

REVIEW ARTICLE

Importance of serological cross-reactivity among *Toxoplasma gondii*, *Hammondia* spp., *Neospora* spp., *Sarcocystis* spp. and *Besnoitia besnoiti*

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SUMMARY

Toxoplasma gondii, *Neospora* spp., *Sarcocystis* spp., *Hammondia* spp. and *Besnoitia besnoiti* are genetically related cyst-forming coccidia. Serology is frequently used for the identification of *T. gondii*, *Neospora* spp. and *B. besnoiti*-exposed individuals. Serologic cross-reactions occur in different tests among animals infected with *T. gondii* and *H. hammondi*, as well as among animals infected by *T. gondii* and *N. caninum*. Infections caused by *N. caninum* and *N. hughesi* are almost indistinguishable by serology. *Neospora caninum*, *B. besnoiti* and *Sarcocystis* spp. infections in cattle show some degree of serologic cross-reactivity. Antibody cross-reactivity between *Neospora* spp. and *H. heydorni*-infected animals is suspected, but not proven to occur. We review serologic cross-reactivity among animals and/or humans infected with *T. gondii*, *Neospora* spp., *Sarcocystis* spp., *Hammondia* spp. and *B. besnoiti*. Emphasis is laid upon antigens and serological methods for *N. caninum* diagnosis which were tested for cross-reactivity with related protozoa. Species-specific antigens, as well as stage-specific proteins have been identified in some of these parasites and have promising use for diagnosis and epidemiological surveys.

Key words: Serology, cross-reaction, *Toxoplasma gondii*, *Neospora* sp., *Sarcocystis* sp., *Hammondia* sp., *Besnoitia besnoiti*.

INTRODUCTION

Toxoplasma gondii, *Neospora* spp., *Sarcocystis* spp., *Hammondia* spp. and *Besnoitia besnoiti* are closely related tissue cyst-forming parasites that belong to the family Sarcocystidae (Carreno *et al.* 1998; Mugridge *et al.* 2000). The complete life cycles of Sarcocystidae organisms are complex and involve several parasite stages in definitive and intermediate hosts. In species with known life cycles, carnivore or omnivore definitive hosts harbour sexual reproduction of the parasites in intestinal epithelium. They may shed large numbers of parasite oocysts or sporocysts in their feces. Intermediate hosts acquire infection upon ingestion of sporulated oocysts or sporocysts in food or water. Sporozoites invade intestinal epithelial cells of the intermediate hosts and spread to other tissues as tachyzoites or merozoites. These latter stages may encyst as slow

multiplying forms, called bradyzoites. Ingestion of tissue cysts by carnivores is the main route of infection for definitive hosts, which culminates with the formation of oocysts in their intestinal epithelium (Levine and Ivens, 1981).

Among the cyst-forming parasites, *T. gondii* is the most studied. It induces disease in a wide range of warm-blooded animals, including humans (Tenter *et al.* 2000), and causes abortion in livestock, especially in sheep and goats (Buxton, 1998). *Neospora caninum* was originally described in dogs (Bjerkas *et al.* 1984; Dubey *et al.* 1988a), but it gradually gained more attention from the scientific community as a major cause of neonatal mortality and abortion in mainly cattle but also other ruminants (O'Toole and Jeffrey, 1987; Parish *et al.* 1987; Anderson *et al.* 1991), besides causing neuromuscular disease in dogs (Ruehlmann *et al.* 1995). *Neospora hughesi* was proposed as a new species in the genus *Neospora* and has been associated with myeloencephalitis in horses (Marsh *et al.* 1998). The genus *Sarcocystis* possesses more than 100 species, with cattle as intermediate hosts of at least three species (*S. cruzi*, *S. hirsuta* and *S. hominis*). Among these,

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only *S. cruzi* is mildly pathogenic for cattle and generally non-pathogenic for its definitive host (dog) (reviewed by Dubey and Lindsay, 2006). Additional *Sarcocystis* spp. have been observed in bovine tissues, but their nomenclatures are still in debate (Dubey et al. 2016; Gjerde, 2016). Three parasite species compose the genus *Hammondia* (*H. hammondi*, *H. heydorni* and *H. truffittae*), which have no known association with disease in humans or in naturally infected animals. However, *Hammondia* spp. are closely related to *T. gondii* and *N. caninum* (Mugridge et al. 2000), so diagnostic methods need to discriminate between infections caused by these parasites. *Besnoitia besnoiti* causes a debilitating disease mainly characterized by both a cutaneous and systemic manifestation (Alvarez-Garcia et al. 2013). Reproductive abnormalities in cattle may also occur, such as infertility in bulls and abortion when cows are infected during pregnancy (Cortes et al. 2014). Bovine besnoitiosis was reported first more than a century ago in Southern France and Portugal, but the parasite has spread to several European countries during the last 10 years and besnoitiosis is now considered as a re-emerging disease in cattle, at least in Europe (Alvarez-Garcia et al. 2013).

Toxoplasma gondii and *H. hammondi* have cats as definitive hosts, which shed morphologically indistinguishable oocysts in their feces. Dogs and certain canid species serve as definitive hosts for *N. caninum* (McAllister et al. 1998; Gondim et al. 2004; King et al. 2010; Dubey et al. 2011) and *H. heydorni* (Blagburn et al. 1988; Slapeta et al. 2002; Soares et al. 2009). *Hammondia truffittae* has two species of wild canids (red fox and arctic fox) as definitive hosts (Gjerde and Dahlgren, 2011). Three *Sarcocystis* spp. from cattle, *S. cruzi*, *S. hirsuta* and *S. hominis*, have dogs, cats, and primates as definitive hosts, respectively (reviewed by Gjerde, 2016). *Besnoitia besnoiti* is suspected to have a carnivore as definitive host, but so far no animal has been identified shedding oocysts of the parasite by natural or experimental infections (Basso et al. 2011).

Infections caused by *T. gondii*, *Hammondia* spp., *Neospora* spp., *Sarcocystis* spp. and *B. besnoiti* are assessed by a great variety of diagnostic tools, depending on the purpose of the analysis and available biological sample. In clinically affected individuals, detection of parasite-specific antibodies in serum or other body fluids is the most commonly employed diagnostic approach, except for *Hammondia* spp., and *Sarcocystis* spp. from cattle. Continuous cultivation of bovine *Sarcocystis* spp., like *S. cruzi*, is difficult (Andrews et al. 1990). To date, *Hammondia* spp. cannot be continuously grown in cell culture, which impedes production of parasite antigens needed to produce serologic tests for these parasites (Riahi et al. 1995; Schares et al. 2003; Gondim et al. 2015). In this review, serologic cross-reactivity is

reviewed in detail among infections caused by *T. gondii*, *Hammondia* spp., *Neospora* spp., *Sarcocystis* spp. and *B. besnoiti*. Special emphasis is put on serologic cross-reactivity among animals infected with *N. caninum* and related pathogens. Further consideration is given to the discovery and production of species-specific and stage-specific antigens, which promise to improve diagnostic specificity and may enable discrimination between different modes of parasite acquisition.

SEROLOGY FOR *T. GONDII* AND CROSS-REACTIVITY WITH RELATED PATHOGENS

Toxoplasma gondii vs *H. hammondi*

During the first decades after *T. gondii* was discovered, several scientists have attempted to develop serologic tests with high sensitivity and specificity to diagnose *T. gondii* infection, as well as to understand the antigenic composition of the parasite. The development and improvement of serological tests, such as the Sabin–Feldman dye test (DT) (Sabin and Feldman, 1948; Beverley and Beattie, 1952), direct agglutination test (Fulton and Turk, 1959; Desmonts and Remington, 1980), complement fixation test (CFT) (Sabin, 1949), enzyme-linked immunosorbent assay (ELISA) (Walls et al. 1977), immunofluorescence antibody test (IFAT) (Kelen et al. 1962), indirect haemagglutination test (IHA) (Lunde and Jacobs, 1958) and Western blot (WB) (Araujo et al. 1984) favoured a great advance in the study of toxoplasmosis.

Cross-immunity studies between T. gondii and H. hammondi. Mice and hamsters that were experimentally infected with *H. hammondi* (CR-4 strain) oocysts developed immunity and did not die after challenged with lethal doses of oocysts from a mouse-virulent *T. gondii* strain (M-7741 strain) (Frenkel and Dubey, 1975). In contrast, cats that were experimentally infected with *H. hammondi* and shed oocysts were not immunized against excretion of *T. gondii* oocysts (Frenkel and Dubey, 1975). An additional study approached cross-immunity between *T. gondii* and *H. hammondi*, by using six *H. hammondi* strains in an infection model of mice and hamsters (Christie and Dubey, 1977). The authors observed that 103 of 108 mice that were orally inoculated with *H. hammondi* oocysts survived a lethal challenge dose (10^5 oocysts) of *T. gondii* (strain M-7741). The *H. hammondi*-inoculated hamsters also developed immunity against a lethal dose of *T. gondii* oocysts, but this immunity was variable depending on the *H. hammondi* strain. The two most immunogenic *H. hammondi* strains conferred protection to fatal toxoplasmosis in 100 and 83% of the hamsters, respectively, after challenging with *T. gondii* oocysts (Christie and Dubey, 1977).

Immunization of goats with *H. hammondi* had a protective effect against abortion induced by *T. gondii*; however, the immunization did not prevent transplacental transmission of *T. gondii* in pregnant does (Munday and Dubey, 1988). Partial immunity against toxoplasmosis was also obtained in Tamar wallabies (*Macropus eugenii*), that were orally infected with 1×10^5 oocysts of *H. hammondi* (Reddacliff *et al.* 1993).

Serologic cross-reactivity between T. gondii and H. hammondi. When *H. hammondi* was first described, some rodent species experimentally infected with this parasite developed cross-reacting antibodies against *T. gondii* antigens in the DT (Frenkel and Dubey, 1975). Further studies were carried out and confirmed that serologic cross-reactivity between *T. gondii* and *H. hammondi* occurred with sera from other animals, besides rodents. Weiland *et al.* (1979) investigated cross-reactivity between *T. gondii* and *H. hammondi* in four animal species (120 mice, six dogs, six rabbits and six pigs) by using five serological tests (DT, CFT, ELISA, IFAT and IHA). Half of the animals were orally inoculated with *T. gondii* oocysts and the other half received *H. hammondi* oocysts by the same route. Sera from *H. hammondi*-infected mice reacted with *T. gondii* antigens in three tests (DT, ELISA and CFT). Sera from dogs infected with *H. hammondi* recognized *T. gondii* antigens by DT and ELISA. Sera from rabbits exhibited cross-reaction between the two parasites by ELISA. The sera of pigs infected with *H. hammondi* did not cross-react with *T. gondii* in any of the five serological tests. In this study, the IFAT was considered the most *Toxoplasma*-specific method. During the course of infection, the animals infected with *T. gondii* presented higher titres in the tests when compared with those infected with *H. hammondi* (Weiland *et al.* 1979). Munday and Dubey (1986) observed that sheep that were inoculated with *H. hammondi* oocysts presented cross-reactivity with *T. gondii* antigen by IFAT. Before infection with *H. hammondi*, the sheep had no detectable antibodies to *T. gondii*. After oral inoculation with *H. hammondi* oocysts, the animals presented antibody titres of 1:16 to *T. gondii* by IFAT. Goats infected with *H. hammondi* were shown to produce antibodies against *T. gondii* tested by DT, with titres up to 1:64 (Dubey, 1981).

The antigenic similarity between *T. gondii* and *H. hammondi* was investigated using sera from experimentally infected mice (Araujo *et al.* 1984). The authors employed *T. gondii* tachyzoites (RH strain) for two antigen detection procedures: (1) the antigen was labelled with ^{125}I , immune-precipitated with sera from *T. gondii* or *H. hammondi*-infected mice, run by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and autoradiographed; (2) the

antigen was lysed, separated by SDS-PAGE and tested by immunoblot. The sera from mice that were orally inoculated with *H. hammondi* oocysts recognized *T. gondii* antigens of MW 92.5 kDa (two antigens), 66.2 kDa, between 66.2 and 45 kDa, and between 31 and 21.5 kDa; compared with the *T. gondii*-positive reaction only a *T. gondii* antigen of 21.5 kDa was not recognized by the *H. hammondi*-positive mouse serum (Araujo *et al.* 1984). This study confirmed that *T. gondii* and *H. hammondi* have similar antigenic components in their tachyzoites.

To facilitate scientific communication among research groups and laboratories, Sibley *et al.* (1991) proposed a nomenclature system for naming mutants, genes and gene products of *T. gondii*, which was based on the system used for the yeast *Saccharomyces cerevisiae*. As an example, the surface protein (P30) was designated SAG1, which is the product of the *SAG1* gene. However, as it was observed more recently that *SAG1* genes belong to a superfamily of related genes, named SRS (SAG1-related sequences), which encode a superfamily of structurally related surface proteins from *T. gondii*, the name of SAG1 (P30) has been changed to SRS29B (Wasmuth *et al.* 2012).

Riahi *et al.* (1998) studied the antigenic similarity of *T. gondii* and *H. hammondi* employing five monoclonal antibodies (MAbs) against *T. gondii* surface antigens and a polyclonal mouse serum to *H. hammondi*. In order to produce enough *H. hammondi* antigen, the authors used an *in vitro* model that allows the production of *H. hammondi* cysts up to 3 months in cell culture (Riahi *et al.* 1995). The cyst formation, confirmed by ultrastructural characteristics of the organism, started from 6 days after sporozoites were inoculated into feline kidney cells (CRFK). At 4 days of infection, the authors assumed that the multiplying *H. hammondi* zoites were tachyzoites (Riahi *et al.* 1998).

Tachyzoite antigens from *T. gondii* and *H. hammondi* were tested by IFAT and WB. By combining the two serologic techniques (IFAT and WB), five *T. gondii* antigens (MW of 30, 32, 35, 66 and 90 kDa) were recognized using polyclonal anti-*H. hammondi* serum. An interesting finding obtained by Riahi *et al.* (1998) was the recognition of the SAG1 (SRS29B) antigen by the anti-*H. hammondi* serum, as this protein is a major antigen of *T. gondii* (Burg *et al.* 1988) and had been considered to be a specific marker for the parasite (Mineo *et al.* 1993). Despite the antigenic similarity between *T. gondii* and *H. hammondi*, MAbs targeted against *H. hammondi* antigens were produced, and five of them did not cross-react with *T. gondii* antigens (Riahi *et al.* 2000). These findings are promising for the characterization of *H. hammondi*-specific antigens or epitopes, which could enable development of serologic tests for this parasite that would not cross-react with *T. gondii*.

The genome of a German strain of *H. hammondi* was sequenced and the genomic synteny between this parasite and *T. gondii* was higher than 95% (Walzer et al. 2013). It was found that orthologues of key *T. gondii* mouse virulence genes are functionally conserved in *H. hammondi*, but these data were not enough to explain the phenotypic differences observed between both parasites (Walzer et al. 2013). In a recent work, the genomes of 62 strains of *T. gondii* were compared with those from *H. hammondi*, *N. caninum* and *Sarcocystis neurona* (Lorenzi et al. 2016); these authors demonstrated that *T. gondii* possesses an expansion of parasite-specific secretory pathogenesis determinants (SPDs) when compared with the three latter parasites. The SPDs encompass genes encoding secretory proteins from micronemes, dense granules, rhoptries and surface antigens, whose expansion and diversity are associated with the patterns of transmission, host range and pathogenicity of *T. gondii* (Lorenzi et al. 2016). It was reported that *T. gondii* shares a high number of orthologues with *H. hammondi* (7095) and *N. caninum* (6308) (Lorenzi et al. 2016).

The search for more practical and efficient methods to detect specific antibodies against *T. gondii* pushed the establishment of tests based on antigenic fractions, which are gradually replacing traditional tests based on whole organisms (agglutination, DT and IFAT) or total extracts of parasite antigens. Recognized immunodominant antigens of *T. gondii*, including the tachyzoite surface antigens SAG1 (p30) (Kasper, 1987; Mineo et al. 1993), SAG2 (p22) (Prince et al. 1990), SAG3 (P43) (Cesbron-Delauw et al. 1994), SAG4 (p18) (Odberg-Ferragut et al. 1996) and several other proteins from dense granules, rhoptries and micronemes are being produced as recombinant proteins (reviewed by Holec-Gasior, 2013).

So far, it is not known whether humans may be infected with *H. hammondi*, and in case it happens, the possibility of serologic cross-reactivity with *T. gondii* antigens cannot be ruled out. Cats seem to shed *T. gondii* and *H. hammondi* in similar proportions as reported in a recent study from Germany (Schaes et al. 2016), and humans and animals of many species are potentially exposed to *H. hammondi*. The establishment of *H. hammondi* infections in most animal species has not been rigorously investigated; however, the life cycle of *H. hammondi* seems to lack avian hosts (Dubey and Sreekumar, 2003).

Toxoplasma gondii vs *N. caninum*

The serologic differentiation between *T. gondii* and *H. hammondi* infections has clinical and epidemiological relevance. Since *H. hammondi* is not known to induce disease in animals or humans, the major concern about the serologic differentiation of these

parasites seems to be to avoid *T. gondii* false-positive results in individuals potentially infected with *H. hammondi*. The identification of *N. caninum* (Bjerkas et al. 1984; Dubey et al. 1988a) imposed a new challenge for the scientific community, because this non-zoonotic Toxoplasmatinae parasite is able to infect and cause disease in some mammalian animals that are also susceptible to *T. gondii* infection (Buxton, 1998). The comparison of the whole-genome sequences from *T. gondii*, *H. hammondi* and *N. caninum* showed that these three related tissue-cyst-forming coccidian parasites have a similar total genome size of 62–65 Mb, and many similar orthologous groups of proteins involved in key biological functions (Lorenzi et al. 2016).

Neospora caninum can be maintained as tachyzoites in cell culture (Dubey et al. 1988b), which has enabled the development of a range of serological tests and antibodies against the parasite (Björkman and Uggla, 1999). Neosporosis research was accelerated by the prior accumulation of knowledge about and procedures for *T. gondii*.

The nomenclature system adopted for mutants, genes and gene products of *T. gondii* (Sibley et al. 1991) was proposed for *N. caninum* (Howe and Sibley, 1999). In the comparison of *T. gondii* and *N. caninum* homologous antigens (e.g. SAG1), the use of a Tg or Nc prefix was recommended to distinguish these gene products (e.g. TgSAG1 and NcSAG1) (Howe and Sibley, 1999). Also, a cluster of cell-surface genes was found in *N. caninum*, as described for *T. gondii*, and these gene products were re-named as members of the SRS superfamily (Wasmuth et al. 2012).

Cross-immunity studies between T. gondii and N. caninum. Cross-protection of mice immunized with *N. caninum* and challenged with *T. gondii* is probably *T. gondii* strain- and dose-dependent. In one study, mice were immunized with *N. caninum* and died after challenging with the highly virulent RH strain of *T. gondii* (Lindsay et al. 1990). When mice were immunized with *N. caninum* and challenged with a less-virulent *T. gondii* strain (PLK, a clone from the ME49 strain), the animals had 100% protection against death; immunization with a higher dose of *N. caninum* tachyzoites improved protection against *T. gondii*-induced disease (Kasper and Khan, 1998). Similar levels of protection were observed when mice were immunized with *N. caninum* tachyzoites and then challenged with oocysts of *T. gondii* from a moderately virulent strain (Lindsay et al. 1998). In another study, pregnant sheep were immunized with a *T. gondii* sheep vaccine (Toxovax[®], Intervet, Cambridge, UK) and challenged with a high dose of *N. caninum* tachyzoites (10⁷ tachyzoites per animal) at 90 days of gestation (Innes et al. 2001). No protection against fetal death was observed. The authors speculated that if

the sheep were challenged with a lower dose of *N. caninum* tachyzoites, there perhaps would be some degree of cross-immunity (Innes *et al.* 2001). The confirmation of cross-immunity between *T. gondii* and *N. caninum* in some studies suggests that these parasites share antigens which may also be involved in serologic cross-reactivity.

Serologic cross-reactivity between T. gondii and N. caninum. In the initial observation of *N. caninum* in dogs, sera from five animals that were naturally infected with the parasite tested negative for *T. gondii* by the DT (Bjerkas *et al.* 1984). In addition, dogs that were naturally or experimentally infected with *N. caninum* did not cross-react to *T. gondii* by IFAT, when using 1:50 dilutions as cutoff (Dubey *et al.* 1988b). Accordingly, the same or higher dilution cutoffs by IFAT have been found to be appropriate to avoid cross-reactivity between *N. caninum* and *T. gondii* in serum samples from different hosts (Lobato *et al.* 2006; Silva *et al.* 2007; Benetti *et al.* 2009). Apical reactions, i.e. reactions limited to the apex of the parasite were regarded as non-specific in *N. caninum* IFAT. Cross-reactivity with apical antigens is potentially caused by the high conservation of antigens in the apical organelles of a variety of Apicomplexan parasites, including *T. gondii*. In contrast, a complete peripheral fluorescence of the parasite was considered as a positive response (Pare *et al.* 1995b).

Cross-reactive antigens between *N. caninum* and *T. gondii* have been observed by immunohistochemistry using tissues of naturally- or experimentally infected animals. A rabbit anti-*N. caninum* serum cross-reacted with *T. gondii* in tissue sections from mice (Barr *et al.* 1991). A bradyzoite antigen from *T. gondii*, designated as BAG1 (synonymous to BAG5) (Weiss *et al.* 1992; Parmley *et al.* 1995), was used to produce hyperimmune serum in rabbit, which cross-reacted with bradyzoites of *N. caninum* (McAllister *et al.* 1996). Polyclonal sera against *T. gondii* tachyzoites induced strong cross-reactivity with *N. caninum* tachyzoites by immunohistochemistry (Sundermann *et al.* 1997). The use of MAbs specific to *T. gondii* (Sundermann *et al.* 1997) or a combination of two MAbs specific to *N. caninum* (Uzêda *et al.* 2013), were demonstrated to avoid immunohistological cross-reactivity between these protozoa.

Although species-specific MAbs have been developed, serologic cross-reactions between *T. gondii* and *N. caninum* have been shown to occur also by means of MAbs. Sundermann *et al.* (1997) generated MAbs against *T. gondii* tachyzoites and observed among 26 MAbs tested by IFAT, five antibodies that cross-reacted with *N. caninum* tachyzoites. Kobayashi *et al.* (2013) cloned the *NcBAG1* gene and generated MAbs against its recombinant protein, which recognized TgBAG1. Liao *et al.*

(2005a) produced 384 MAbs against *N. caninum* by immunizing mice with *N. caninum* tachyzoites; 10 of the 384 MAbs were also reactive against *T. gondii* tachyzoites. Similarly, Sohn *et al.* (2011) developed 46 MAbs using a mouse immunized with a mixed fraction of *N. caninum* organelles and some of the MAbs cross-reacted with *T. gondii*. MAbs generated to oocyst antigens of *T. gondii* cross-reacted by immunofluorescence with the sporocyst wall (Dumetre and Darde, 2007) and tissue the cyst wall of *N. caninum* (Gondim *et al.* 2016). These findings show that cross-reactive antigens between *T. gondii* and *N. caninum* are present in tachyzoites, tissue cysts and oocysts of these parasites.

Affinity-purified antibodies raised against a 38 kDa microneme-associated protein of *N. caninum* (NcMIC3) also recognized a 45 kDa protein in tachyzoite extracts of *T. gondii* (Sonda *et al.* 2000). MAbs raised against *T. gondii* reacted with *N. caninum* tachyzoites by IFAT, but only at low titres (10–40) (Latif and Jakubek, 2008). A number of reports demonstrated the usefulness of MAbs against *T. gondii*, which do not cross-react with *N. caninum* (Baszler *et al.* 2001; Uchida *et al.* 2004; Srinivasan *et al.* 2006; Cunha-Junior *et al.* 2010).

Certain surface antigens of *N. caninum*, although not identical to those from *T. gondii*, were homologous to them (Hemphill *et al.* 1997; Howe *et al.* 1998; Howe and Sibley, 1999). Two surface antigens of *N. caninum*, similar to SAG1 and SRS2 from *T. gondii*, were called NcSAG1 and NcSRS2. MAbs against NcSAG1 (6C11, Ncmab-4) did not cross react with *T. gondii* (Björkman and Hemphill, 1998; Howe *et al.* 1998). Evaluation of several MAbs against NcSRS2 (5H5, Ncmab-10, 5·2·15) revealed no cross-reactions with *T. gondii* antigens in immunoblot (Björkman and Hemphill, 1998; Howe *et al.* 1998; Schares *et al.* 1999a).

Antigens of *N. caninum* tested by WB using monoclonal or polyclonal antibodies, resulted in the identification of a limited number of specific immunodominant bands (often referred to as ‘immunodominant antigens’) and other less reactive bands (Table 1). The comparison of these antigens based on their molecular weights is difficult, as differences on the SDS-PAGE conditions, especially the use of reducing or non-reducing conditions, cause variation in the estimated molecular weights. Most likely, some of these immunodominant bands observed in WBs may represent more than a single protein.

In WB, non-reduced antigens exhibit much stronger reactivity than reduced antigens (Barta and Dubey, 1992) which is a clear indication that most of the epitopes recognized on these antigens are conformational epitopes. Under non-reduced conditions a large number of researchers observed mainly four areas with specific immunodominant bands in *N. caninum* tachyzoite antigen:

Table 1. Immunodominant bands recognized by *Neospora caninum*-infected or immunized animals in tachyzoite antigen

Antigen treatment	Molecular weight in kDa of antigenic bands (antigen treatment) ^a	Source of <i>N. caninum</i> antibodies	Parasite species used to examine specificity	Reference
Non-reduced	16, 29, 31, 32, 37, 46, 51, 56, 79 (SDS-PAGE sample buffer soluble total lysate)	Hyperimmune serum from a rabbit	Not examined	(Barta and Dubey, 1992)
	17, 29, <u>30</u> , 37, <u>46</u> , <u>88</u> , <u>97</u> (aqueous buffer and detergent soluble total lysate)	Sera from several naturally and experimentally <i>N. caninum</i> infected (cattle, dog, sheep, goat, pig) or immunized animal species (rabbits)	<i>T. gondii</i> , <i>S. cruzi</i> , <i>H. hammondi</i> ; faint reactions to 46 kDa observed in pre-immune serum of rabbits; 46, 88, 97 kDa antigens clearly and 30 kDa faintly and inconsistently recognized by <i>T. gondii</i> antiserum; no cross-reactivities reported with anti- <i>S. cruzi</i> or anti- <i>H. hammondi</i>	(Bjerkas et al. 1994)
	17–18, <u>30–45</u> , >51 (soluble part of sonicated total lysate and iscom incorporated antigen)	Serum from experimentally infected or immunized rabbits and from a naturally infected dogs	<i>T. gondii</i> , <i>S. cruzi</i> ; anti- <i>T. gondii</i> showed faint reactions with 40 kDa; anti- <i>S. cruzi</i> : no reactions	(Björkman et al. 1994)
	28–35, 45–52, 64–78 (ISCOM incorporated antigen)	Sera and milk from naturally and experimentally infected bovines	<i>T. gondii</i> ; serum of a <i>T. gondii</i> -infected calf did not react	(Björkman et al. 1997)
	28, 31, <u>40</u> , 83, 91 (soluble part of sonicated total lysate)		<i>T. gondii</i> , <i>S. cruzi</i> ; 40 kDa faintly stained by anti- <i>T. gondii</i> ; anti- <i>S. cruzi</i> showed negligible reactions	(Stenlund et al. 1997)
	16/17, 29/30, 30–36, 37, 45 (SDS-PAGE sample buffer soluble total lysate)	Sera from experimentally infected calves and naturally infected cattle	<i>T. gondii</i> ; no cross-reactions with anti- <i>T. gondii</i>	(Atkinson et al. 2000)
	17, 29, 30, 33, 37, 40 (SDS-PAGE sample buffer soluble total lysate)	Sera from naturally infected bovine fetuses	Not examined	(Sondgen et al. 2001)
	<u>29</u> , <u>36</u> (SDS-PAGE sample buffer soluble total lysate)	Sera from naturally infected bovines	<i>T. gondii</i> ; no cross-reactions in 14 of 17 naturally seropositive cattle, faint reactions in the remaining (true infection status regarding <i>N. caninum</i> unknown)	(Staubli et al. 2006)
	14, 29, 30, 33, 35, 40, 55, 60, 77, 84, 97, 170 (aqueous buffer and detergent soluble total lysate)	Sera from experimentally and naturally infected dogs	Not examined	(Jesus et al. 2007)
	17, 29–32 (aqueous buffer soluble total lysate)	Sera from naturally infected dogs and mice	<i>T. gondii</i> ; cross-reactivity with 45–50, 60, 82, 88, 97 and 120 kDa antigens as determined by immunoblot inhibition assays	(Silva et al. 2007)
Reduced	17, 31, 34, 37, 40·5, 47, 50, 55, 65 (SDS-PAGE sample buffer soluble total lysate)	Hyperimmune serum from a rabbit	Not examined	(Barta and Dubey, 1992)
	<u>14</u> , <u>20</u> , <u>31</u> , 35, <u>42</u> , 54, 65, 76, 116 (aqueous buffer soluble total lysate)	Sera of cattle naturally or experimentally infected with <i>N. caninum</i> , <i>T. gondii</i> , <i>S. cruzi</i> , <i>S. hirsuta</i> , <i>S. hominis</i>	<i>T. gondii</i> , <i>S. hirsuta</i> : 14, 31, 37, 42, 55, 80 kDa recognized by anti- <i>T. gondii</i> ; 11, 25, 34, 37 kDa recognized by anti- <i>S. hirsuta</i> serum.	(Baszler et al. 1996)
	36·5–38, 45·5–48·5, 52–53·5, 58, 58·5, 59·5, 60·5, 62, 63·5, <u>64</u> , <u>66·5</u> , <u>67</u> , <u>67·5</u> , <u>68·5</u> , <u>69·5</u> (total lysate antigen)	<i>N. caninum</i> or <i>T. gondii</i> experimentally infected cattle, sheep and goats	<i>T. gondii</i> ; 63·5, 64, 66·5, 67, 67·5, 68·5, 69·5 kDa antigens recognized	(Harkins et al. 1998)
	17–18, 34–35, 37, 60–62 (aqueous buffer soluble total lysate)	Sera from naturally infected pregnant cattle and aborted fetuses	Not examined	(Alvarez-Garcia et al. 2002)
	18, 25, 33, 35–36, 45–46, 47, 60–62 (SDS-PAGE sample buffer soluble total lysate)	Sera from naturally infected bovines	Not examined	(Okeoma et al. 2004)

^a Cross-reacting immunodominant antigen bands underlined.

14–19 kDa, 29–32 kDa, 30–36 kDa, and 36–40 kDa. However, other areas with major bands of reactions were also observed with non-reduced antigens, but differed widely between studies (Table 1).

Sera from animals immunized with recombinant forms of the major *N. caninum* antigens NcSAG1 and NcSRS2 provided evidence that these antigens are among those recognized in the range 29–32 kDa and 36–40 kDa bands (Howe *et al.* 1998) (Table 2).

Under reduced conditions there is a dominant band between 17–19 kDa which most likely represent reactions against NcGRA7 (Alvarez-Garcia *et al.* 2007) and other antigens (Table 2). However, as demonstrated by MAbs (4·7·12; Ncmab-7) in combination with immunoprecipitation, surface biotinylation and immuno-electron microscopy, a surface antigen might also be among those migrating in WB at 17–19 kDa under reduced conditions (Björkman and Hemphill, 1998; Schares *et al.* 1999a). Further immunodominant banding areas are at 34–36 and 37–46 kDa, eventually also representing reactions to NcSAG1, NcGRA6, NcGRA7 and NcSRS2 (Table 2). When tested with polyclonal antibodies against *T. gondii*, only minor reactions were observed with antigen bands regarded as specific for *N. caninum* in WB (Barta and Dubey, 1992; Björkman *et al.* 1994, 1997).

For the diagnosis of *T. gondii* infection in veterinary investigations, the use of recombinant and synthetic antigens, developed using novel molecular techniques, have expanded diagnostic options as alternatives to native antigens directly isolated from cultivated parasites. For diagnosis of *T. gondii* infection in cats, sheep and pigs, some species-specific ELISAs are available that have performed well when compared with previous reference serological techniques, as reviewed by Wyrosdick and Schaefer (2015).

ELISAs for *N. caninum* antibodies in sera from dogs and cattle have been developed in several studies (Table 3). Conventional ELISAs using crude soluble antigen showed higher levels of serologic cross-reactivity to *T. gondii* when compared with IFAT (Björkman *et al.* 1994; Silva *et al.* 2007). Serologic cross-reactivity with *T. gondii* was also observed when polyclonal mouse and cat sera were tested by a *N. caninum* ELISA using crude antigen (Nishikawa *et al.* 2002). In contrast, the same mouse and cat sera did not cross-react by an ELISA based on *N. caninum* recombinant antigen (NcSRS2) (Nishikawa *et al.* 2002). ELISAs prepared with *N. caninum* tachyzoite antigen associated with immunostimulating complexes (ISCOM), and using MAbs as secondary antibodies, presented better specificity than conventional ELISAs (Björkman *et al.* 1994, 1997). The ISCOM particles have affinity for surface proteins, which minimizes interference by internal non-specific antigens (Björkman *et al.* 1994). Moreover, none of the MAbs developed against *N. caninum* ISCOM incorporated antigens (including

also Ncmab-4, and Ncmab-10 mentioned above) cross-reacted with *T. gondii* (Björkman and Lunden, 1998). An ELISA based on immuno-affinity-purified native NcSRS2 showed no significant cross-reactions when tested with sera from cattle experimentally infected with a variety of protozoan parasites including also ten cattle infected with *T. gondii* (Schaes *et al.* 2000). In addition, the TgSAG2A molecule has been demonstrated to be specific to *T. gondii*, considering that no cross-reactivity has been shown with *N. caninum* when using recombinant protein or even mimotopes derived from this molecular marker, as characterized by A4D12 MAb (Bela *et al.* 2008; Carvalho *et al.* 2008; Cunha-Junior *et al.* 2010; Santana *et al.* 2012; Macedo *et al.* 2013).

Nowadays it is becoming clear that there is a need to characterize new molecular markers that are species-specific for *T. gondii* and *N. caninum* for the development of new diagnostic tools (Zhang *et al.* 2011; Regidor-Cerrillo *et al.* 2015). In this context, the identification of cross-reactive and species-specific antigens between *N. caninum* and *T. gondii* tachyzoites is mandatory and the proteomics approach constitutes an appropriate strategy for this purpose (Zhang *et al.* 2011). These authors demonstrated the usefulness of proteomics to immuno-screen for cross-reactive or species-specific antigens from both parasites. Moreover, they showed that there was significant homology in the antigenic proteome profiles between the two parasites (Zhang *et al.* 2011). Taking together, these findings shed light on the process to design new diagnostic tools in order to avoid cross-reactivity between *N. caninum* and *T. gondii* diagnostic tests.

The characterization of cross-reactive antigens between *T. gondii* and *N. caninum* has been achieved in some studies. An NTPase identified in *N. caninum* tachyzoites was antigenically cross-reactive to the NTPases of *T. gondii* (Asai *et al.* 1998). Protein disulphide isomerase (PDI), heat-shock protein 70 (HSP70) and ribosomal protein P1 (RP1), were identified as cross-reactive antigens between the two parasites even when using MAbs, due to the high degree of homology among these parasite components (Liao *et al.* 2005a). Zhang *et al.* (2007a) demonstrated that antibodies raised against the apical membrane antigen 1 of *T. gondii* (TgAMA 1) also recognize recombinant NcAMA 1. The ribosomal phosphoprotein (P0) was shown to be a cross-reactive antigen between *T. gondii* and *N. caninum* (Zhang *et al.* 2007b); antibodies raised against rNcPO inhibited the growth of both *T. gondii* and *N. caninum* tachyzoites. A protease with 42 kDa was localized in the rhoptry of *T. gondii* by means of a MAb; this MAb also reacted to a 42 kDa protein in *N. caninum*, which was also localized in the rhoptry of this parasite (Ahn *et al.* 2001).

Table 2. *Neospora caninum*-recombinant antigens and cross-reactions tested against *Toxoplasma gondii* and related protozoan parasites

<i>N. caninum</i> -recombinant antigens established for diagnostic purposes (other names or variants of the recombinant proteins)	Localisation (estimated size, reference)	Parasite species against which cross-reactivity was tested	References which examined cross-reactivity
rNcSAG1 (rNcp29, NcSAG1t)	Surface (29–38 kDa) (Howe <i>et al.</i> 1998; Sonda <i>et al.</i> 1998)	<i>T. gondii</i>	(Howe <i>et al.</i> 2002; Chahan <i>et al.</i> 2003; Liao <i>et al.</i> 2005b; Huang <i>et al.</i> 2007; Ybanez <i>et al.</i> 2013)
rNcSRS2 (Ba/SRS2p44, NcSRS2t, tNcSRS2)	Surface (35–43 kDa) (Hemphill and Gottstein, 1996; Hemphill <i>et al.</i> 1997; Howe <i>et al.</i> 1998)	<i>Sarcocystis</i> spp. <i>T. gondii</i>	(Howe <i>et al.</i> 2002) (Nishikawa <i>et al.</i> 2001, 2002; Gaturaga <i>et al.</i> 2005; Huang <i>et al.</i> 2007; Liu <i>et al.</i> 2007)
rNcSAG4	Surface (18 kDa) (Fernandez-Garcia <i>et al.</i> 2006)	<i>H. heydorni</i> <i>T. gondii</i> <i>B. besnoiti</i>	(Nishikawa <i>et al.</i> 2001, 2002) (Aguado-Martinez <i>et al.</i> 2008) (Aguado-Martinez <i>et al.</i> 2008)
rNcGRA6 (Nc14·1, NcDG2, NcGRA6d, NcGRA6s)	Dense granules (68 kDa, 37 kDa) (Liddell <i>et al.</i> 1998)	<i>Sarcocystis</i> spp. <i>T. gondii</i>	(Aguado-Martinez <i>et al.</i> 2008) (Lally <i>et al.</i> 1996; Jenkins <i>et al.</i> 1997)
rNcGRA7 (Nc4·1, NcDG1, N57, MBPNcGRA7, rNcGRA7t, rNcGRA7)	Dense granules (33 kDa, 17–18 kDa) (Lally <i>et al.</i> 1997; Hemphill <i>et al.</i> 1998; Alvarez-Garcia <i>et al.</i> 2007; Fuchs <i>et al.</i> 1998)	<i>Sarcocystis</i> spp. <i>T. gondii</i>	(Lally <i>et al.</i> 1996; Jenkins <i>et al.</i> 1997) (Lally <i>et al.</i> 1996; Jenkins <i>et al.</i> 1997; Aguado-Martinez <i>et al.</i> 2008; Hamidinejat <i>et al.</i> 2015)
rNcMIC10 (NcMiC10 M, NMIC10N, NcMIC10C)	Micronemes (18 kDa) (Yin <i>et al.</i> 2012)	<i>B. besnoiti</i> <i>Sarcocystis</i> spp.	(Aguado-Martinez <i>et al.</i> 2008) (Lally <i>et al.</i> 1996; Jenkins <i>et al.</i> 1997; Aguado-Martinez <i>et al.</i> 2008)
rSubtilisin-like serin protease (N54, NcSUB1, NcSUB1t, NcSUB1tr)	Unknown (97, 87, 77, 67 and 64 kDa) (Louie <i>et al.</i> 1997)	Others, including <i>Babesia</i> spp., <i>Tritrichomonas fetus</i> , <i>Leishmania infantum</i> <i>T. gondii</i>	(Louie <i>et al.</i> 1997; Hara <i>et al.</i> 2006; Huang <i>et al.</i> 2007) (Yin <i>et al.</i> 2012)
		<i>T. fetus</i>	(Louie <i>et al.</i> 1997)

Table 3. Cross-reactions tested for *Neospora caninum* in published in-house ELISAs

<i>N. caninum</i> ELISA	Parasitic species for which cross-reactivity was examined	Reference
Whole tachyzoite lysate antigen ELISA (various extraction protocols)	<i>T. gondii</i>	(Pare <i>et al.</i> 1995a; Dubey <i>et al.</i> 1996)
	<i>Hammondia</i> spp.	(Dubey <i>et al.</i> 1996)
	<i>Sarcocystis</i> spp.	(Pare <i>et al.</i> 1995a; Dubey <i>et al.</i> 1996) ^a
	<i>Eimeria</i> spp.	(Pare <i>et al.</i> 1995a; Dubey <i>et al.</i> 1996)
	<i>Cryptosporidium</i> spp.	(Pare <i>et al.</i> 1995a; Dubey <i>et al.</i> 1996)
	<i>T. gondii</i> , <i>Sarcocystis</i> spp., <i>Babesia</i> spp.	(Osawa <i>et al.</i> 1998)
Fixed whole tachyzoites ELISA	<i>T. gondii</i> , <i>Sarcocystis</i> spp., <i>Eimeria</i> spp., <i>Babesia</i> spp., <i>Cryptosporidium</i> spp.	(Wouda <i>et al.</i> 1998)
	<i>T. gondii</i>	(Silva <i>et al.</i> 2007)
	<i>Sarcocystis</i> spp.	(Williams <i>et al.</i> 1997)
	<i>Eimeria</i> spp.	(Williams <i>et al.</i> 1997) ^a
	<i>Babesia</i> spp.	(Williams <i>et al.</i> 1997) ^a
ISCOM incorporated antigen, indirect	<i>Cryptosporidium</i> spp.	(Williams <i>et al.</i> 1997)
	<i>T. gondii</i>	(Björkman <i>et al.</i> 1994) ^b , (Björkman <i>et al.</i> 1997), (Björkman and Hemphill, 1998) ^c , (Björkman and Lunden, 1998) ^d , (Björkman <i>et al.</i> 1994) ^b , (Björkman <i>et al.</i> 1997)
Native NcSRS2, indirect	<i>Sarcocystis</i> spp.	(Björkman <i>et al.</i> 1994) ^b , (Björkman <i>et al.</i> 1997)
	<i>Eimeria</i> spp., <i>Babesia</i> spp.	(Björkman <i>et al.</i> 1997)
	<i>T. gondii</i>	(Schaes <i>et al.</i> 2000; Hosseinijad <i>et al.</i> 2010)
Antigen-capture indirect (polyclonal antiserum)	<i>Sarcocystis</i> spp., <i>Eimeria</i> spp., <i>Babesia</i> spp., <i>Cryptosporidium</i> spp.	(Schaes <i>et al.</i> 2000)
	<i>Leishmania infantum</i>	(Hosseinijad <i>et al.</i> 2010)
Antigen-capture competitive ELISA (Mabs 5B6-25 and 4A4-2)	<i>T. gondii</i> , <i>Sarcocystis</i> spp., <i>Eimeria</i> spp., <i>Babesia</i> spp., <i>Cryptosporidium</i> spp.	(Schaes <i>et al.</i> 1999b)
	<i>T. gondii</i> , <i>Sarcocystis</i> spp.	(Baszler <i>et al.</i> 2001)
Competitive ELISA (4A4-2)	<i>T. gondii</i>	(Baszler <i>et al.</i> 1996) ^e
	<i>Sarcocystis</i> spp.	(Baszler <i>et al.</i> 1996) ^e
Competitive ELISA (rabbit polyclonal antibody)	<i>T. gondii</i> , <i>Sarcocystis</i> spp., <i>Eimeria</i> spp., <i>Babesia</i> spp.	(McGarry <i>et al.</i> 2000)

^a Cross-reactions observed in serum dilutions lower than the cutoff.

^b Rabbit sera against *T. gondii* and *S. cruzi* do not react in WB with ISCOM antigen.

^c MAb against ISCOM antigens do not recognize *T. gondii* in WB.

^d Mouse serum immunized with ISCOM antigen does not recognize *T. gondii*.

^e Some sera showed elevated levels of cross-reactions in WB.

The genome of *N. caninum* (NC-Liverpool strain) was compared with the available genome of the ME-49 strain of *T. gondii* (Reid *et al.* 2012). The authors found a high synteny between the two genomes and pointed out that most divergences occurred within the SRS antigens. Transcriptome analysis suggested that *N. caninum* uses fewer SRS antigens than *T. gondii* (Reid *et al.* 2012). Therefore, selecting those species-specific parasitic surface antigens for the establishment of serologic tests, such as SRSs, may favour the specificity of these tests.

Serological tests for *N. caninum* and cross-reactivity with *Sarcocystis* spp., *B. besnoiti*, *N. hughesi* and *Hammondia* spp.

Sera from *Sarcocystis* spp.-infected cattle have been shown to cross-react with several *N. caninum*

antigens by WB (Baszler *et al.* 1996). However, antibodies against *Sarcocystis* spp. did not cross-react with *N. caninum*-immunodominant antigens (19, 29, 30 and 37 kDa) (Bjerkas *et al.* 1994).

Sera from calves that were experimentally infected with *Sarcocystis* spp. tested positive by a conventional ELISA using crude *N. caninum* antigen (Dubey *et al.* 1996); the same sera tested negative by *N. caninum* IFAT. In contrast, positive sera against several *Sarcocystis* spp. (*S. cruzi*, *S. hirsuta*, *S. hominis* and *S. neurona*) did not result in positive reactions in ELISAs based on *N. caninum*-selected antigens (ISCOM, whole-fixed tachyzoites, affinity-purified and recombinant) (Björkman *et al.* 1994; Baszler *et al.* 1996, 2001; Lally *et al.* 1996; Schaes *et al.* 2000; Howe *et al.* 2002). Serologic cross-reactivity between infections caused by *N. caninum* and *Sarcocystis* spp. seems to be negligible when *N. caninum*-specific antigens are employed (Table 3).

Besnoitia besnoiti and *N. caninum* have cattle as their major hosts and may co-infect a high proportion of animals in regions where these parasites are endemic (Jacquet et al. 2010). Serologic tests such as indirect ELISA and IFAT had been developed over several decades for *B. besnoiti* antibodies (Frank et al. 1970; Neuman, 1972; Janitschke et al. 1984; Shkap et al. 1984), but at that time, the closely related parasite *N. caninum* was unknown, so the specificity of those tests could not have been ascertained. Later, it was shown that sera from *N. caninum*-positive cattle and gerbils recognized *B. besnoiti* antigens by IFAT when a less stringent cutoff (1:64) was used (Shkap et al. 2002); the authors also observed that these animal sera reacted against two bands of *B. besnoiti* antigens under reducing conditions by WB. In another study, a more stringent cutoff (1:200) for *B. besnoiti* IFAT did not show cross-reactions with sera from *N. caninum*-infected animals, whereas serum dilutions of 1:100 showed some level of cross-reactivity (Schaes et al. 2010).

Besnoitia besnoiti WBs and indirect ELISAs were developed for detection of antibodies in cattle and were tested for cross-reactivity with sera from animals infected with *N. caninum* and *T. gondii* (Cortes et al. 2006; Fernandez-Garcia et al. 2010; Schaes et al. 2011). Cross-reactivity with *N. caninum* was observed in the tested ELISAs and WBs, especially with animals exhibiting high antibody titres for *N. caninum*. A novel ELISA for *B. besnoiti* was developed with affinity-purified antigens of confirmed relevance, mostly localized on the surface of tachyzoites. This ELISA has shown a lower degree of cross-reactivity with *N. caninum* (Schaes et al. 2013). A recent study demonstrated that cattle exhibiting high antibody levels to *N. caninum* and/or *Sarcocystis* spp. presented a higher number of false-positive reactions for *B. besnoiti* by an in-house ELISA (Garcia-Lunar et al. 2015); thus, infection with *Sarcocystis* spp. and *N. caninum* may augment cross-reactions with *B. besnoiti*.

No serologic cross-reactivity has been observed between *N. caninum* and *H. heydorni*. A few serum samples from mice, dog and sheep that were infected with *H. heydorni* did not cross-react with *N. caninum* antigens by IFAT, ELISA or WB (Nishikawa et al. 2002; Gondim et al. 2015). However, further studies using larger numbers of sera from *H. heydorni*-infected animals are necessary to confirm the absence of cross-reactivity between *N. caninum* and *H. heydorni*.

Neospora hughesi was proposed as a new species based on seven nucleotide differences in the internal transcribed spacer 1 (ITS1) of the rDNA, as well as on ultrastructural and antigenic differences when compared with *N. caninum* (Marsh et al. 1998). The acceptance of *N. hughesi* as a new species was reinforced by differences observed in amino acid

sequences of two surface antigens (SAG1 and SRS2) to those from *N. caninum* (Marsh et al. 1999). However, polyclonal serum from a *N. caninum*-infected rabbit recognized NcSAG1, NhSAG1, NcSRS2 and NhSRS2 by immunoblot (Marsh et al. 1999). Differences also have been found in gene sequences between the dense granule proteins GRA6 and GRA7 of *N. caninum* and *N. hughesi*, although polyclonal serum to *N. caninum* recognized GRA6 and GRA7 antigens from both *N. caninum* and *N. hughesi* by immunoblot (Walsh et al. 2001).

An ELISA for *N. hughesi* antibodies was developed using a recombinant NhSAG1 as antigen (Hoane et al. 2005); animals infected with *N. hughesi* presented a higher antibody reactivity to rNhSAG1 than to rNcSAG1, but the test was not able to unambiguously differentiate infections caused by *N. hughesi* or *N. caninum*. In another study, pre- and post-infection sera from dog and cattle that were experimentally inoculated with *N. caninum* were tested simultaneously by IFAT using tachyzoites of *N. caninum* or *N. hughesi* (Gondim et al. 2009); all sera that tested positive for *N. caninum* also reacted with *N. hughesi* tachyzoites, although the antibody titres for *N. hughesi* IFAT were slightly lower as compared with the IFAT for *N. caninum*. To date, infections caused by *N. caninum* and *N. hughesi* cannot be serologically discriminated. As horses may be infected by both *N. caninum* and *N. hughesi* (Marsh et al. 1999; Pitel et al. 2003; Veronesi et al. 2008), a species-specific serologic test for equines is desired.

Serological tests based on chimeric antigens, synthetic peptides and stage-specific antigens

Chimeric antigens. Almost 30 years ago, the production of recombinant polypeptides derived from genes encoding *T. gondii* antigens revolutionized the search for more efficient serologic methods (Johnson et al. 1989; Johnson and Illana, 1991). The development of ELISAs based on a mixture of recombinant antigens, rather than the use of a single recombinant protein, has been presumed to increase the sensitivity of the ELISA for human sera, while maintaining the desired specificity (Johnson et al. 1992; Aubert et al. 2000). ELISAs containing a mixture of recombinant antigens were also tested for antibodies against *T. gondii* in sheep and cats (Tenter et al. 1992).

A chimeric antigen is the fusion of gene fragments constructed as a single gene and expressed to form a hybrid protein (Yang et al. 2004). The use of chimeric antigens for *T. gondii* serology is promising. Chimeric proteins are usually larger than single recombinant antigens resulting in a better binding to microtitre plates. In addition, as a chimeric antigen preparation consists of a single

antigen, it may be easier to standardize than mixtures of recombinant antigens (Beghetto *et al.* 2006; Lau *et al.* 2011; Holec-Gasior *et al.* 2012a).

Several chimeric antigens have been tested by WB or ELISA for the detection of human antibodies against *T. gondii*. Among the developed chimeric antigens tested by serology, are preparations including parts of well-characterized immunodominant proteins, such as SAG1, SAG2, MIC1, MIC2, MIC3, MAG1, M2AP, GRA1, GRA2, GRA3 and ROP1 (Beghetto *et al.* 2006; Lau *et al.* 2011; Holec-Gasior *et al.* 2012a, b; Ferra *et al.* 2015a). The standardization of a chimeric-antigen-based test for *T. gondii* antibodies depends on various factors, including an optimal selection of antigens and proper expression of all desired epitopes. In addition to humans, a recent paper reports the first trial of chimeric antigens employed for *T. gondii* serology in farm animals (horses, swine and sheep) (Ferra *et al.* 2015b); the authors validated their antigen with more than 400 sera and also included 15 sera, which were serologically positive for *N. caninum* but negative for *T. gondii*. It is interesting to note that each animal species responded differently to the chimeric-antigen preparations used in the ELISAs, but the SAG2–GRA1–ROP1_L construct reached the best overall specificity and sensitivity for the three tested species (Ferra *et al.* 2015b). Despite progress in the development of chimeric antigens for *T. gondii* serology, in particular for humans, inadequate information is available about the serologic cross-reactivity potential of those tests with sera from animals infected with other Toxoplasmatinae parasites.

Synthetic peptides, serotyping and stage-specific antigens. The combination of molecular engineering and chemical synthesis of antigens has been applied for the development of serological techniques with improved sensitivity and specificity. Synthetic peptides representing several epitopes of numerous antigens have been used in microarray assays for serotyping of viral and bacterial diseases (Neuman de Vegvar *et al.* 2003; Nahtman *et al.* 2007).

In *T. gondii* infections, the humoral response has been demonstrated to be partially strain-specific. In one study, MAbs produced against SAG2A from naturally infected mice recognized the surface antigens encoded by the SAG2 allele of type I and III strains, but not of type II strains (Parmley *et al.* 1994). Another study identified a MAb showing differences in the recognition of type II and III strains (Bohne *et al.* 1993). Kong *et al.* (2003) screened nucleotide sequences from types I, II and III of *T. gondii* for the identification of polymorphic regions from genes coding selected antigens. Allele-specific peptides were synthesized and screened by ELISA using sera from mice and humans. Synthetic peptides based on SAG2A,

GRA3, GRA6 and GRA7 were able to discriminate type II from non-type II infections (Kong *et al.* 2003). In subsequent studies, serotyping for *T. gondii* infections using new recombinant polypeptides or new or improved synthetic peptides, as well as target populations from different regions, have been performed by ELISAs (Peyron *et al.* 2006; Sousa *et al.* 2008, 2009). In two studies, peptide-microarrays were validated to discriminate the serological responses against clonal-type *T. gondii* strains in samples from humans (Maksimov *et al.* 2012b) and cats (Maksimov *et al.* 2013). The latter study used sera from experimentally infected cats for validation and showed significant type-specific differences in the IgG response against the tested peptide panel. However, in many peptides, reactions were not clonal type-specific (Maksimov *et al.* 2013).

A peptide microarray was developed and validated for serotyping *T. gondii* infections in humans, aiming to differentiate between different manifestations of *T. gondii* infection (Maksimov *et al.* 2012a). Thirty eight *T. gondii* synthetic peptides, consisting of 18 peptides characterized in previous studies, and 20 novel peptides, predicted by bioinformatics approach, were tested to differentiate acute, latent and ocular infections. Some peptides based on dense granule and microneme antigens (GRA2-28, MIC3-282 and MIC3-191) showed promising results for differentiation between acute and latent infections (Maksimov *et al.* 2012a).

The use of synthetic peptides for *T. gondii* serotyping may have a great potential for discriminating between *T. gondii* infections and infections caused by other parasite species expected to induce some degree of serologic cross-reactivity. Of course, one important prerequisite is that peptides applied in such tests are not specific for particular clonal-types or genotypes of *T. gondii* or related parasite species. In addition, potential cross-reactivities need to be addressed; for instance, synthetic peptides considered to react specifically with antibodies generated by *T. gondii* infections need to be tested with sera from animals exposed to *H. hammondi*, as this parasite has a very close genetic relationship to the former (Walzer *et al.* 2013, 2014). The use of *T. gondii*-specific peptides for diagnosis in animals should also be tested with *N. caninum*, which may cross-react, as shown in Table 3. To our knowledge, there are no published studies using synthetic peptides to differentiate infections in animals cause by *T. gondii*, *H. hammondi* or *N. caninum*.

Stage-specific antigens (native or recombinant) from *T. gondii* have been investigated for decades by several research groups, including oocysts and sporozoites which can be produced in cats (Kasper *et al.* 1984; Ferguson *et al.* 2000; Dumetre and Darde, 2007; Possenti *et al.* 2010, 2013; Bushkin *et al.* 2012; Fritz *et al.* 2012). In contrast, antigens

from *Neospora* spp. are mostly obtained from tachyzoites. Only small numbers of studies have produced *N. caninum* tissue cysts in cell culture or in animals (Weiss et al. 1999; Tunev et al. 2002; Risco-Castillo et al. 2004; Vonlaufen et al. 2004), or have induced oocyst production in canid definitive hosts (McAllister et al. 1998; Schares et al. 2001; Gondim et al. 2002).

The identification of the first bradyzoite-specific gene of *N. caninum* (NcSAG4), an orthologue to TgSAG4, allowed the production and characterization of the recombinant protein rNcSAG4 (Fernandez-Garcia et al. 2006). This antigen has been applied for the development of a stage-specific ELISA for bovine neosporosis (Aguado-Martinez et al. 2008).

The production of *Hammondia* spp. antigens is even more difficult, because there is no permanent culture for these parasites (Riahi et al. 1995; Schares et al. 2003; Gondim et al. 2015). Therefore, most antigens from *Hammondia* spp. are derived from oocysts or from parasite cysts from intermediate hosts (Riahi et al. 1998, 1999, 2000; Walzer et al. 2013). Generation of recombinant antigens from *Hammondia* spp. is lacking. It would enable studies on exposure of animals and humans to these parasites, as well as on serologic cross-reactivity with related protozoa.

A serologic test for humans based on a *T. gondii* sporozoite-specific protein has been evaluated and seems to represent a promising method to discriminate between oocyst-induced infections from those transmitted by ingestion of infected meat (Hill et al. 2011), which is an important consideration in epidemiological investigations. This study was the first to use a sporozoite protein, called *T. gondii* embryogenesis-related protein (TgERP) in a serologic test for *T. gondii*. In a recent study another sporozoite-derived protein called CCp5A was successfully used in an ELISA to identify oocyst-infected animals (Santana et al. 2015); this test was able to identify antibodies in sera from humans, pigs, mice and chickens that were naturally or experimentally infected with *T. gondii* oocysts and discriminate between animals infected from ingestion of oocysts or by carnivorousism. Further investigations are necessary for validation and to confirm whether the sporozoite proteins TgERP (Hill et al. 2011) and CCp5A (Santana et al. 2015) cross-react with sporozoite proteins derived from *Hammondia* spp. and *Neospora* spp. It is also important to test whether low infectious dose with oocysts will elicit detectable antibodies to TgERP or CCp5A.

Type or stage-specific antigens have a wide spectrum of applications, including investigations of infection outbreaks and identification of risk factors in selected populations (e.g. ingestion of oocyst or tissue cysts). Those antigens should be used with caution and only after rigorous validation in epidemiological studies.

CONCLUDING REMARKS

Most currently available serologic tests for *T. gondii* may show some level of cross-reactivity with related coccidia, in particular with *H. hammondi* and *N. caninum*. Serological cross-reactivity between *T. gondii* and *N. caninum* has been observed when crude antigen ELISAs are employed. Several ELISAs based on recombinant species-specific antigens did not present cross-reactivity with *N. caninum*. Therefore, *T. gondii* shares more surface antigens with *H. hammondi* than with *N. caninum*.

When detecting *T. gondii* antibodies in animals, IFAT seems to be more specific than the ELISAs based on crude antigen. Since *N. caninum* is not considered to be a human pathogen, the major concern regarding serologic cross-reactivity would be potential exposure to *H. hammondi*, although it is unknown whether this parasite is able to induce infection in humans.

Antibodies from cattle infected with *B. besnoiti* cross-react with *N. caninum* antigens by IFAT in serum dilutions lower than the recommended cutoff (1:200). Novel *B. besnoiti* ELISAs (e.g. ELISA based on purified surface antigens) showed a lower degree of cross-reactivity with sera from cattle infected with *N. caninum* or other related parasites. Antibodies against bovine *Sarcocystis* sp. present negligible cross-reactions with *N. caninum*-immunodominant antigens. The development of ELISAs for *N. caninum* antibodies based on chimeric peptides specific for the parasite seems to be promising. Chimeric antigens would enable a better standardization of serologic tests, as a single protein is used. Moreover, chimeric antigens would potentially present higher sensitivity than single recombinant antigens. Despite the close phylogenetic relationship between *N. caninum* and *H. heydorni*, no evidence of serologic cross-reactivity between these protozoa has been confirmed to date. *Toxoplasma gondii* infections cause more confusion with *N. caninum* serology than those infections induced by *H. heydorni*. *Neospora caninum* and *N. hughesi* infections cannot be serologically differentiated, because currently available serologic tests are genus rather than species-specific for these protozoa.

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