Tropical Biomedicine 25(3): 225–231 (2008)

Determination of the specificities of monoclonal and polyclonal antibodies to *Neospora*, *Toxoplasma* and *Cryptosporidium* by Fluorescent Antibody Test (FAT)

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Received 30 April 2008; received in revised form 7 October 2008; accepted 10 October 2008

Abstract. Flourescent antibody test (FAT) was applied to determine the cross-reactivities of monoclonal (mAb), polyclonal (pAb) antibodies to *Neospora*, *Toxoplasma* and *Cryptosporidium* and antisera from cattle naturally infected with *Neospora canium* against antigens from a number of sources. Both mAb and pAb to *Neospora* reacted strongly (FAT titre up to 2560) with the homologous antigens and demonstrated weak titre (80) or no reaction with both *Toxoplasma* and *Cryptosporidium* antigens. Also mAb and pAb to *Toxoplasma gondii* reacted at titres of 80 - 640 with homologous antigens and at titres of 10-40 with *N. caninum*. No cross-reactions with either mAb or pAb antibodies to *N. caninum* and *T. gondii* were observed with *Cryptosporidium parvum*. The same results were observed with *C. parvum* mAb when tested with both *N. caninum* and *T. gondii* antigens. Sera from cattle naturally infected with *N. caninum* had titres ranging from 80- 640 with *N. caninum* antigens, and 10- 40 with *T. gondii* and *C. parvum* antigens. At low dilutions, the complete surfaces of *Neospora* and *Toxoplasma* parasites were fluorescent, while in higher dilutions only dotted fluorescence appeared on the apical complex. These results indicated the presence of cross-reactivity between *Neospora* and *Toxoplasma* but not with *Cryptosporidium*. Accordingly the recommended cut-off antibody titre for diagnosis of neosporosis is 80.

INTRODUCTION

Neospora caninum is a worldwide coccidian parasite which causes encephalitis and abortion in cattle and is closely related to *Toxoplasma gondii* (Brindly *et al*., 1993). Cattle can also be infected with *Cryptosporidium parvum* (Casemore *et al*., 1997). Indirect fluorescent antibody (IFA) and ELISA assays were previously used to determine the cross-reactivity between different genera of suborder Eimeriina (William *et al*., 1997; Lorenzo *et al*., 1998). Many workers reported the existence of cross-reactions between *Neospora* and *Toxoplasma* and between *Cryptosporidium* and *Eimeria* (Stibbs & Ongerth, 1986; Ortega- Mora *et al*., 1992; Conrad *et al*., 1993, Dubey & Lindsay, 1993; McAllister *et al*., 1996; Sundermann *et al*., 1997). In contrast, other investigators showed no cross-reaction between the above mentioned genera (Anusz *et al*., 1990; Haeber *et al*., 1992; Cole *et al*., 1993, Dubey *et al*., 1997; Osawa *et al*., 1998). The aims of this study were to detect the cross-reactivities of monoclonal and polyclonal antibodies (to *Neospora*, *Toxoplasma* and *Cryptosporidium*) and sera from naturally infected cattle with *Neospora* against *Neospora*, *Toxoplasma*, and *Cryptosporidium* antigens; and also to determine the IFA cut-off antibody titre for positive sera in cases of neosporosis.

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MATERIALS AND METHODS

Parasites

Neospora caninum tachyzoites (Nc-1 and Nc Sweb1 isolates) were propagated by serial passage in Vero cells using modified DMEM medium supplemented with 5% normal horse serum, 2 mM glutamine, penicillin (60 µg/ml), dihydrostreptompycin (50 µg/m1) at 37°C in a 5% CO2 inside a humidified incubator as described by Stenlund *et al*. (1997). *Toxoplasma gondii* tachyzoites (RH- strain) were cultivated under the same conditions as for *N. caninum*. *Cryptosporidium parvum* was obtained as an aliquot of 2 x 10^8 oocysts in 2 ml from Moredun Research Institute, Edinburgh, Scotland.

Monoclonal and polyclonal antibodies and animal sera

i. Six monoclonal antibodies (Nc-1mAb; 4,7,10,13,17,24) to *Neospora* Nc-1 isolate, each with an estimated protein concentration of 0.4 mg/ml (Bjorkman & Hemphill,1998).

ii. Three monoclonal antibodies (Tx1, Tx2, Tx3) to *Toxoplasma* RH-strain, each with an estimated protein concentration of 0.4 mg/ml.

iii. One monoclonal antibody (OW-IGO) to *C. parvum* was supplied by Professor Alain Bonnin / France.

iv. Two polyclonal antisera (Ra α Nc-1 A and B) to Neospora Nc-1 isoloate (protein concentration 32 mg/ml and 26 mg/ml respectively) .

v. Four polyclonal antibodies (Ra αTx A, B, C, D) to *Toxoplasma* RH-strain (protein concentration approximately 35 mg/m1)

vi. Ten sera from cattle naturally infected with *N. caninum* were obtained from Swedish cattle farms.

vii. Normal mouse, rabbit, and cow sera served as control sera.

All monoclonal and polyclonal antibodies to *Neospora* and *Toxoplasma* were prepared at the Department of Parasitology, National Veterinary Institute, Uppsala, Sweden.

The monoclonal and polyclonal antibodies and tested sera were diluted in two-fold serial dilutions from 1:10 to 1:5120 in phosphate buffer saline (PBS), pH 7.4.

FA test

1. Preparation of antigens

Harvested *Neospora* and *Toxoplasma* tachyzoites were centrifuged at 2000 x g for 5 min. The supernatants were discarded and the pellets were washed once with PBS and resuspended in PBS. Different numbers of tachyzoites were applied for each circle on ethanol-cleaned microscope slides (Kebo, Stockholm, Sweden) to determine the best results with the immunofluorescent reaction. The optimum volume of each suspension was 5-10 µl containing approximately 500 parasites, and it was applied onto each of the 10 circles.

For *Cryptosporidium*, approximately 300 oocysts / 30 µl were placed in each circle. The slides were allowed to dry completely at room temperature, fixed in methanol for 5 min, air dried, wrapped in tissue paper and aluminum foil, placed in self-sealed polythene bags containing crystals of silica gel and store at -20°C until used.

2. Conjugated sera

Rabbit anti-mouse, rabbit anti-cow, and swine anti-rabbit immunoglobulins conjugated with fluorescein isohiocyanate (DAKO A/S, Denmark) were used at 1:40 dilution in PBS.

3. Test procedure

Antigens slides of the different test parasites were removed from -20°C storage and placed at 4°C for one hour and were maintained at room temperature for another hour before the staining procedure. Thirty µl of each dilution of the different mAb, pAb and sera were dropped on to each circle on the slide and the slides were incubated in a humid chamber for 30 min at room temperature. These slides were then rinsed three times in PBS (5 min each time). Thirty µl of fluorescein-conjugated antisera were dropped onto each circle, and the slides were incubated for 30 min at room temperature and the washing procedure was repeated.

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The dried slides were mounted in Fluoprep (BioMerieux Ref75 521)and examined with a fluorescent microscope (Olympus, BH-2) at x 400 magnification.

RESULTS

The best results of fluorescent reaction (4+) were obtained with about 500 tachyzoites in 5-10 µl of suspension of *Neospora* or *Toxoplasma* per circle on microscope slide (Table 1).

The results of cross-reactivities of mAb and pAb against *N. caninum*, *T. gondii* and *C. parvum* are summarized in Tables 2 and 3. Monoclonal antibodies raised to *N. caninum* (Nc-1 isolate) reacted with both isolates(Nc-1 , Nc-Sweb) in titres of 320 – 2560, and no fluorescent reaction was observed with both *T. gondii* and *C. parvum* antigens. Monoclonal antibodies to *T. gondii* reacted at titres 80 -640 with homologous antigen and at titres of 10 – 40 with both *N. caninum* isolates. Furthermore, mAb against *C. parvum* reacted only with homologous antigen. Similar fluorescent reactions (intensity and titres of 320- 2560) were observed with pAb to *Neospora* (Ra α Nc-1A ,1B) with both isolates of *N. caninum* and in titres of 40-80 with *T. gondii*. While polyclonal antibodies against *T. gondii* reacted only with homologous antigen, no

Table 1: The effect of the number of the tachyzoites of *N. caninum* and *T. gondii* on the intensity of fluorescent reaction

No. of parasites circle/field	Quantity of suspension (µ1)	Intensity of fluorescence
320/26	5	4+
640/32	10	4+
1280/104	20	3+
1920/156	30	2+

1+ = weak, 2+ = good, 3+ = very good, 4+ =excellent.

Table 2: Cross reactivity of monoclonal (mAb) and polyclonal (pAb) antibodies against *Neospora caninum*, *Toxoplasma gondii* and *Crytosporidium parvum* antigens by FA test

Antibodies mAb and pAb	Nc-1	AntigensNc-Sweb	*Toxoplasma*	*Cryptosporidium*
4	2560	1280	0	0
7	640	640	0	0
10	640	320	0	0
13	320	320	0	0
17	640	640	0	0
24	640	640	0	0
Tx 1	0	20	80	0
Tx 2	0	40	640	0
Tx 3	10	10	160	0
OW-IGC	0	0	0	160
Raα Nc-1 A	2560	2560	80	0
Raα Nc-1 B	320	320	40	0
Raα Tx A	0	0	5120	0
Raα Tx B	0	0	5120	0
RaαTx C	0	0	5120	0
RaαTx D	0	0	5120	0

Tx1, Tx2, Tx3 are *Toxoplasma* RH-strain; OW-IGO for *C. parvum*; Raα Nc-1 and B to *Neospora* Nc-1; RaαTx A,B,C,D to *Toxoplasma* RH-strain.

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Table 3: Sensitivity of FA test for differentiation of antibodies and antisera to *Neospora*, *Toxoplasma* and *Cryptosporidium*

Antisera	Nc-1	Nc-Sweb1	AntigensToxo	Crypto	cutoff titre
mAb α *Neopora*	320-2560	320-1560	0	0	10
mAb α *Toxoplasma*	10	10-40	80-640	0	80
mAb α Crypto	0	0	0	160	10
pAb α *Neospora*	2560	2560	80	0	160
pAb α Toxo	0	0	5120	0	10
Naturally infected cattle	160-640	80-640	20-40	10-40	10

Table 4: Maximum FA titres of sera from cattle naturally infected with *Neospora caninum* against *Neospora*, *Toxoplasma* and *Cryptosporidium* antigens

Serum No.	Nc-1	Antigens		
		Nc-Sweb 1	*Toxoplasma*	*Cryptosporidium*
1	320	160	40	20
2	160	80	40	40
3	320	80	40	20
4	1280	640	20	20
5	160	160	40	0
6	160	160	40	0
7	640	640	40	10
8	320	320	40	20
9	160	80	40	10
10	320	640	40	0

fluorescent reaction was observed with mAb and pAb raised to *N. caninum* and *T. gondii* with *C. parvum* antigen. At low dilutions, the complete surfaces of *Neospora* and *Toxoplasma* antigens were fluorescent, while in higher dilutions only a dotted fluorescence appeared on the apical complex.

Sera from cattle naturally infected with *N.caninum* showed titres 80 – 1280 with both isolates of *N. caninum* and 10 – 40 with *T. gondii* and *C. parvum* antigens (Table 4). No cross-reactions were detected with normal control sera of mouse, rabbit and cow at a dilution of 1:10.

DISCUSSION

Differentiation of *N. caninum* infection from other genera of coccidian protozoa is considered as an important goal for the researchers and diagnosticians. There is a problem of nonspecific binding of antibodies during the immunodiagnosis of *Neospora*, *Toxoplasma*, and *Cryptosporidium* (Dubey & Lindsay, 1993). Bjorkman *et al.* (1994) overcomes some of these cross-reactivity problems by combining soluble extracts of *N. caninum* with immunostimulating complex (iscoms) using an enzyme-linked immunosorbent assay (ELISA).

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In FA test, the problem of cross-reactivity of *N. caninum* with other related protozoa such as *T. gondii* may be due to the presence of common antigens associated with the apical complex structure of the Apicomplexa group. Taylor *et al.* (1990) and Conrad *et al.* (1993) showed in the IFA test that the genera of Apicomplexa possess apical structure and a common antigen was restricted to the apical 3rd of the invasive stages. In the present study, the whole parasite was fluorescent at low dilutions, but it was restricted to the apical end in high dilutions. Cross-reaction was observed only at low dilutions with heterologous antigens. Howe *et al.* (1998) identified two surface proteins of (29 and 35 kDa) from *N. caninum* and they showed that they are most similar to the *T. gondii* surface antigen 1 (SAG1). The same workers showed the similarity of surface antigens of five different isolates of *Neospora* and the monoclonal recognized epitopes of these isolates, but did not react with either *T. gondii* or *Sarcocytis neurona*. In this study, mAb raised against *N. caninum* exhibited nearly the same titres (320 – 2560) with both isolates of *Neospora* but yielded no reaction with *T. gondii* or *C. parvum* antigens. On the other hand, mAbs to *Toxoplasma* reacted at tires of 80-640 with *T. gondii* antigen and at low titres (10-40) with *N. caninum*. The presence of cross-reactivity between mAbs of *Toxoplasma* and *Neospora* isolates may be attributed to the cysteine residues that have been identified in the surface antigens of both *Neospora* and *Toxoplasma* (Manger *et al.* 1998). These cysteines are probably involved in intramolecular disulfied bonding. Their conservation may indicate that all of these proteins have a similar folding pattern (Howe *et al.*, 1998).

Monoclonal antibodies to *C. parvum* reacted only with *C. parvum* antigen and no cross-reaction was detected with either *N. canium* or *T. gondii* tachyzoites. Campbell & Current (1983) reported no cross-reaction of antisera to *Cryptosporidium* sp. with *Toxoplasma*, *Sarcocystis* and *Isospora* by the FA test. Romand *et al.* (1998) showed the existence of cross-reaction between antisera of *Sarcocystis* and *Neospora* antigen at dilution 1:20 by direct agglutination test and < 1:50 in FA test. In the present study, polyclonal antibodies to Nc-1 isolate reacted up to 320-2560 with both *Neospora* isolates and only in low titres (40-80) with *Toxoplasma* antigen. No reaction occurred with *Crytosporidium*. Barr *et al.* (1991) and Conrad *et al.* (1993) reported slight cross-reactivity of rabbit polyclonal antibodies to *N. caninum* with *T. gondii*. Polyclonal antibodies to *T. gondii* reacted only with homologous antigen and no reaction was observed either with *Neospora* or *Cryptosporidium*.

Sera from cattle naturally infected with *N. caninum* had titres >80 with *N. caninum* antigens (both isolates) and titres 10 – 40 with both *T. gondii* and *C. parvum* antigens. Conrad *et al.* (1993) showed that cattle infected with *N. caninum* had titres between 320 -5120 with *Neospora* and <160 with *Toxoplasma* antigens in the IFA test. This is in agreement with Dubey & Lindsay (1993) and Romand *et al.* (1998) who reported that sera to *N. caninum* had titre <50 with *T. gondii* in the IFA test. Cattle may be simultaneously infected with one or more coccidian parasites. Additionally, cross-reaction exists between the mentioned genera. This cross-reactivity implies that care should be taken when using immunodiagnostic assays for coccidian protozoa. The present study indicates that only titre of 80 or over should be regarded as positive indicators of neosporosis in FA testing.

Acknowledgements. Thanks are extended to Department of Parasitology, Uppsala, Sweden for providing the facilities and antisera during the sabbatical leave of the first author and special thanks to Professor Arvid Uggla for his support and friendship. The supply of *C. parvum* monoclonal antibody by Professor Alain Bonnin of France is greatly appreciated.

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