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Original Article

Development and Standardization of Dot – ELISA for Detection of *Neospora caninum* Antibodies in Cattle and Comparison with Standard Indirect ELISA and Direct Agglutination Test (DAT)

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Abstract

Background: *Neospora caninum* is a protozoan parasite from phylum apicomplexa and an important agent causing abortion in cattle which produce notable economic loss all around the world.

Methods: Dot-Elisa was set up performing checker board procedure and then 178 sera of cattle examined with commercial indirect ELISA and direct agglutination test (DAT) were also evaluated by dot-ELISA afterwards.

Results: Kappa statistical analysis revealed that Dot-ELISA has good agreements with ELISA as well as the DAT and also, Mc Nemar´s analyzing showed that this procedure has acceptable ability to discriminate positive results. Relative sensitivity and specificity of Dot-ELISA were respectively 92.63% and 89.16% and 93.4% and 90.8% in comparison with ELISA and DAT.

Conclusion: Since the dot-ELISA is easy, inexpensive and not needed high experience to interpret the results, it is superior to ELISA and DAT when we aim to screen the cattle on the farm and slaughterhouses or when the laboratory equipment is not available**.**

Introduction

*N*eospora caninum (Apicomplexa: Eimeriina: Sarcocystidae) is a cyst forming protozoan parasite closely related to *Toxoplasma gondii*. The parasite is one of the most important etiological agents of abortion and neonatal mortality in cattle worldwide (1-3). First recognition of the parasite was in 1984 when Bjerkas et al. reported unidentified sporozoan protozoa in dogs in Norway (4).

Confirmed definitive hosts of *N. caninum* are dogs and coyotes, but other wild canids may act a role on this issue. The parasite infects a wide range of warm-blooded animals including dogs as intermediate host (5-7). *Neospora caninum* may be transmitted to intermediate hosts following ingestion of oocysts via contaminated feed or water or may be passed vertically from mother to fetus via the placenta (5). Abortion is the only observed clinical sign in adult cows. *N. caninum* abortions can occur at any time of gestation but the majority of abortions are at 5-6 months of gestation (8, 9).

There are three infection stages of the parasite: tachyzoites, bradyzoites, and sporozoites. Tachyzoites and bradyzoites occur in tissues of intermediate hosts whereas sporozoites are present in oocysts that are excreted in the feces of the definitive hosts (10).

At present, serological assays including Direct agglutination test (DAT) using preserved whole tachyzoites and the ELISA have been evaluated as high sensitive and specific tools for diagnosis of *N. caninum* (1, 11).

Since, there are little informative details in the literatures about validation of dot-ELISA for detection of antibodies against *N. caninum* in naturally infected cattle for epidemiological and screening purposes, thus the presented study aimed to develop a dot-ELISA for diagnosis of anti- *N. caninum* antibodies in naturally acquired cattle and to estimate the value of the test in comparison with commercially available ELISA and DAT.

Materials and Methods

Parasite

Neospora caninum tachyzoites were prepared in Razi Institute, Shiraz branch, Iran. Nc-1 strain, were grown in monolayers of Vero cells cultured in RPMI 1640 medium (Sigma Co., USA) supplemented with2% of fetal calf serum and penicillin (10,000 U/ml), streptomycin (100 g/ml) and amphotericin B (25 g/ml) at 37 ℃ with 5% CO_2 in tissue culture flasks. Tachyzoites were harvested by scraping the monolayer when 80% of the cells infected and then purified by sequential passage of the cell monolayer through a 27-gauge needle and centrifuged to remove host cell debris. The supernatant was collected, centrifuged and washed twice in phosphate-buffered saline (PBS, pH 7.2).

Serum Samples

One hundred seventy eight serum specimens from cattle were evaluated. Blood samples were randomly collected from husbandry farms in Fars Province, south of Iran. The abortion history of the animals was unknown. Blood samples were centrifuged at 1000×g and then the supernatants were frozen at −20°C until the examinations have performed (12).

ELISA

ELISA was utilized according to the manufacturer's instructions (IDEXX, USA), the test samples were first diluted 1:100 with a sample diluent. Then, 100 µl of negative and positive controls and samples were dispensed into wells. The plate was incubated for 30 min at room temperature. After that, each well was washed with approximately 300 µl of phosphate buffered wash solution four times, and then 100 µl of anti-bovine conjugate was dispensed into each well. After incubation for 30 min at room temperature, the washing of each well was done. The volume of 100 µl of TMB

substrate solution was dispensed into each test plate well and incubated for 15 min at room temperature then 100 µl of stop solution has dispensed into each well to stop the reaction. The absorbance was measured and recorded at 650 nm by the ELISA reader.

In order to the assay to be valid, the difference between the positive control mean (PCx) and the negative control mean (NCx) should be greater than or equal to 0.15. In addition, the NCx must be less than or equal to 0.20. The presence or absence of antibody to *N. caninum* was determined by sample to positive (S/P) ratio.

S/P= *sample – NCx / PCx - NCx*

Each sample with S/P ratio below 0.50 was classified as negative for *N. caninum* antibodies. If the S/P ratio was greater than or equal to 0.50, the sample was classified as positive for *N. cani*num antibodies.

DAT

IgG antibodies were also assayed by Direct agglutination test based upon the direct agglutination of fixed tachyzoites of *N. caninum*, incorporating 2-mercaptoethanol to prevent non-specific IgM agglutination (13). Sera were started at 1:10 serum dilution for *N. caninum*. A titer of 1:20 and higher was indicative for *N. caninum* infection in cattle. A complete agglutination was considered as positive result. Clear and cut button-shaped was interpreted as a negative reaction.

Sera were also tested for the presence of anti-*T. gondii* antibodies using the modified agglutination test (MAT) with sera pre-treated with 2 mercaptoethanol (14-16).

Dot-ELISA

To set up the suitable concentration of antigen and the best dilutions of sera and conjugate, checkerboard procedure using positive and negative controls was performed. Positive and negative controls had been prepared in another performed work by authors of this manuscript. These sera had been confirmed by two commercial ELISA kits (IDEXX and IDVET) and also indirect fluorescent antibody test (IFAT).

Antigens were dotted onto strips of nitrocellulose membranes (Cut into 0.7 cm ×5 cm) on square zones and after air drying, fixed on PBS for 30 minutes. The dismounted free binding sites of membranes were blocked with skim milk in 0.05% PBS-Tween (pH=7.4) for 60 minutes at 37 °C. Sera were diluted in PBS-T and incubated with the strips for 60 minutes at 37 ^{0}C with constant shaking and then washed four times with 0.05% PBS-Tween. After that, rabbit anti - bovine IgG peroxidase conjugate (Sigma) was added in incubation circumstances same as previous stage and also washed as described. The strips were soaked in 5 mL of a chromogen substrate consisted of 6 mg of 4-chloro-α-naphthol diluted in 2 mL of absolute methanol, 5 µL of 100-vol hydrogen peroxide and 10 mL of TBS for 10 minutes and at last the reaction has stopped by washing with distilled water. A clearly defined bluish spots indicated as positive. No or very pale colored dot demonstrated the negative reactions (Fig. 1).
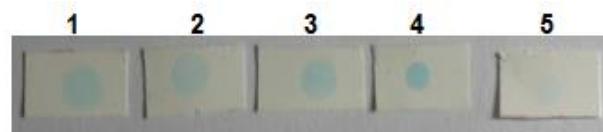

Fig.1: 1-4 reveal Positive reactions of Dot-ELISA and Negative reaction revealed in 5 as a very pale bluish dot

Statistical analyzes

Kappa statistical test (*k*) was utilized to assess the agreement between Dot- ELISA and two other mentioned procedures. Strength of agreements was evaluated as follows: <0.2 = slight agreement; 0.2–0.4 = fair; 0.4–0.6 = moderate; 0.6– 0.8 = substantial; >0.8 = almost perfect (17). Mc Nemar´s analyzing was used for detection the agreement of Dot-ELISA with ELISA as well as the DAT to reveal the positive results. Revealed in Table 2, sensitivity and specificity of Dot- ELISA in

comparison with ELISA and DAT have also calculated. Sensitivity was calculated as the percentage of positive sera in the Dot- ELISA that had also been positive by the ELISA. Specificity was also defined as the percentage of negative sera in ELISA that were also negative in the Dot- ELISA. For measuring the sensitivity and specificity of Dot- ELISA in comparison with DAT, these calculations were also performed according to DAT results (18).

Results

Using positive and negative serum samples, 2500 whole tachyzoites per microliter, the dilution of 1/20 for sera and 1/1000 for conjugate were determined as the best amounts on dot-ELISA. Based on these settings, out of 178 examined sera 54.4% were positive for *N. caninum* antibodies whereas 53.3% and 56.1% were positive utilizing ELISA and DAT respectively (Table 1). Only two samples reacted with *T. gondii* using the DAT.

According to the Kappa statistical analysis, Dot-ELISA had good agreement with ELISA as well as the DAT. Furthermore, Mc Nemar´s analyzing revealed that Dot-ELISA has acceptable potential to discriminate the positive results.

Relative sensitivity and specificity of Dot-ELISA were respectively 92.63 and 89.16% in comparison with ELISA and 93.4% and 90.8% compared with the DAT (Table 2).

Table 1: Occurrence of anti-*N. caninum* antibodies in examined cattle using dot-ELISA, ELISA and DAT

	Dot-ELISA N(%)	ELISA N(%)	DAT N(%)
Positive	97 (54.4)	95 (53.3)	100 (56.1)
Negative	81 (45.6)	83 (46.7)	78 (43.8)
Total	178	178	178

Table 2: Relative sensitivity and specificity of Dot- ELISA in comparison with standard ELISA and DAT

Dot-ELISA	ELISA		Dot-ELISA	DAT	
	Positive	Negative		Positive	Negative
Positive	88 (a)	9 (b)	Positive	85 (a)	8 (b)
Negative	7 (c)	74 (d)	Negative	6 (c)	79 (d)
Total	95 (a + c)	83 (b + d)	Total	91 (a + c)	87 (b + d)
Sensitivity%	92.63			93.4	
Specificity%	89.16			90.8	

Sensitivity = a/ (a + c); Specificity = d/ (b + d)

Discussion

The main aim of most diagnostic procedures is diagnosis of the infections and screening or epidemiological studies. For instance, *Neospora*-associated abortions are mainly identified by examination of the aborted fetus or fetal fluids by immunohistochemistry or PCR (19-21). However, in many situations when the aborted fetuses are not available for screening and or epidemiologic studies, serological investigations in herd populations are methods of choice. Many serologic procedures including ELISA, indirect fluorescent antibody test (IFAT) or DAT and etc. are just carrying out in laboratory sites but some procedures such as dot-ELISA are performable in both laboratory and field (22).

Currently, several serologic tests have established to detect anti-*N. caninum* antibodies in cattle or other hosts (23-27) but there is a little information about dot-ELISA for this pur-

pose. Dot- ELISA has a good potential to diagnose the neosporosis in aborted cattle with acceptable sensitivity and remarkable specificity (28).

In the presented study, we aimed to assess the dot-ELISA in comparison with traditional ELISA and the DAT which does not require species-specific conjugates and is utilized as routine procedure in seroepidemiology of neosporosis in various hosts (29).

In our study, the DAT was slightly better than ELISA and dot-ELISA to detect positive results but, the differences between three performed procedures to identify the positive reaction against *N. caninum* was not statistically significant and dot-ELISA revealed a good and acceptable competence to distinguish the positive results in comparison with ELISA and DAT.

Detected high sensitivity and specificity of dot-ELISA in our study to identify the naturally infected cattle with *N. caninum* clarified that this test could be considered as a useful tool. Nine sera which revealed negative reactions by ELISA had positive results utilizing dot-ELISA maybe due to cross-reactive antibodies. From those nine ELISA negative sera, only 1 had DAT positive result to *N. caninum* and no reaction to *T. gondii* were seen using the MAT. These results also, replicating a good sensitivity of dot-ELISA to detect the anti- *N. caninum* antibodies and to discriminate them from *T. gondii* as a highly cross reacting agent with this parasite.

DAT has 100% sensitivity and 97% specificity in comparison with the ELISA (30). Therefore, it seems that this situation is due to false-positive reactions in testing sera with dot-ELISA. Pinheiro et al. reported 94% sensitivity and 73% specificity for dot-ELISA for detection of anti-*N. caninum* antibodies in dogs in comparison with IFAT at a cut-off point of 1:50 (31).

One of the preferences of the ELISA and DAT is that a huge number of animal sera can be examined simultaneously and in a same condition and maybe it interprets some false

positive results of dot-ELISA. On the other hands, ELISA is often expensive tool and requires an ELISA reader and also huge numbers of tachyzoites are needed for the DAT.

The accuracy of the ELISA as well as the DAT in this condition is critically important because the evaluation of dot-ELISA was dependent upon the accuracy of these methods.

In our study, overall prevalence of anti - *N. caninum* antibodies was 54.4%, 53.3% and 56.1% using Dot-ELISA, ELISA and DAT respectively. These findings indicate that the variations in different screening studies are affected with either applying different serologic tests or detecting variable cut-off points. Thus, there are serious difficulties for comparing the prevalence among different studies. In Iran, prevalence of neosporosis in cattle reported 38.8% in Tehran (32), 21% in Ahvaz (33), 10.5% in Tabriz (34), 46% in Mashhad (35) and 12.6% in Kerman (36). Therefore we can conclude that this disease can cause serious problems in our district especially when occur simultaneously with other infectious diseases. For instance, 9.4% of dogs in Meshkin-Shahr infected with *N. caninum* had afflicted with *Leishmania infantum* infection (37).

Conclusion

We suggest that the dot-ELISA test may be superior to ELISA when we aim to screen the cattle on herd populations and slaughterhouses or when the laboratory equipment is not available. This procedure is easy, inexpensive and do not need high experienced person to read the results. Also, since the DAT needs enough time and elucidating the results of this test is difficult, the dot-ELISA could be considered as alternative.

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this MSc. research. The authors also declare that there is no conflict of interests.

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