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Scientific Report

Study on pattern of *Neospora caninum* tachyzoite proteins by SDS-PAGE and Western blotting in aborted cows

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Summary

The intracellular parasite *Neospora caninum* is prevalent in several countries and is increasingly recognized as an important cause of abortion and stillbirth in cattle. For characterizeing the tachyzoite antigens of *Neospora caninum* in aborted cows, sera were obtained from 116 cows which were aborted in the third semester of the pregnancy period and had antibodies to *Neospora caninum* in ELISA. To obtain the protein content of *Neospora*, purified tachyzoites were lysed, electrophoretically separated and blotted to nitrocelloluse membrane for immunostaining. Minimum 9 and maximum 13 protein bands ranging from 10 to 90 kDa were observed after immunostaining. It seems that, in almost all of the cows, two protein bands with a molecular weight of 45 and 41 kDa, have a prominent reaction in Western blotting. According to our findings, these two protein bands are the most important antigens observed after Western blotting, in seropositive aborted cows.

Key words: Abortion, Cow, *Neospora caninum*, Tachyzoite, Western blotting

Introduction

The coccidian protozoan *Neospora caninum* is a parasite of great veterinary importance, which infects different animal species such as cattle, dogs, horses, sheep and goats (Dubey, 1999). Demonstration of *Neospora caninum* antibodies, using different serological techniques such as indirect fluorescent antibody test (IFAT) and different ELISAs, is routinely used to detect infection in adult animals and as a complementary technique in the diagnosis of neosporiosis in aborted foetuses (Bjorkman *et al*., 1994). Identification of immuno-dominant antigens by host sera has proved to be a useful tool in diagnosis of bovine neosporiosis, although it is rarely used in naturally infected animals (Atkinson *et al*., 2000). In naturally infected cattle, proteins with a molecular weight of 25, 65 and 116 kD have been reported to be recognized by sera from cows with confirmed Neospora-induced abortion and aborted foetuses and recognition of three or four immuno-dominant tachyzaoite antigen (17, 29, 30, 37 and 46 kD) by sera from naturally infected cows has been considered as a confirmation of infection (Schares *et al*., 1998). Furthermore, other researchers have suggested the 17 kD immunodominant antigen as a possible antigenic marker, since changes in its recognition could be related to acute infection or reactivation of a chronic infection (Alvarez-Garcia *et al*., 2002). The aim of this study is to compare the pattern of recognition of *Neospora caninum* antigen by SDS-PAGE and Western blot in naturally infected cows.

Materials and Methods

Blood was obtained from 116 cows which aborted in the third semester of the

pregnancy period. Sera were harvested by spinning in 300 g for 10 Min. Sera were alqucated in microtubes and kept at -20°C.

By using a commercial ELISA kit (IDEXX), sera were tested for the presence of antibodies to *Neospora caninum* according to manufacturer instructions. Sera with a positive reaction were conducted to Western blotting.

To obtain the protein content of *Neospora caninum*, purified tachyzoites (4×10^8) of *Neospora caninum* (NC-1 isolate) were incubated in 150 µl of ice cold lysis buffer (pH = 7.2) for 2 h and then were centrifuged at 8500 × g for 5 min at 4°C. Electrophoresis of *Neospora caninum* proteins (2.5 µg/10 µl) were performed in two separate polyacrylamide gel (4% stacking gel and 12.5% resolving gel) in 70 v for 150 min. Proteins were electrophortically transferred to nitro-cellulose membrane for Western blot. To reveal the protein bands silver nitrate staining was used. In the second gel selected for Western blotting, protein bands were blotted on Nitrocellulose membrane (Macherey Negel) by electric current (300 V) for 3 h. The membrane was cut into several strips and immunostaining performed by applying 8 sera (1:100) in PBS-Tween (0.5%) from naturally aborted and infected cows which were diagnosed as positive by a commercial ELISA. Horse radish peroxidase (HRP) conjugated antibodies to bovine Ig diluted 1:2500 in PBS-T were added to the strips for 2 h. After four steps of washing, the reaction was revealed by a precipitant chromogen, Diaminobenzidine (DAB). All the pictures were recorded by a digital camera. Relative factor was calculated for the estimation of molecular weights.

Results

Twenty three of the 116 sera samples had a positive reaction in ELISA. Regardless of the multiple protein bands, two of them with molecular weights of 41 and 45 kDa were obvious in SDS-PAGE after silver nitrate staining (Fig. 1). Minimum 9 and maximum 13 protein bands ranging from 10 to 90 kDa were observed in the strips after immunostaining.

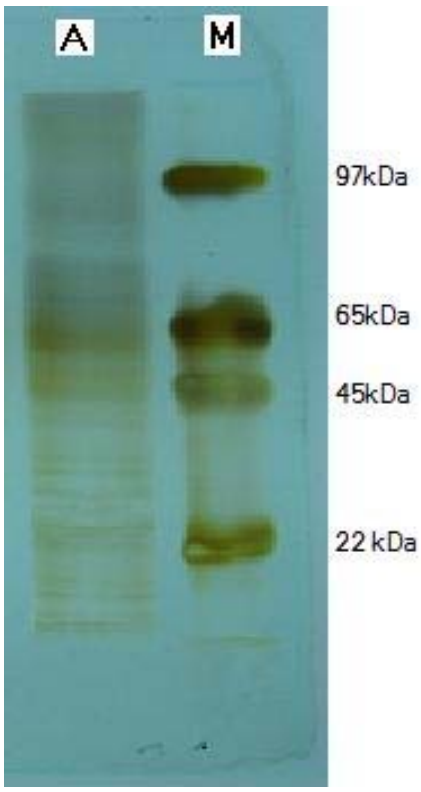

Fig. 1: Protein profile of *Neospora caninum* tachyzoites after SDS-PAGE and silver staining (A) protein size marker (M)

Three patterns of antigen recognition were recorded in Western blotting.

Pattern A: in 11 of 23 strips, the most obvious bands were two antigens with a molecular weight of 41 and 45 kDa.

Pattern B: in 7 of 23 strips, the prominent antigen was a 19 kDa protein and/or 41 and 45 kDa bands were observable.

Pattern C: in 5 of 23 strips, all the bands were pale.

In most of the stained strips, the densities of these two protein bands were more than the other bands. In general 13 strong to weak reactions with molecular weights of 10, 19, 24, 32, 41, 45, 50, 64, 72, 79, and 90 kDa were recorded on the strips.

Discussion

Based on the observations, three patterns of antigen recognition were found in the sera of aborted cows. Besides, two medium molecular weight antigens (41 and 45 kDa) were present in 70% (16 of 23 strips) of the

pre-confirmed (by ELISA) samples. Also, these two protein bands were found with high density in SDS-PAGE. Thus we concluded that both of them might be the most immunodominant antigens in *Neospora caninum* tachyzoites. It must be emphasized that slight differences in antigen recognition observed in each pattern could be due to the host and/or parasite variations. Researchers reported that four immunodominant antigens (17-18, 34-35, 37 and 60-62 kDa) of tachyzoite proteins were identified in infected cows (Bjerkas *et al*., 1994; Lally *et al*., 1996; Alvarez-Garcia *et al*., 2002). Based on the frequency of presence and intensity of recognition, although all of these antigens were recorded in the present work, only two of the aforementioned antigens (41 and 45 kDa), that might represent 34-35 and 37 kDa antigens in that report, were considered immunodominant antigens. These proteins are membrane associated proteins and are located on the surface of the parasite (Bjerkas *et al*., 1994). The difference among molecular weights could be the result of using different techniques for calculation of molecular weight.

Bjorkman *et al*. (1999) reported a 17 kDa protein that belongs to the body part of rhoptries. The rhoptries are club shaped organelles found in infective stages of coccidian and believed to be associated with penetration and early establishment of infection in *Neospora caninum*. A protein with a similar molecular weight (TgMIC10) was also demonstrated by others to be present in tachyzoites of *Toxoplasma gondii* (Hoff *et al*., 2001). Although Alvarez-Garcia *et al*. (2006) recognized the 17 kDa antigen with high intensity in all of the positive samples examined, we observed the antigen (with molecular weight of 19 kDa) in 17 of 33 positive samples.

The reason for this discrepancy might be due to: 1) occurrence of the abortion in the chronic phase of the infection, as Hoff *et al*. (2001) reported the lower level of expression of the protein in bradyzoites, 2) fluctuation of antibody concentration during pregnancy (Alvarez-Garcia *et al*., 2006) and more importantly, 3) the presence of co-infection, since previous exposure to different antigens, specially those from closely related coccidian species such as *Toxoplasma gondii*, could interfere the pattern of antigen recognition in neosporosis. In fact, the best way to study the pattern of antigen recognition in neosporosis is to pre-exclude the presence of such exposures.

We found no relationship among the different patterns of antigen recognition and age of cows or semester of abortion (data not shown). However, to the best of our knowledge, 41 and 45 kDa protein bands are the most important antigens, observed after Western blotting, in seropositive aborted cows.

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References

Alvarez-Garcia, G; Lopez-Perez, I; Innes, E; Collantes-Fernandez, E; Fernandez-Garcia, A; Gomez-Bautista, M and Ortega-Mora, LM (2006). Use of an immunodominant P17 antigenic fraction of *Neospora caninum* in detection of antibody response in cattle. Mem. Inst. Oswaldo Cruz., 101: 529-534.

Alvarez-Garcia, G; Pereira-Bueno, J; Gomez-Bautista, M and Ortega-Mora, LM (2002). Pattern of recognition of *Neospora caninum* tachyzoite antigens by naturally infected pregnant cattle and aborted foetuses. Vet. Parasitol., 107: 15-27.

Atkinson, R; Harper, PAW; Reichel, MP and Ellis, JT (2000). Progress in serodiagnosis of *Neospora caninum* infections of cattle. Parasitol. Today. 16: 110-114.

Bjerkas, I; Jenkins, M and Dubey, JP (1994). Identification and characterization of *Neospora caninum* tachyzoite antigens useful for diagnosis of neosporosis. Clin. Diagn. Lab. Immunol., 1: 214-221.

Bjorkman, C; Lunden, A; Holmdahl, J; Barber, J; Tress, AJ and Uggla, A (1994). *Neospora caninum* in dogs: detection of antibodies by ELISA using an iscom antigen. Parasite Immunol., 16: 643-648.

Bjorkman, C; Näslund, K; Stenlund, S; Maley, SW; Buxton, D and Uggla, A (1999). An IgG avidity ELISA to discriminate between recent and chronic *Neospora caninum* infection. J. Vet. Diagn. Invest., 11: 41-44.

Dubey, JP (1999). Recent advances in *Neospora* and neosporosis. Vet. Parasitol., 84: 349-367.

Hoff, EF; Cook, SH; Sherman, GD; Harper, JM; Ferguson, DJP; Dubremetz, JF and Carruthers, VB (2001). *Toxoplasma gondii*: molecular cloning and characterization of a novel 18-kDa secretory antigen, TgMIC10. Exp. Parasitol., 97: 77-88.

Lally, NC; Jenkins, MC and Dubey, JP (1996). Evaluation of two *Neospora caninum* recombinant antigens for use in an enzyme-linked immunosorbent assay for the diagnosis of bovine neosporosis. Clin. Diagn. Lab. Immunol., 3: 275-279.

Schares, G; Peters, M; Wurn, R; Barwald, A and Conraths, FJ (1998). The efficiency of vertical transmission of *Neospora caninum* in dairy cattle analysed by serological techniques. Vet. Parasitol., 80: 87-98.