. The first PCR assay for the diagnosis of neosporosis was based upon using the *N. caninum* 14-3-3 gene by Lally NC in 1996 (Lally *et al*., 1996b). Since then, several PCR-based methods have been developed targeting the parasite ITS1 region (Payne and Ellis, 1996) and the repeated Neospora-specific Nc5 sequence (Kaufmann *et al*., 1996) with different modifications, such as nested, semi-nested or real-time (using a double-stranded DNA-binding dye named SYBR Green I) PCR test, trying to increase the sensitivity and specificity of the techniques (Kaufmann *et al*., 1996; Medina *et al*., 2006).

One of the most commonly used diagnostic PCRs includes a set of primers that are targeted to the repetitive genomic sequence Nc5 (Müller *et al*., 2001). Nc5 *Neospora* PCR was first developed to define the *N. caninum* infection in bovine By Kaufmann in 1996 (Kaufmann *et al*., 1996). The sequence Nc5 was chosen as the target for the assays for several reasons: this sequence has apparently not been found in other taxa; it is a repetitive sequence (Yamage *et al*., 1996) and finally, it has shown excellent sensitivity when used in a quantitative-competitive detection system for Neospora in mice (Liddell *et al*., 1999) and for the diagnoses of bovine aborted fetuses (Kaufmann *et al*., 1996). In the real-time PCR for the Nc5 sequence, as little as 0.1 tachyzoites per reaction, which is approximately equal to 10 fg of genomic Neospora DNA, could be detected (Collantes-Fernandez *et al*., 2002). Several Nc5 PCR assay for quantitative detection of *N. caninum* were established, such as a quantitative-competitive PCR (QC-PCR) (Liddell *et al*., 1999) real-time fluorescent PCR (Müller *et al*., 2002) and real-time PCR (Okeoma *et al*., 2005) and so on. The Nc5 PCR analyses showed that brain was the most reliable tissue for PCR analysis to detect *N. caninum*-infected fetuses.

A sensitive and specific PCR detection assay for *N. caninum* DNA would be useful to augment the diagnosis of *N. caninum* abortion where pathologic changes in fetal tissues are consistent with neosporosis but can not be consistently confirmed by IHC or serology. Hence, PCR techniques have been useful as diagnostic tools for

detecting the parasite in bovine aborted fetuses (Gottstein *et al*., 1998). PCR assays also can detect *N. caninum* DNA in formalin-fixed, paraffin-embedded tissues, fresh tissues, serum and colostrum (McInnes *et al*., 2006; Moskwa *et al*., 2007). In addition, *Neospora*-specific PCR products can be amplified from DNAs of different bovine tissues, including brain, spinal cord, heart, lung, kidney, diaphragm, skeletal muscle and placenta, as well as amniotic fluid samples of infected cattle. Recently, 44 fetal brains from 8 aborted dairy fetuses in Aguascalientes, a state in the central part of Mexico were analyzed by Medina *et al*. (2006). A single tube nested PCR was designed to probe the presence of *N. caninum* in fetal brain tissue with primers NF1, NS2, NR1 and SR1, with histopathology as the reference technique (Medina *et al*., 2006). From the 44 bovine fetuses studied, 35 (80%) were diagnosed as being infected by *N. caninum* using PCR probe and 20 (45%) were considered positive by histopathology, because they had lesions suggestive of bovine neosporosis. Fair agreement was observed (31%) between both probes.

DISCUSSION

Neosporosis, which has a worldwide distribution, has been reported as one of the most serious diseases affecting dairy and beef cattle industry and as the major cause of bovine abortion in several developed countries such as USA, Japan and Canada. *N. caninum*-abortion is reported as a significant cause of economic loss in dairy and beef cattle herds worldwide. Researches on the diagnosis of *Neospora*-infection are hotly.

Clinical diagnosis is a significant method for the pre-death diagnosis of neosporosis in cattle. When abortion in dam and the birth of a feeble abnormal calf are detected in one or more herd-based cattle, *N. caninum*-infection is mentioned. Because no pathogens of *N. caninum* can be isolated and there are many cases which had a mild, subclinical *N. caninum* infection can not be diagnosed, so this method is often below the real detection and just plays as a reference for identification of nosporosis in cattle.

Both histopathology and immunohistochemical examination can accurately detect the parasite of *N. caninum*. The most characteristic lesion is focal encephalitis characterized by necrosis and nonsuppurative inflammation. But both are relatively insensitive techniques for detecting the parasite in host tissues sometimes, due to the low quality of the fetal tissue that could be autolyzed, mummified, or macerated.

AT is proved to be highly sensitive and specific for both naturally and experimentally infected animals, highly reproducible between and within readers, easy to use on large sample sizes without requiring special equipment and useful in testing serum from any species without modification. In addition, such a test could be used in extensive seroprevalence studies to help pinpoint possible definitive hosts for *N. caninum*, particularly for those species have been found in dairies with endemic *N. caninum* infection. This would in turn allow for the development and implementation of proper preventative measures against *N. caninum*. But the validation of this method is limited by the high require of antigen preparation, the intact tachyzoite is needed. If the concentration of the tachyzoites in the antigen was too low, reactions were not detectable. Conversely, if the antigen concentration was too high, the negative buttons began to appear fuzzy, looking more like weakly positive reactions than like true-negative reactions and resulted in false-positive readings (Desmonts and Remington, 1980). As stated by Desmonts and Remington (1980) for the *T. gondii* antigen, preservation of the parasite integrity and reduction of cell culture debris in the antigen preparation were very important for the *N. caninum* antigen to work properly. The parasites had to retain a normal crescent shape to maintain optimum test sensitivity. The reduced specificity of AT was due to false-positive reactions in testing fetal fluids with particulate matter or severely hemolyzed serum. AT performed perfectly for the experimentally infected animals, but gave either false-positive or false-negative results for the naturally infected-animal samples. Furthermore, results from AT kit for *T. gondii* could be read after letting the test plate sit at room temperature undisturbed for 5 h, but AT for *N. caninum* results were readable only after an overnight incubation, which remains the only major drawback to this method.

In a word, AT allows for the determination of *N. caninum* seroprevalence in wildlife or domestic animals due to not requiring species-specific secondary antibody, hardly use to detected the samples that were originated from natural infectious animal in the same species. Continued use of AT to screen a large number of samples from as many different species as possible may help researchers to target those species which have a greater probability of being exposed to *N. caninum* and possibly being the definitive host.

Nowadays, in addition to AT, IFAT and ELISA are also widely used to detect serum antibody to *N. caninum* and highly diagnostic sensitivity and diagnostic specificity are dated.

IFAT is the first serological test used for the demonstration of antibodies to *N. caninum*, since then, it has been used extensively for diagnosing *Neospora*-infection in dogs and cattle and is often used as a golden reference test for *N. caninum* antibodies with which other assays are compared. The specificity of the IFAT test for *N. caninum* used in our review had been confirmed in a report by Dubey and Lindsay, which no serological cross-reactivity was found among calves experimentally infected with *Sarcocystis* sp., *Eimeria bovis*, *T. gondii* and *Cryptosporidium parvum* (Dubey and Lindsay, 1996). In IFAT assay, titer and absorbance values are dependant on antigen composition, secondary antibodies and other reagents. False-negative or false-positive results can be exhibited, so results should be interpreted with caution. In addition, performance of the test requires training and experience and because of visual, individual interpretation of reactions, IFAT results are to a certain degree subjective. It is imperative that the optimal dilution of the fluorescence-labelled secondary antibody (the conjugate) is carefully established with known positive and negative control sera along with the particular microscope used. Furthermore, the cut-off titre can be arbitrarily selected to provide sensitivity and specificity requested for the particular application. Comparisons of IFAT results from different laboratories are extremely difficult, given the different antigen preparations, reagents and serum dilutions used and the diversity in cutoff selected, the method must give a further optimal modification to detect *N. caninum* infection in cattle.

To date, various ELISAs have been reported for the diagnosis of neosporosis in cattle. Many of them are commercially available such as Biovet and IDEXX ELISAs. *Neospora*-ELISA provided a simple, rapid and versatile method to detect sera antibody to *N. caninum* infection in cattle. Compared with IFAT, one of the advantages of the ELISA is that the registration of reactions is done objectively and the assay can easily be automated. It is therefore, a technique well suited for screening of large numbers of samples, e.g. in serological surveys. In addition, another advantage is that bovine milk can be tested for antibodies against *N. caninum* by the ELISA based on a surface or rebombinant antigen of *N. caninum* tachyzoites. Excellent agreement has been observed between milk and serum results.

However, a problem with *N. caninum* serological detection is that, at present, no ultimate, generally accepted and standardized test is available. Besides, serology is not applicable in young fetuses, because the bovine fetus is not capable of producing antibodies in response to an antigenic challenge before about 5 months of age.

Molecular diagnostic techniques have the advantage of being highly specific and sensitive, as well as being able to amplify small amounts of parasites in a larger quantity of tissue than is generally available for histopathologic examination on a microscope slide. Hence, PCR test is far more sensitive than standard histology or immunohistochemistry in detecting the distribution of *Neospora* infection in tissues and is therefore a useful tool in disease pathogenesis studies. PCR assays can detect *N. caninum* DNA in formalin-fixed, paraffin-embedded tissues, fresh tissues, frozen extended semens and the bloods. Furthermore, the PCR probe detection system could also be a valuable tool for studies focused on the identification of the definitive host for *Neospora* sp. and be useful in epidemiological surveys of both domestic and wild canine hosts. One of the main disadvantages of PCR for routine diagnosis of infectious diseases is amplicon contamination, which may lead to false-positive tests. The resultant amplicon should be as short as practical, since, DNA in some clinical samples may be degraded due to autolysis, aging, or chemical fixation. Although, multiple PCR methods have been described for detection of *N. caninum* DNA in bovine tissues, *N. caninum* PCR has been tested infrequently for the routine diagnosis of naturally occurring *N. caninum* abortion. In addition, this technique is labor-intensive, low throughput and requires post-PCR analysis. At the same time, due to the costs, time, equipment and expertise required to perform these tests, PCR techniques are not suitable for practical needs. At present, PCR-based detection systems are primarily used as investigative tools in a relatively limited number of diagnostic and research laboratories. The PCR provide a powerful supplementary method for the diagnosis of *Neospora* infection in the aborted fetuses of bovines. However, it may become more applicable as this technology continues to become more widely used and cost-effective in the diagnosis of other diseases of importance in cattle and veterinary medicine.

CONCLUSION

In summary, serological tests are useful in determining whether an animal has been infected with *N. caninum*, histopathology examinations are accurate identification *N. caninum* in cattle, especially, the pathogens could be isolated in infected organism. It is

recognized that PCR has been proven insufficiently robust to serve as a diagnostic test alone, although when used in conjunction with other diagnostic techniques, it does prove to be a useful aid. Compared among these methods, IFAT and ELISA are more applicable for specific-*N. caninum* diagnosis, both of them are the better choices for detecting neosporosis in cattle.

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