

Short Communication

Serological Evaluation of Experimental *Toxoplasma gondii* Infection in Cats by Using Immunoblotting Based on an Affinity Purified Surface Antigen

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ABSTRACT

Toxoplasma gondii is an apicomplexan parasite that infects human and almost all warm-blooded animals. The life cycle of the parasite includes an asexual reproduction in intermediate hosts (Mammals and birds) and a sexual reproduction in definitive hosts (Felidae). Cats are both the intermediate and the definitive host for *T. gondii*. The aim of this study was to investigate anti-*T. gondii* antibodies in cats by using an immunoblot method based on a major surface antigen; SAG1. Six sero-negative kittens were infected intraperitoneally by Rh strain of *T. gondii*. Serum samples were collected and evaluated for the presence of IgG antibodies by using two techniques; immunoblotting and indirect fluorescent antibody test (IFAT). SAG1 based immunoblotting was able to detect anti-*T. gondii* antibodies at least eight days after infection. Comparative evaluation showed that this method is as sensitive as IFAT to diagnose *T. gondii* infection in cats.

Keywords: Cats, Immunoblot, P30, SAG1, *Toxoplasma gondii*

INTRODUCTION

Infection with the protozoan *T. gondii* is one of the most common parasitic infections of man and other warm-blooded animals. This Apicomplexan protozoa is an obligate intracellular parasite which causes congenital defects or abortion and fatal diseases in immunocompromised patients. Other than zoonotic importance, *T. gondii* is responsible for economic losses because it leads to abortions in sheep and neonatal deaths of lambs. The parasite *T. gondii*

multiplies asexually in all the hosts, while the only known definitive hosts belong to the family Felidae, which includes the domestic cat, iriomote cat, leopard, tiger and lions. Therefore, cats are both the intermediate and definitive hosts for this apicomplexan parasite. As intermediate hosts, immune-suppression of cats due to viral infections or cancer could lead to serious disseminated toxoplasmosis (Dubey & Lappin 2006, Lukesova & Literak 1998). As definitive hosts, cats infected with *T. gondii* serve as important source of oocyst infection to humans, birds and all other animals, through contamination of the environment with feces

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(Smith *et al* 1992). Base on these data and regarding the importance of cats in *T. gondii* life cycle, we selected cats to investigate the serological pattern that cats response when encounter to Rh strain of the parasite which is the most common and virulent type of the parasite.

Serological diagnosis of *T. gondii* infection is the most practical assays among other diagnostic methods in infected cats. Multiple diagnostic tests have been developed for serological diagnosis of *T. gondii* including the Sabin-Feldman dye test, indirect fluorescent antibody test (IFAT), indirect hemagglutination test (IHA), latex agglutination test (LAT), modified agglutination test (MAT), enzyme linked immunosorbent assay (ELISA) (Kimbita *et al* 2001) and Immunoblotting (Wastling *et al* 1994). Each test has its superiority or weak points when compare to others.

Immunoblotting is a technique that combines electrophoresis of toxoplasmic antigens, an electrotransfer, and a specific antibody test and has been used to diagnose the infection in human and animals (Wastling *et al* 1994). Reactivity to SAG1; the major surface protein has been shown to be a sensitive tool in diagnosis of *T. gondii* infection in cats or dogs (Hosseininejad *et al* 2009, Kimbita *et al* 2001, Silva *et al* 2002). The aim of this study was to evaluate an SAG1 based immunoblotting in order to diagnose early *T. gondii* infection in cats in comparison to IFAT (a reference and standard diagnostic test). In the other words, the performance of this new serological test was evaluated in different stages of humoral response of cats to Rh strain of *T. gondii*.

MATERIALS AND METHODS

Preparation of Parasites and Antigens. The strain which has been used in our study either for experimental infection or antigen purification was *T. gondii* Rh strain. Tachyzoites were grown in vitro using Vero cell mono-layers in RPMI-1640 supplemented with 2% fetal bovine serum and a mixture of

penicillin/streptomycin antibiotics. The cultures were incubated at 37 °C in a 5% CO₂ environment. Tachyzoites were used immediately for IFAT and inoculation or frozen at -80 °C until required for antigen preparation.

Affinity purification of SAG1 antigen. For affinity purification of antigen, mouse monoclonal antibodies (Mab Clone P30/3, Immune Systems Ltd, UK) were covalently bound to rProtein A Sepharose 4B (Pharmacia Biotech). Prior to affinity purification, cell-culture derived *T. gondii* tachyzoites were extracted in phosphate-buffered saline (PBS), 0.5% Triton X-100 (4 °C). After sonication on ice for 90 sec (50% active cycle, output control level 2; VibraCell Ultrasonicator, USA), the suspension was centrifuged at 13,000g for 10 min at 4°C. The supernatant was applied to the immunosorbent which had been equilibrated with PBS, 0.1% Triton X-100. After 2 hrs the immunosorbent was washed with 100 volumes PBS, 0.1% Triton and eluted with 0.1 M glycine, pH 2.6. The eluate was neutralized using 1M Tris and 10 times PBS. The purity of SAG1 was checked by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), Western blotting and India ink staining (Hosseininejad *et al* 2009, Wastling *et al* 1994).

Animals. Eight clinically healthy household kittens of 45±5 days old from both sexes were used in this study. Indirect fluorescent antibody tests (IFAT) were performed for the kittens and relevant queens (their mothers) to ensure that they do not have detectable antibodies against *T. gondii*. All Kittens were dewormed using 22 mg/kg Mebendazole (MehrDarou®, Iran) for five consecutive days and caged separately during the experiment. Feedings were performed using pasteurized milk and cooked food during the study period. Cat serum samples were collected every other day after injections and frozen until being used. 10⁴ 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. Kittens were evaluated for any clinical manifestation of the disease at the beginning of the

experiment and during it. Survey chest radiographs were taken and evaluated for any respiratory involvement.

Indirect Fluorescent Antibody Test. 4×10^7 *T. gondii* Rh strain tachyzoites were harvested from Vero cell cultures and fixed in 0.2% formaldehyde in PBS. 10 well slides were coated using this suspension and were maintained in -20 °C until used. Immediately before use, prepared slides were soaked in cold acetone for 10 minutes followed by 10 minutes washing in PBS. 20 μ l of diluted serum samples (diluting was started from 1:32 in PBS) were add to each well and incubated in 37 °C incubator for 30 min. Slides were washed and incubated in anti-fading solution for 10 minutes. Isothiocyanate-labeled goat anti-cat IgG (H+L) (102-095-003, Jackson ImmunoResearch, USA) were diluted (1:30 in PBS-1% Evan's blue) and 20 μ l of this mixture was added to each well and incubated in 37 °C for 30 min. slides were washed and incubated in anti-fading solution and PBS; 10 minutes for each one. Slides were covered and test results were recorded. Positive serum samples were diluted two times more and tested again until being negative. Positive and negative controls were obtained from friedrich-loeffler institute, Germany. They were pools of sera collected from seropositive or seronegative cats. Presence or absence of anti- *T. gondii* antibodies were evaluated using different serological methods.

Immunoblotting. The immunoblot procedure was basically carried out as described by Schares et al. for closely related protozoan parasite; *Neospora caninum* (Schaes *et al* 1998). PVDF membranes with transferred purified SAG1 antigen striped and empty spaces were blocked using PBST-G (PBST- 2% (v/v) fish gelatine liquid (Serva-Germany)) for 15 minutes. PBST-G was removed and each strip was incubated with a test serum diluted 1:100 in PBST-G for 2 hrs. Stripes were washed four times using PBST followed by incubation with HRP conjugated goat anti-cat antibody (102-035-003, Jackson ImmunoResearch, USA) for 2 hours. Stripes were then washed four times using PBST and three times using PBS (to remove

tween) and incubated with a substrate solution (40 μ l H₂O₂ (30% (v/v)) and 30 mg 4-chloro-1-naphthol (Sigma-Aldrich) in 40 ml PBS, 20% (v/v) methanol) for 20 minutes. 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. Test results were regarded as positive when a 30 kDa Immunodominant antigen was detectable.

RESULTS AND DISCUSSION

Clinical signs of the infection were seen 5-8 days post-inoculation in kittens that had received tachyzoites and were included mild increased rectal temperature and anorexia. Survey radiographs showed increase of radio-opacity and diffuse interstitial pattern and mild alveolar pattern in dorsal parts of caudal lobes of the lungs during 3rd week of infection in two cats. Analysis of the serum samples using indirect fluorescent antibody test and immunoblotting during the first 7 days post inoculation showed no detectable antibodies at the initial dilutions (1:16 for IFAT or 1:100 for Immunoblotting) in none of cats. Anti- *T. gondii* antibodies were detectable from day 8 afterwards; Day 8 (two cats), 12 (three cats) and 14 (one cat) post-inoculation in dilutions of 1:16 or 1:32. Antibody titers increased gradually and reached to 1:128 to 1:512 at the last sampling time on day 26. Antibodies were detectable using IFAT at titers starting from 1:16 or 1:32 from day 8 and reached to 1:128 to 1:512 on day 20 (Figure 1). Immunoblotting using affinity purified SAG1 antigen was able to detect antibodies in all cases in which antibodies were detectable using IFAT (Fig. 1). Detectable positive lane was regarded as specific for SAG1 antigen when it was on the area of 30 kDa marker protein. Weak unspecific bands were seen in some other regions of the stripe were probably resulted from remained un-blocked regions or un-specific reactions were ignored. The results of comparative analysis using these two methods showed a 100% concordant between them. Ante-mortem diagnosis of

clinical toxoplasmosis in cats can be performed base on evaluation of clinical signs, laboratory findings, serologic examinations and response to anti-toxoplasmic treatment, while serologic examination is the only practical way for diagnosis of *T. gondii* infection in clinically healthy cats.

starting 7 days p.i. and up to 6 yr p.i. Cats seroconverted 10 days p.i. and high titers persisted even after 6 years (Dubey 1995). Other researchers have also induced *T. gondii* infection in cats in order to evaluate immune responses, find suitable diagnostic ways or devise appropriate medications (Davidson *et al* 1996, Hawkins *et al* 1997, Lappin *et al* 1989). In routine diagnosis, several techniques are used to confirm *T. gondii* infection. Immunodominant surface antigen 1 (SAG1, P30) of *T. gondii* is considered as an important antigen for the development of effective diagnostic tests or subunit vaccines (Handman *et al* 1980). SAG1 antigen is stage specific and only detectable in the tachyzoite stage (Kasper *et al* 1984). Recombinant surface antigens like SAG1 or SAG2 have also been shown to be useful in the sero-diagnosis in cats or human (Coceres *et al* 2010, Holec-Gasior & Kur 2010, Huang *et al* 2002, Kimbita *et al* 2001) and purified SAG1 has been used to design ELISA test (Hosseininejad *et al* 2009). In apicomplexan parasites, surface antigens are regarded more specific than intracellular antigens (Bjorkman & Ugglä 1999). IFAT tests detect antibodies directed towards antigens present on the cell surface of tachyzoites and the results of this test are comparable to the diagnostic tests performed based on surface antigens. IFAT is regarded as an important reference test for sero-diagnosis of *T. gondii* infection (Dubey *et al* 1995). Affinity purified SAG1 has its superiority in compare to recombinant antigen because no translational or post-translational modifications or errors take place and it keeps its native characters. Immunoblotting based on a single antigen has high specificity because of the minimized possibility for cross-reactions and interpretation can be performed much more easier than immunoblots based on crude antigens. The results of this study shows that Immunoblotting using affinity purified *T. gondii* SAG1 antigen is a sensitive diagnostic test and is capable to diagnose early infection in cats. The results obtained from immunoblotting shows that it is comparable with the standard IFAT test so that infection is detectable in

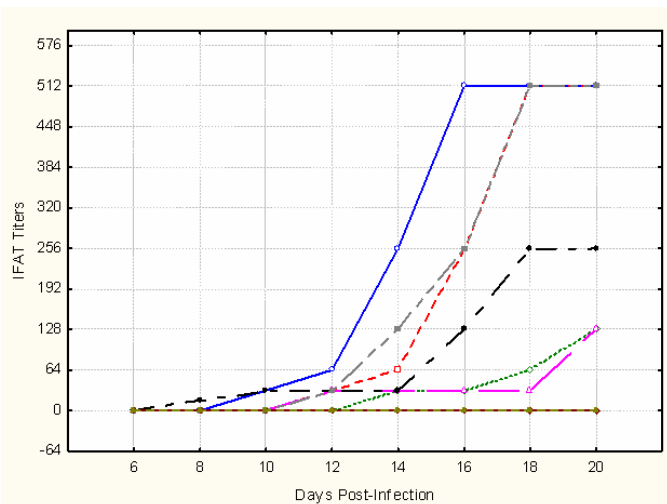


Figure 1. Serological response of cats to infection with Rh strain of *T. gondii* tachyzoites when evaluated with IFAT.

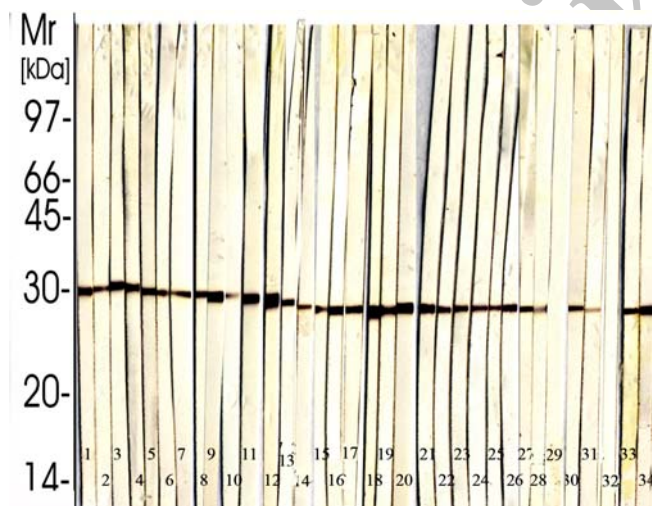


Figure 2. Immunoblots based on affinity purified SAG1 antigen reveal 32 positive cats sera. Lanes 29 and 32 are negative.

Cats can easily be infected with *T. gondii* in laboratory and response serologically (Sato *et al* 1993). Serological responses of cats after oral infection with tissue cysts were studied previously with MAT, IHA, DT, and ELISA; The cats were bled sequentially

early stages of the disease when serum samples are evaluated with this immunoblotting method.

Ethics

Animal welfare codes were followed as described by animal right organization in: 2006: Aledavoud S.J., Javazadeh blouri A., Sadeghi H., Sabzghabaie M.A.M., Latifi S.M., Nourouzian R. and Larijani B.: Preparation of Ethic codes for studies on laboratory animals. JBUMS (Journal of Babol University of Medical Sciences- Medical Ethic Supplement, Jan & Feb 2006, Vol 8, Sup 3, 55-64. 2005: The first national symposium for ethics in medical research, Tehran, Iran.

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