Iranian Journal of Veterinary Research, Shiraz University, 2013, Vol. 14, No. 1, Pages 61-64

Short Paper

Evaluation of an immunoblotting test based on whole tachyzoite lysate for diagnosis of humoral response of domestic cats infected with *Toxoplasma gondii*, Rh strain

Hosseininejad, M.

Department of Clinical Sciences, Faculty of Veterinary Medicine, Shahrekord University, Shahrekord, Iran

Correspondence: M. Hosseininejad, Department of Clinical Sciences, Faculty of Veterinary Medicine, Shahrekord University, Shahrekord, Iran. E-mail: hosseini-m@vet.sku.ac.ir

(Received 30 Jul 2012; revised version 1 Sept 2012; accepted 24 Sept 2012)

Summary

This study was aimed to detect anti-*Toxoplasma gondii* antibodies in cats infected with Rh strain of *T. gondii* by using an immunoblotting method. For this, cats were experimentally infected using tachyzoites harvested from Vero cell cultures. Tachyzoites were then lysed and transferred to polyacrylamide gels followed by blotting to PVDF membranes. An immunoblotting was performed using these membranes to detect IgG antibodies. Protein bands were detected in regions related to the presence of *T. gondii* surface antigens, dense granular or rhoptry proteins. No positive bands were detected in serum samples of kittens that received only Vero cell lysates (controls). Positive or negative results were in concordance to the results of IFAT. The results of this study revealed that whole tachyzoite antigen based immunoblotting is an appropriate diagnostic test for serological detection of anti-*T. gondii* antibodies in recently infected kittens with this Apicomplexan parasite.

Key words: Immunoblotting, Cat, *Toxoplasma gondii*

Introduction

Toxoplasma gondii, an obligate intracellular protozoan parasite infects human and all warm blooded animals. It has been found worldwide with different seropositivity. Infection to *T. gondii* is among the most common parasitic infections of animals and human in the world (Tenter *et al*., 2000). Cats are both the intermediate and definitive hosts for *T. gondii*; they can get the clinical disease due to multiplication of tachyzoites in their different cell types or they can shed oocysts that may be infectious for the other intermediate hosts (Frenkel *et al*., 1970; Mosallanejad *et al*., 2007). Infection following ingestion of *Toxoplasma* oocysts may cause abortion, which is important in human and sheep (Hartley and Marshall, 1957).

Three practical ways to diagnose *T. gondii* infection in living cats include: parasite isolation, fecal examination for oocysts and serologic tests. Isolation of the parasite is difficult, costly and time consuming and it is not easy to perform in routine diagnostic laboratories. Oocyst shedding period is very short (less than 3 weeks, mostly for 1 week) and after this period most cats remain infected without excreting the oocysts (Dubey, 1995). Therefore the most suitable remaining method for detecting *T. gondii* infection is serological evaluation of body fluids, especially serum samples. Once infected, animals harbor Toxoplasmic tissue cysts for life, which stimulate a long term humoral immune response in infected adults (Dubey and Lappin, 2006; Hosseininejad and Hosseini, 2011).

Immunoblotting is a diagnostic test in which pathogen antigens are firstly separated in gels and then transferred to special membranes. Samples suspected to have antibodies are then exposed to the membranes and possible reactions of

antigens and antibodies are detected using color changes following enzymatic reactions (Wastling *et al*., 1994). This study was performed to investigate the possibility of using crude tachyzoite antigen based immunoblotting to diagnose early *T. gondii* Rh strain infection of kittens.

Materials and Methods

Animals used in this assay were eight clinically healthy household kittens 45 ± 5 days old from both sexes. To ensure that the kittens were serologically negative against *T. gondii*, indirect fluorescent antibody test (IFAT) was performed for the kittens and relevant queens. Antihelming treatment was carried out using 22 mg/kg Mebendazole (MehrDarou®, Iran) for five consecutive days. Kittens were caged separately during the experiment and fed using pasteurized milk and cooked food during the study period. 10,000 tachyzoites were suspended in sterile PBS and injected intra-peritoneally to 6 kittens. Two other kittens (controls) received Vero cell lysates suspended in sterile PBS. Serum samples were collected every other day after inoculation and frozen until used. IFATs were performed as described previously, detection of anti *T. gondii* in dogs except Isothiocyanate-labeled goat anti-cat IgG (H+L) (102-095-003, Jackson Immunoresearch, USA) were used as conjugates (Hosseininejad *et al*., 2009).

Immunoblotting was carried out basically identical to what has been performed by Wastling *et al*. (1994). To prepare crude antigen extract, purified *T. gondii* tachyzoites were suspended in a buffer containing tris (hydroxymethyl) aminomethane, sodium dodecyl sulphate (SDS), glycerol and bromophenol blue. The pH of this buffer was adjusted to 6.8. Suspended tachyzoites were heated for 10 min at 94°C. The whole RH extracts were loaded on 12.5% SDS-PAGE gels. The gels were run at 120 volts until the bromophenol blue front leaves the gels. Separated antigens were then transferred to PVDF membranes using a semi-dry blotting instrument.

The membranes containing whole tachyzoite lysates were stripped and empty spaces were blocked using PBST-G (PBST-2% (v/v) fish gelatine liquid (Serva-Germany)) for 15 min. PBST-G was removed and each strip was incubated with a test serum diluted 1:100 in PBST-G for 2 h. Strips were washed four times using PBST followed by incubation with HRP conjugated goat anti-cat antibody (102-035-003, Jackson Immunoresearch, USA) for 2 h. Strips were then washed four times using PBST and three times using PBS (to remove tween) and incubated with a substrate solution (40 µl H_2O_2 (30% (v/v)) and 30 mg 4-chloro-1-naphthol (Sigma-Aldrich) in 40 ml PBS, 20% (v/v) methanol) for 20 min. Relative molecular masses were determined by comparison with a low molecular weight standard (LMW marker, Amersham, UK) run under the same conditions as the samples. Serum samples with known status of presence or absence of anti-*T. gondii* antibodies were always used as positive or negative controls. IFAT was performed for all of the samples to compare the concordance.

Results

Whole tachyzoite lysates containing strips were able to detect the infection in kittens from day eight onward. Bands of 22 to more than 97 kDa weights were detectable in positive samples. The most common band in positive samples and the early strong detected band was a 30 kDa protein region pertaining to the major surface antigen of tachyzoite. A 22 kDa band, pertaining to another surface antigen, SAG2 was detectable in about 50 percent of positive samples. Other detectable bands were 22-30 kDa (of dense granular proteins) and 50-70 kDa (of rhoptry proteins) (Fig. 1). IFAT results showed complete agreement with the results obtained from immunoblotting.

Discussion

Cats can be infected experimentally with *T. gondii* in many ways. The age of cats, the stage of the parasite, and the route of inoculation can influence the outcome of the infection. However, cats of any age may die or develop severe disease following

parenteral inoculation with tachyzoites, bradyzoites, or oocysts, especially when an immuno-compromising disease such as feline immunodeficiency disease presents. Experimental toxoplasmosis has been inducted in cats using 10,000 tachyzoites (Davidson *et al*., 1996).

Serologic tests including the Sabin-Feldman dye test, indirect fluorescent antibody test (IFAT), indirect hemagg-lutination test (IHA), latex agglutination test (LAT), ELISA and modified agglutination test (MAT) have been used previously for detection of antibodies against *T. gondii* (Hosseininejad *et al*., 2009).

MAT, IHA, DT, and ELISA have also been used previously to detect anti-*T. gondii* antibodies in cats experimentally infected with tissue cysts (Dubey, 1995). Chumpitazi *et al*. (1995) have found that IB has a sensitivity of the same order as or higher than that of the other techniques used in diagnosis of congenital toxoplasmosis.

As it is shown in Fig. 1, the most common band in positive samples was a 30 kDa protein region pertaining to the major surface antigen of *T. gondii* tachyzoite. SAG1 protein is considered an important antigen for development of effective diagnostic tests or subunit vaccines (Handman *et al*., 1980). P30 antigen is stage specific and only detectable in the tachyzoite stage and plays an essential role in the early stages of parasite entry into the host cells (Kasper *et al*., 1984; Grimwood and Smith, 1996).

Sohn and Nam (1999) have investigated 198 sera collected from stray cats by using a western blotting method and compared it with other diagnostic tests. Out of 198 sera assayed, 26 sera (13.1%) showed blot patterns against *T. gondii*. They found 30, 22, 22-30 and 50-70 kDa proteins and some minor bands as the most recognized proteins in infected cats (Sohn and Nam, 1999).

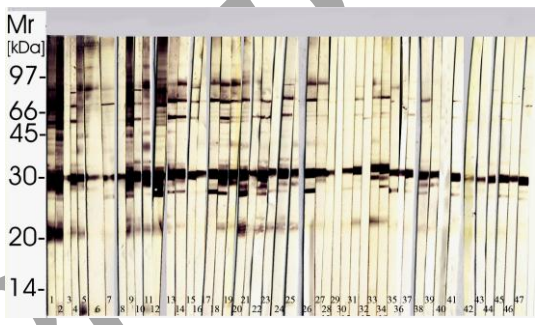

Fig. 1: Immunoblots performed using whole tachyzoites of *T. gondii* for detection of IgG antibodies in experimentally infected kittens using Rh strain of parasite. Bands 29, 32 and 40 kDa show negative and other bands show positive results. Positive bands are located mostly on 22, 30, 22-30 and 50-70 kDa regions

Table 1: Immunoblot results in comparison with IFAT titers on different days post-infection

Days		No.							
		1	2	3	4	5	6	7	8
>8	IFAT titers	0	0	0	0	0	0	0	0
	IB	-	-	-	-	-	-	-	-
8	IFAT titers	0	0	0	0	16	0	0	0
	IB	-	-	-	-	+	-	-	-
10	IFAT titers	32	0	0	0	32	0	0	0
	IB	+	-	-	-	+	-	-	-
12	IFAT titers	64	32	0	32	32	32	0	0
	IB	+	+	-	+	+	+	-	-
14	IFAT titers	256	64	32	32	32	128	0	0
	IB	+	+	+	+	+	+	-	-
16	IFAT titers	512	256	32	32	128	256	0	0
	IB	+	+	+	+	+	+	-	-
18	IFAT titers	512	512	64	32	256	512	0	0
	IB	+	+	+	+	+	+	-	-
20	IFAT titers	512	512	128	128	256	512	0	0
	IB	+	+	+	+	+	+	-	-
21	IFAT titers	512	512	128	256	256	512	0	0
	IB	+	+	+	+	+	+	-	-
22	IFAT titers	512	512	128	256	512	512	0	0
	IB	+	+	+	+	+	+	-	-

In another work, the *T. gondii*-specific target antigens for feline immunoglobulin M (IgM) and immunoglobulin G (IgG) immune responses were studied longitudinally using western blot immunoassay in cats infected with *T. gondii* strain ME49. On week 20 PI, the 19-kDa (6/8 samples), 26-kDa (8/8 samples), 28-kDa (8/8 samples), 31-kDa (7/8 samples), 35-kDa (6/8 samples), 51-kDa (6/8 samples), 55-kDa (7/8 samples), and 65-kDa (7/8 samples) antigens were recognized most commonly in the IgG western blot immunoassay (Lappin *et al*., 1994).

As shown in Table 1, the results of immunoblotting using crude *T. gondii* antigens are in agreement with the results of standard IFAT test, showing the high performance of immunoblotting to detect infection caused by Rh strain of the parasite. Other researchers have revealed at least an equal or even higher sensitivity of immunoblotting compared to other diagnostic tests to detect congenital toxoplasmosis (Chumpitazi *et al*., 1995). Gross *et al*. (2004) showed that immunoblotting helps to provide more precise results to show the clinical infection resulted from *T. gondii* infection.

In conclusion, Immunoblotting using whole tachyzoite antigen is a sensitive diagnostic way to diagnose *T. gondii* infection in early stages in which tachyzoites multiply rapidly in different cell types.

References

Chumpitazi, BF; Boussaid, A; Pelloux, H; Racinet, C; Bost, M and Goullier-Fleuret, A (1995). Diagnosis of congenital toxoplasmosis by immunoblotting and relationship with other methods. J. Clin. Microbiol., 33: 1479-1485.

Davidson, MG; Lappin, MR; Rottman, JR; Tompkins, MB; English, RV; Bruce, AT and Jayawickrama, J (1996). Paradoxical effect of clindamycin in experimental, acute toxoplasmosis in cats. Antimicrob. Agents Chemother., 40: 1352-1359.

Dubey, JP (1995). Duration of immunity to shedding of *Toxoplasma gondii* oocysts by cats. J. Parasitol., 81: 410-415.

Dubey, JP and Lappin, M (2006). Toxoplasmosis and neosporosis. In: Greene, C (Ed.), *Toxoplasmosis and neosporosis*. W. B. Saunders Co., PP: 754-775.

Frenkel, JK; Dubey, JP and Miller, NL (1970). *Toxoplasma gondii* in cats: fecal stages identified as coccidian oocysts. Science. 167: 893-896.

Grimwood, J and Smith, JE (1996). *Toxoplasma gondii*: the role of parasite surface and secreted proteins in host cell invasion. Int. J. Parasitol., 26: 169-173.

Gross, U; Holpert, M and Goebel, S (2004). Impact of stage differentiation on diagnosis of toxoplasmosis. Ann. Ist. Super Sanita. 40: 65-70.

Handman, E; Goding, JW and Remington, JS (1980). Detection and characterization of membrane antigens of *Toxoplasma gondii*. J. Immunol., 124: 2578-2583.

Hartley, WJ and Marshall, SC (1957). Toxoplasmosis as a cause of ovine perinatal mortality. N. Z. Vet. J., 5: 119-124.

Hosseininejad, M; Azizi, HR; Hosseini, F and Schares, G (2009). Development of an indirect ELISA test using a purified tachyzoite surface antigen SAG1 for sero-diagnosis of canine *Toxoplasma gondii* infection. Vet. Parasitol., 164: 315-319.

Hosseininejad, M and Hosseini, F (2011). Seroprevalence of *Neospora caninum* and *Toxoplasma gondii* infection in dogs from west and central parts of Iran using two indirect ELISA tests and assessment of associate risk factors. Iranian J. Vet. Res., 12: 46-51.

Kasper, LH; Bradley, MS and Pfefferkorn, ER (1984). Identification of stage-specific sporozoite antigens of *Toxoplasma gondii* by monoclonal antibodies. J. Immunol., 132: 443-449.

Lappin, MR; Bush, DJ and Reduker, DW (1994). Feline serum antibody responses to *Toxoplasma gondii* and characterization of target antigens. J. Parasitol., 80: 73-80.

Mosallanejad, B; Malmasi, A; Mohebali, M and Tabatabayi, M (2007). Anterior uveitis in a kitten infected with *Toxoplasma gondii* (Tehran strain). Iranian J. Vet. Res., 8: 91-93.

Sohn, WM and Nam, HW (1999). Western blot analysis of stray cat sera against *Toxoplasma gondii* and the diagnostic availability of monoclonal antibodies in sandwich-ELISA. Korean J. Parasitol., 37: 249-256.

Tenter, AM; Heckeroth, AR and Weiss, LM (2000). *Toxoplasma gondii*: from animals to humans. Int. J. Parasitol., 30: 1217-1258.

Wastling, JM; Harkins, D and Buxton, D (1994). Western blot analysis of the IgG response of sheep vaccinated with S48 *Toxoplasma gondii* (Toxovax). Res. Vet. Sci., 57: 384-386.