Global Veterinaria 7 (2): 149-152, 2011
ISSN 1992-6197

Adaptation of Dot-Elisa for Serodiagnosis of *Neospora caninum* Infestation in Aborted Cows

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Abstract: *Neospora caninum* is a protozoan parasite that is considered a major cause of abortion in dairy cattle. Several serologic tests can be used to detect *N. caninum* antibodies including ELISA, the indirect fluorescent antibody test (IFAT) and the direct agglutination test. However there is a lack of a quick method for detection of this parasite. This study was done for developing a dot-ELISA as a test for diagnosis or screening of cattle. Blood samples were collected from 32 aborted cows. All the sera were tested for serodiagnosis of *Neospora caninum* by using a commercial ELISA (*Neospora caninum* antibody test kit, IDEXX, US). An in house dot-Elisa was developed and the some sera were conducted to this assay. Results clearly show good agreement between dot-Elisa and commercial ELISA. The relative sensitivity and specifity of dot-Elisa were 71% and 100% respectively.

Key words: Dot – ELISA · *Neospora caninum* · Serodiagnosis · Aborted · Cow

INTRODUCTION

Neospora caninum is a protozoan parasite that is considered a major cause of abortion in dairy cattle and has been identified from many parts of the world including North America, Mexico, Europe, Asia, Africa, Australia and South America [1]. The protozoa affect both dairy and beef cattle. Based on serologic surveys, up to 100% of cattle in some herds have been exposed to *N. caninum*. As stated earlier, major effects of N. caninum infections in cattle is abortion and in some geographic regions up to 42.5% of abortions are attributable to neosporosis. The economic impact will depend on the indirect costs, as well as on the value of fetuses lost. Dogs can serve both as intermediate and definitive hosts. Clinical signs have only been reported in individual calves younger than two months of age. Cows of any age may abort from 3 months of gestation to term. Most *Neospora*-induced abortions occur at 5–6 months of gestation. Fetuses may die in uterine, be reabsorbed, mummified, autolyzed, stillborn, born alive but diseased or born clinically normal but chronically infected. Within herds, abortions may be clustered, sporadic or epidemic. Presence of specific antibodies in serum from an aborted cow is only indicative of exposure to *N. caninum* [2]. Several serologic tests can be used to detect *N. caninum* antibodies including ELISA, the indirect fluorescent antibody test (IFAT) and the direct agglutination test. However there is a lack of a quick method for detection of this parasite [3].This study was done for developing a dot-ELISA as a test for diagnosis or screening of cattle.

MATERIALS AND METHODS

Animals and Samples: Blood samples were collected from 32 cows which aborted in third semester of pregnancy period from different herds during summer and winter of 2010 in vicinity of Tabriz (North West of Iran). Sera harvested by centrifugation at 2000 rpm for 20 minutes and kept in -20°C until test.

ELISA for Detection of Antibodies to *Neospora caninum*: All the sera were tested for serodiagnosis of *Neospora caninum* by using a commercial ELISA (*Neospora caninum* antibody test kit, IDEXX, US).To estimate the antibody titer, the positive sera were applied in the same ELISA with different dilutions: 1:100, 1:200 and 1:400.

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Antigen Preparation: Tachyzoites of Neospora 5×10^6 (NC-1strain) were lysed by a buffer (0.15 M Nacl, 0.05 M Tris-HCl pH=7.2,0.1% sodium dodecyl sulphate,1% Triton X-100, 0.1% Sodium deoxycholate, 10^{-4} M PMSF) after a short spinning (8000 g at room temperature for 2 minutes) the supernatant (500 µl) aliquted in 50 µl volumes and stored at -20°C. We considered each µl of the antigen preparation equivalent to the protein content of 10^4 tachyzoites [4].

Dot-ELISA: Antigen preparation with different protein content equivalent to 1×10^4, 5×10^4 and 1×10^5 tachyzoites in volume of 5 µl were load in the predefined points on nitrocellulose membrane (Macherey–Nagel, Porablot, Germany). The membranes were cut and let to dry at room temperature for 10 minutes. Blocking was done by adding a 3.5% skim milk powder (in PBS) for 1 h at RT. After three washes in 0.05% PBS-Tween (PBS-T), different dilutions of sera (1:50, 1:100, 1:200) were added to the membranes and incubated at 37°C for 2 h. After three washes with PBS-T the membranes were incubated with peroxides-conjugated rabbit anti cow IgG (Koma biotech, S. Korea) for 2 h at 37°C. The reaction was revealed by adding TMB substrate in a dark place. After an appropriate time, the reaction was stopped by adding plenty of PBS-T. The final color reaction was recorded by digital camera.The results of this test categorized qualitatively by a blind person as: +, ++ and +++ according to the density of precipitated color. By assuming the commercial ELISA as a reference test, the relative sensitivity specify, positive and negative predictive value (PV) of the test calculated according to the following equations:

Relative sensitivity% = (# positive by both methods) / (# positive by both methods + # positive by reference test and negative by the method being compared with the reference test) ×100

Relative specifity% = (# negative by both methods) / (# negative by both methods + # negative by the reference test and positive by the method being compared with the standard) ×100.

$$PV_+ = [TP \div (TP+FP)] \times 100$$
$$PV_- = [TN \div (TN+FN)] \times 100$$

In order to evaluate the agreement between two diagnostic methods (ELISA and dot-ELISA), Kappa measurement (SPSS, USA) was employed. The Kappa statistic varies between 0 (change agreement) and 1 (prefect agreement).

Table 1: Comparison of positive results of commercial ELISA wit dot-ELISA in different dilution of sera

Positive sera by commercial ELISA	Different dilution of sera											
	1:50			1:100			1:200			1:400		
1	+++		+++		1.49	++		1.02	+		0.53
2	+++		++		0.76	+		0.36	-		0.11
3	+		+/-		0.31	-		0.18	-		0.05
4	-		-		0.26	-		0.07	-		0.05
5	+++		++		1.19	++		0.73	+		0.33
6	-		-		0.23	-		0.09	-		0.04
7	+		+		0.31	-		0.10	-		0.6
Positive control serum of commercial kit	No data		+/-		0.23	-		0.08	-		0.04

Table 2: Comparison of the intensity of reaction, using different concentration of tachyzoites

	Different antigen preparations equivalent to protein content of:		
Sera No.	1×10^4 tachyzoites	5×10^4 tachyzoites	1×10^5 tachyzoites
2 (1:100)	+++ 	+++ 	++ 
7 (1:50)	+ 	+ 	+ 

RESULTS

Commercial ELISA: Neospora caninum antibody test kit showed that 7 of 32 (20%) sera from aborted cows had a positive reaction. The remaining (25 of 32) had negative results.

Dot-ELISA: The results clearly shows, even the test with an antigen preparation equivalent to 1×10^4 tachyzoites can detect the antibodies to *Neospora caninum* in different dilution of serum (Table 1).Comparison between commercial ELISA and dot–ELISA revealed that of 7 sera that tested positive by commercial ELISA (with recommended dilution of kit, 1:100), 5 were also positive by dot-ELISA. The rest (2 sera) were considered negative by dot-ELISA even with dilution 1:50 of the sera.

There was no difference ($p<0.01$) among various antigen preparation in regard to the obtained results with the constant dilution of positive sera (No. 2 and No. 7) (Table 2). So the antigen preparation equivalent to protein content of 1×10^4 tachyzoites was considered for the rest of the study.

In dilution 1:200, 3 and 2 of 7 positive sera were positive in commercial ELISA and dot-ELISA respectively. But in highest dilution (1:400) the results were the same with both tests. What were showed to be negative in commercial ELISA (25 cases) were also revealed to be negative by dot-ELISA. The relative sensitivity and specifity of dot-ELISA were 71% and 100% respectively. Positive and negative predictive values estimated as 71% and 92% respectively.

DISCUSSION

Currently the diagnosis of neosporosis is based mostly on Enzyme Linked Immunosorbent Assay (ELISA) and Indirect Fluorescent antibody Test (IFAT). Since ELISA has quantities results and can cover a large number of samples simultaneously it has same advantages over other immunoassay such as Western blotting and IFA. Although dot-ELISA has the disadvantage of being qualitative, a tiny amount of reagents are used in the assay and in other word upon the optimization of the assay it could be applied for a large number of samples [2].

We tried to establish a dot-ELISA for detection of antibodies against *Neospora caninum*. Meanwhile, the results of the established test were compared with a commercial ELISA, using a panel of same sera from aborted cows. Purified *Neospora caninum* (NC-1strain) (in Vero cell line) was used as for antigen. Preparation of whole antigen of tachyzoites was blotted on the nitrocellulose papers with considering the serum dilution of 1:100. The dot-ELISA which was developed in this study has a sensitivity of 95% and specifity of 88% in comparison to the results of the commercial ELISA as a reference test. Regarding the above mentioned dilution (1:100), the results of the present work are in line with the results of Pare *et al*. [8] who developed ELISA for detection of antibodies against *Neospora caninum* in dog's blood.

The prominent antigens from the view point of chromogenic reaction and intensity were two protein bands of 41 and 45 KDa molecular weight and based on the results of western blotting; three pattern of antigen recognition was observed by the same sera of aborted cows, which was applied in the present research. All of five positive sera in dot-ELISA had the ability to react with different protein bands of the tachzoites ranging from 10 to 90 KDa [5, 6].

At the time of the manuscript preparation there is no data available regarding the negative and positive predictive values calculation in literature. The negative and positive predictive values are 92% and 71% respectively, which indicated a strong correlation between dot-ELISA and commercial ELISA. The dot-ELISA developed for the detection of canine neosporosis presented 71% sensitivity and 100% specificity**.** These specificity and sensitivity demonstrate the adequacy of this standardized test for the screening of neosporosis in

the veterinary medicine [7]. Sensitivity and specificity of 98 and 96%, respectively, have also been reported for other tests such as the Immunostimulatory Complex (ISCom) ELISA [3] and of 89 and 97% for ELISA using crude antigen [8]. Other studies reported a sensitivity ranging from 92 to 98% and a specificity ranging from 87 to 100% for the same antigen preparation used in another system [9-11].

Calculated Kappa value between (0.72%) dot-ELISA and commercial ELISA indicated a good agreement in 1:250 and 1:200 dilution of serum when 1×10^4 lysed tachyzoites were spotted on the nitrocellulose paper. In other word by applying the lowest number of the tachyzoites the results of dot-ELISA is still comparable to commercial ELISA and it is helpful for lowering of the cost of the test.

Positive predictive values of dot-ELISA shows infected animals with *Neospora caninum* which have positive reaction in dot-ELISA. Based on the calculated PV_+, it might be calculated that using dot-ELISA will lowering the false positive results and it might be helpful for developing a screening assay for detection of the antibodies to *Neospora caninum* in cows in the areas which the frequency of the disease is low.

In summary, the available commercial ELISA which was used in this study could be replacing with dot-ELISA which was described in the present report.

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