Short Communication

Exploration of immunoblot profiles of *Neospora caninum* probed with different bovine immunoglobulin classes

Yong-Seung Shin[1], Eung-Goo Lee[2], Tae-Sung Jung[1],*

[1]*Institue of Animal Medicine, College of Veterinary Medicine, Gyeongsang National University, Jinju 660-701, Korea*
[2]*Department of Molecular Parasitology, School of Medicine, Sungkyunkwan University, Suwon 440-746, Korea*

   The present study was attempted to compare the *Neospora caninum* (*N. caninum*) antigenic bands recognized by different bovine immunoglobulin classes. A total 10, 5, 2, and 6 antigenic bands were exhibited on immunoblot profiles against bovine IgM, IgE, IgA, and IgG, respectively. A 46 kDa band was probed as a common antigenic band except IgA; 69 kDa band was bovine IgM and IgE; 33, 37, 55, and 79 kDa bands were bovine IgM and IgG; 72 kDa band was found IgM and IgA profiles. Based on the analysis, it appeared that different immunoglobulin classes recognizing different antigenic molecules were cooperating to cope with neosporosis.

Key words: antigenic protein, bovine immunoglobulin classes, immunoblotting, *Neospora caninum*

   Neospora caninum (*N. caninum*), an obligate intracellular protozoan parasite, is known to have a great similarity to *Toxoplasma gondii* (*T. gondii*), which is belonging to the same phylum Apicomplexa [4]. This parasite has been identified as a contagious pathogen in animals worldwide, particularly cattle and dogs [5].

   Antigenic proteins from *N. caninum* have been identified and tested for diagnosis by a number of investigators [6,7], but, there are very few reports available on the humoral immune responses against *N. caninum* as opposed to *T. gondii* studied well at different stages of infection. Briefly, *T. gondii* specific IgG usually appears within 1-2 weeks after infection and reaches its peak concentration within 1-2 months, after which it gradually declines, though it generally persists for life [18]. IgM appears earlier and declines more rapidly than IgG during toxoplasmosis [11]. IgA and IgE also show in the early stage of infection [14, 15, 19]. These reports suggest that an examination of each immunoglobulin class may contribute to understanding of the immune reaction in neosporosis [1].

   In this study, *N. caninum* antigenic proteins recognized by bovine IgM, IgE, IgA, and IgG were explored with sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) immunoblotting by comparing their molecular weights and the intensity of their antigenic bands.

   The *N. caninum* KBA-2 isolate [8] was maintained and subjected according to Lee *et al*. [9]. Purified tachyzoites (approximately 1×10^8) were dissolved in 40 mM Tris-base (Sigma, USA), disrupted three times by freeze-thaw cycles in liquid nitrogen, and then sonicated (XL-2020; Misonix, USA) at 5.5 Watts for 15 seconds, 8 times on ice slurry. Following that, 1% (v/v) Triton X-100 (Sigma, USA) and 50% (v/v) $2 \times$ SDS Sample Buffer™ (Sigma, USA) were added to a suspension of disrupted tachyzoites and boiled for 5 minutes. Protein concentrations of the samples were estimated using the Bradford protein assay kit (Bio-Rad, USA).

   In order to produce anti-*N. caninum* serum, live KBA-2 tachyzoites (1×10^8) suspended in PBS (pH 7.4) were administrated intravenously to one Holstein cow confirmed as *N. caninum* and *T. gondii* negative by indirect fluorescent antibody test (IFAT) [2,12]. The cow was housed in a pen for the duration of the experiment according to the guidelines for experimental animal resources developed by Gyeongsang National University. Serum was collected 13 weeks post-inoculation and stored at -20°C until use. In the collected serum, the antibody titers of *N. caninum* and *T. gondii* were evaluated as 1 : 800 and below 1 : 100 by IFAT, respectively.

   The *N. caninum* tachyzoite lysate was separated in 10% SDS-PAGE gels. The gel was then transferred onto polyvinylidene fluoride (PVDF) membrane (Immobilon-P, Millipore, USA) for immunoblotting. Immunoblotting was performed as described by Mansfield [10]. The PVDF membranes blotted were incubated with bovine anti-*N. caninum* serum in a 1 : 1 dilution ratio with 5% (w/v) skim milk in phosphate buffered saline (pH 7.4). The specific antigenic proteins corresponding to each bovine immunoglobulin class were then further incubated in a 1 : 2,000 dilution of

*Corresponding author
Tel: +82-55-751-5822; Fax: +82-55-751-5803
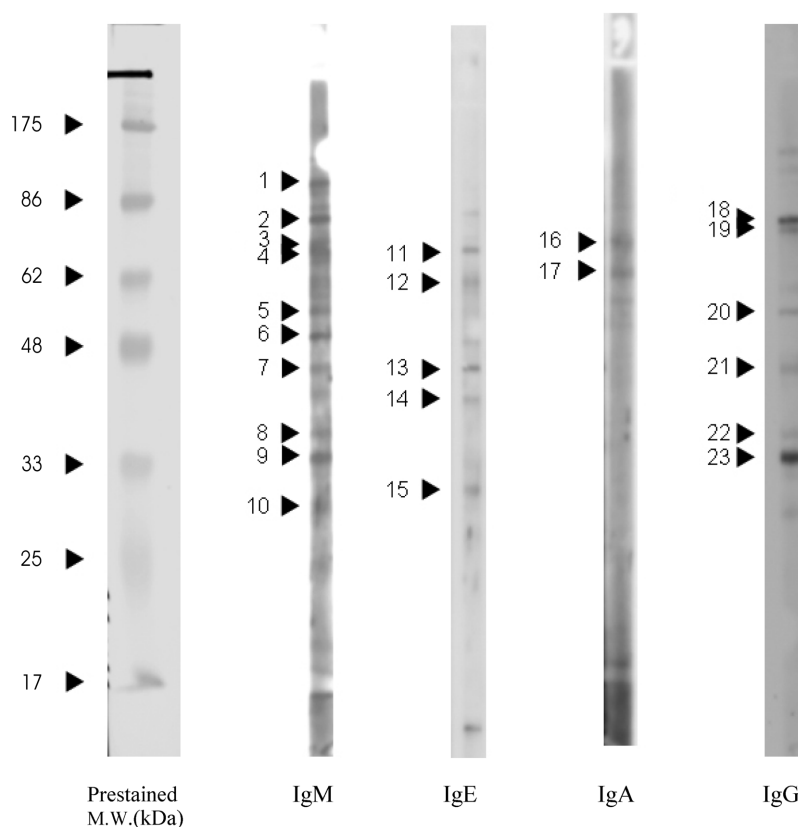E-mail: jungts@nongae.gsnu.ac.kr

Fig. 1. SDS-PAGE immunoblot profiles of *N. caninum* tachyzoites lysate with bovine IgM, IgE, IgA, and IgG.

horseradish peroxidase (HRP)-conjugated anti-bovine IgG, IgM, and IgA (Koma, Korea). For the detection of IgE specific antigenic protein, a 1:40 dilution of mouse anti-bovine IgE monoclonal antibody [16] was incubated for 1 hour and then washed 3 times for 15 minutes with a TBS-T buffer (20 mM Tris, 500 mM NaCl, 0.05% (v/v) Tween 20, pH 7.4). It was then re-incubated in a 1 : 2,000 dilution of HRP-conjugated goat anti-mouse IgG (Jackson, USA) for an additional hour. After washing 5 times with a TBS-T buffer, PVDF membranes were soaked in ECL Solution (Amersham, Sweden) for 1 minute and exposed to X-ray film (Fuji, Japan) for 30 seconds.

Afterwards, the immunoblotted bands were digitalized using an Agfa Arcus 1200™ image scanner (Agfa-Gevaert, Belgium), and the acquired images were analyzed using Phoretix™ 2D software (Ver. 5.01, NonLinear Dynamics, UK). For purposes of more precise discussion, immunobloted image analysis was performed at least 3 times, and several minor bands, which showed non-reproducible exhibition during the image analysis, were not analyzed at all.

The results showed that the SDS-PAGE immunoblot profiles of *N. caninum* tachyzoites lysate were exhibited 10, 5, 2, and 6 antigenic bands with bovine IgM, IgE, IgA, and IgG, respectively (Fig. 1 and Table 1). Bands placed at 46 kDa were commonly recognized by bovine IgM, IgE, and

IgG. The band at 69 kDa was found on both bovine IgM and IgE profiles. The bands at 33, 37, 55, and 79 kDa were exhibited by both bovine IgM and IgG. The band at 72 kDa was also seen in both IgM and IgA profiles. Of these

Table 1. Molecular weights (M.W.) of antigenic bands

M.W. (kDa)	Antigenic bands			
	IgM	IgE	IgA	IgG
103	1			
79	2			18
75				19
72	3		16	
69	4	11		
63			17	
61		12		
55	5			20
49	6			
46	7	13		21
41		14		
37	8			22
33	9			23
30		15		
29	10			

antigenic bands, those placed at 33 (IgM and IgG), 49 (IgM), and 79 kDa (IgM and IgG) were strongly recognized.

Generally, antibodies only bind to antigens that stimulate their production and each immunoglobulin responds to the antigenic proteins of a pathogen according to their own specific features of immunoglobulin class [17]. The analysis of host's each immunoglobulin response against *N. caninum*, therefore, might be helpful to understand the immune response of bovine neosporosis. In this study, all immunoblot profiles showed either common or individual antigenic bands probed with different bovine immunoglobulin classes. The antigenic bands recognized by only one immunoglobulin class seemed to stimulate the host immune system into producing a single class of immunoglobulin. In contrast, the antigenic bands commonly recognized by several immunoglobulin classes might react with the host immune system into producing multiple immunoglobulin classes. Thus, if these commonly recognized antigenic proteins might be use as a vaccine for neosporosis, it seemed to be effective candidates since it might stimulate the host immune system to make a number of immunoglobulin classes against *N. caninum*.

As shown in Fig. 1, the profile probed with IgM exhibited a number of common antigenic bands, either with IgG or IgE. However, bands between IgE and IgG profiles presented fewer commonalities. The *N. caninum* SDS-PAGE immunoblot profiles in this study displayed many bands when probed with IgM (10), as compared to those that were probed with IgG (6), IgE (5) and IgA (2). In the diagnosis of protozoa infection, the activation of host IgM and IgE immune responses were used to interpret that the host was at an early and progressive stage of infection [3, 13]. The donor cow used this study, therefore, was highly suspected to be undergoing progressive infection with *N. caninum* and entering to the point of a chronic phase since the immune responses of IgM and IgG reacted more strongly than those of IgE and IgA.

In conclusion, comparing immunoblot profiles with different immunoglobulin classes might be useful for planning strategies to develop effective vaccine candidates and for making an interpretation of infection stage.

Acknowledgments

References

1. **Ashburn D, Joss AW, Pennington TH, Ho-Yen DO.** Do IgA, IgE, and IgG avidity tests have any value in the diagnosis of Toxoplasma infection in pregnancy? J Clin Pathol 1998, **51**, 312-315. 1.

2. **Baszler TV, Adams S, Vander-Schalie J, Mathison BA, Kostovic M.** Validation of a commercially available monoclonal antibody-based competitive-inhibition enzyme-linked immunosorbent assay for detection of serum antibodies to *Neospora caninum* in cattle. J Clin Microbiol 2001, **39**, 3851-3857.

3. **Decoster A, Darcy F, Capron A.** Recognition of *Toxoplasma gondii* excreted and secreted antigens by human sera from acquired and congenital toxoplasmosis: identification of markers of acute and chronic infection. Clin Exp Immunol 1988, **73**, 376-382.

4. **Dubey JP, Carpenter JL, Speer CA, Topper MJ, Uggla A.** Newly recognized fatal protozoan disease of dogs. J Am Vet Med Assoc 1988, **192**, 1269-1285.

5. **Dubey JP.** Review of *Neospora caninum* and neosporosis in animals. Korean J Parasitol 2003, **41**, 1-16.

6. **Innes EA, Andrianarivo AG, Bjorkman C, Williams DJ, Conrad PA.** Immune responses to *Neospora caninum* and prospects for vaccination. Trends Parasitol 2002, **18**, 497-504.

7. **Jenkins M, Baszler T, Bjorkman C, Schares G, Williams D.** Diagnosis and seroepidemiology of *Neospora caninum*-associated bovine abortion. Int J Parasitol 2002, **32**, 631-636.

8. **Kim JH, Sohn HJ, Hwang WS, Hwang EK, Jean YH, Yamane I, Kim DY.** In vitro isolation and characterization of bovine *Neospora caninum* in Korea. Vet Parasitol 2000, **90**, 147-154.

9. **Lee EG, Kim JH, Shin YS, Shin GW, Suh MD, Kim DY, Kim YH, Kim GS, Jung TS.** Establishment of a two-dimensional electrophoresis map for *Neospora caninum* tachyzoites by proteomics. Proteomics 2003, **3**, 2339-2350.

10. **Mansfield MA.** Rapid immunodetection on polyvinylidene fluoride membrane blots without blocking. Anal Biochem 1995, **229**, 140-143.

11. **Montoya JG.** Laboratory diagnosis of *Toxoplasma gondii* infection and toxoplasmosis. J Infect Dis 2002, **185**, S73-82.

12. **Pappas MG, Lunde MN, Hajkowski R, McMahon J.** Determination of IgM and IgG antibodies to Toxoplasma using the IFA test, ELISA, and Dot-ELISA procedures. Vet Parasitol 1996, **20**, 31-42.

13. **Pinon JM, Toubas D, Marx C, Mougeot G, Bonnin A, Bonhomme A, Villaume M, Foudrinier F, Lepan H.** Detection of specific immunoglobulin E in patients with toxoplasmosis. J Clin Microbiol 1990, **28**, 1739-1743.

14. **Stepick-Biek P, Thulliez P, Araujo FG, Remington JS.** IgA antibodies for diagnosis of acute congenital and acquired toxoplasmosis. J Infect Dis 1990, **162**, 270-273.

15. **Takahashi EE, Rossi CL.** Use of three immunological techniques for the detection of Toxoplasma spIgA antibodies in acute toxoplasmosis. J Clin Pathol 1994, **47**, 1101-1104.

16. **Thatcher EF, Gershwin LJ.** Generation and characterization of murine monoclonal antibodies specific for bovine

immunoglobulin E. Vet Immunol Immunopathol 1988, **18**, 53-66.

17. **Tizard IR.** Veterinary immunology, 5th ed. pp. 5-7. Saunders, Philadelphia, 1996.

18. **Verhofstede C, Van Gelder P, Rabaey M.** The infection-stage-related IgG response to *Toxoplasma gondii* studied by immunoblotting. Parasitol Res 1988, **74**, 516-520.

19. **Villena I, Aubert D, Brodard V, Quereux C, Leroux B, Dupouy D, Remy G, Foudrinier F, Chemla C, Gomez-Marin JE, Pinon JM.** Detection of specific immunoglobulin E during maternal, fetal, and congenital toxoplasmosis. J Clin Microbiol 1999, **37**, 3487-3490.