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▣ BRIEF COMMUNICATION

Development of a Disperse Dye Immunoassay Technique for Detection of Antibodies against *Neospora caninum* in Cattle

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Abstract: In this study a disperse dye immunoassay method was standardized and evaluated for detection of antibodies against *Neospora caninum* in cattle. Sera from 150 cattle with a recent history of abortion were collected and tested by commercial ELISA kit and a standardized in-house dye immunoassay system. The positivity rate for the sera used in this study was 34.6% for the disperse dye immunoassay (DDIA) compared to 32% obtained by ELISA kit. This study showed no significant difference between DDIA and ELISA. The results indicated that the DDIA provide an economic, simple, rapid and robust test for detection of *N. caninum* infection in cattle.

Key words: *Neospora caninum*, Cattle, Dot dye immunoassay, Colloidal dye

The protozoan parasite *Neospora caninum* is a major cause of abortion in cattle worldwide [1,2]. *N. caninum* was first diagnosed in litter of dogs in Norway in 1984 [3]. It has been found in various animal species such as dogs, cattle, horses, sheep and goats [4]. The diagnosis of neosporosis can be confirmed by immunohistochemical identification of parasite in tissue sections and by demonstration of specific antibodies in serum. Several assays are available for detecting antibodies to *N. caninum* in cattle, which are mainly based on detecting antibodies against tachyzoite antigens [4]. The IFAT (Indirect Fluorescent Antibody Test) has been used in surveys for antibody detection raised against *N. caninum* in dog. Although IFAT is a specific test, but it is time consuming when a large number of sera is being analyzed [5,6]. ELISA (Enzyme Linked Immunosorbent Assay) also is known as a sensitive and specific technique for serological surveys [7-9], however, this test requires an equipped laboratory, with proper instruments and trained technicians. In contrast, colloidal dye immunoassay provides an economic, simple and rapid approach for the immunoassay, and does not require specialized training or equipment. Moreover, the components are stable without any need for refrigeration [10-13]. In this study, an indirect dye test was developed to analyze antibodies against *N. caninum* in cattle.

Blood samples were collected from a total of 150 cows with a history of abortion in different period of pregnancy from different dairy farms. Serum samples were stored at -20°C until use. The *N. caninum* NC-1 isolate, was obtained from Razi Vaccine and Serum Research Institute, Shiraz, Iran, which was cultured in Vero cell line with fresh DMEM medium (Sigma Co., USA) and 2% fetal calf serum. DMEM medium was supplemented with an antibiotic/antimycotic solution, containing Penicillin (10,000 U/ml), Streptomycin (100 µg/ml), and Amphotericin B (25 µg/ml) (Invitrogen, USA). The cells were incubated with 5% CO_2 and observed daily. Tachyzoites were harvested when more than 80% of the Vero cells were infected. Then the suspended parasites were pelleted by centrifugation with 1,800 g for 15 min and passed through a 27-gauge needle to rupture the host cells. Finally, the tachyzoites were separated from the host cell debris by centrifugation in a 40% Percoll density gradient. Cell-free tachyzoites were pelleted (800 g for 20 min), washed three times in PBS, resuspended in sterile distilled water, and sonicated for six 30-s pulses (MSE Soniprep 150, SANYO, Japan). Cell debris and intact cells were removed by centrifugation (1,000×*g* for 20 min at 4°C) [14,15]. Disperse dye, BLUE SP (DNBS), was provided from DA-YU Chemical Co. (Taiwan ROC). Rabbit anti-bovine IgG was purchased from Sigma. A nonenzymatic detection reagent was prepared by conjugation of anti-bovine IgG antibody to colloi-

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dal dye particles as described previously [16]. Dye particle suspensions were prepared using a washing/centrifugation procedure. The concentration of dye in the aqueous solution used for antibody conjugation was monitored at its optimum wavelength (λ max) and expressed as the multiples of dye concentration corresponding to an A (absorbance at λ max) = 1. To determine optimum concentration; various concentrations were tested. Ten μg of the antibody per one milliliter dye was mixed and shaken in a 37℃ incubator for 1 hr. The antibody and colloidal dye conjugate was stored at 4℃ until use. To optimize the DDIA, dye-antibody conjugates (various ratios of dye and rabbit anti-bovine antibody) were used to detect the antibody presence in the bovine's sera in order to select the most effective combinations of dye and secondary antibody. To analyze the sensitivity and specificity of the assay, parallel test was applied to compare the results of DDIA and ELISA kit (iscom ELISA kit, Svanova Biotech AB, Sweden). The procedures of antibody detection using DDIA are described as follow. The nitrocellulose membrane was divided into 3 segments for suspected serum, positive and negative control 2 μl of *N.caninum* soluble antigen was dotted on suspected and 2 μl of uninfected Bovine serum was dotted on positive control segment of nitrocellulose membrane. Dots were allowed to dry on the NC strips. The non specific binding sites on the NC surface were blocked with 2% BSA for 1 hr and then washed 3 times with tween 20 solution in PBS (0.5 ml Tween-20 per 1 L PBS). The strips were placed in serum samples which were diluted 1:50 with PBS for 1 hr. The strips were then washed 3 times with PBS tween 20 and were put in dye/antibody reagent for 45 min. The blue color dot on suspected segments was considered as positive.

To determine the optimal conditions, various dye concentrations were used to link the dye and antibodies in the conjugation procedure. The optimum concentration was A (λ max) = 15. The procedure of dye-antibody conjugation should be carefully optimized to achieve high sensitivity and specificity in the assay. According to our results the pH values around 7 the temperature at 37℃ and the dyeing incubation time of 30 min were the most suitable conditions for dyed-antibody preparation. Out of 150 field serum samples collected from cattle, 39 (26%) were positive in DDIA and ELISA tests and 91(66.6%) were negative in both tests. The kappa statistic and apparent correlation rate were used to measure the strength of agreement between the results of the tests by the ELISA and DDIA. A kappa statistic value of >0.75 represents excellent agreement between the results [17]. In the method we used in our study good agreement (Kappa = 0.697) between the prevalence estimated by the DDIA and ELISA tests was found (Table 1).

Table 1. The comparison of DDIA and ELISA for the detection of antibodies against *N. caninum* in cattle

ELISA results	DDIA results		
	No. of positive (%)	No. of negative (%)	Total (%)
No. of positive (%)	39 (26.0)	8 (33.5)	47 (33.3)
No. of negative (%)	12 (8.0)	91 (66.6)	103 (66.6)
Total (%)	51 (34.0)	99 (66.0)	150 (100)

Due to the fact that *N. caninum* is closely related to *Toxoplasma gondii* and the potential cross-reaction might occur the DDIA was evaluated by using 5 bovine confirmed *T. gondii* seropositive samples previously diagnosed by ELISA and IFA tests. The result of DDIA showed no cross reaction with bovine Toxoplasma positive sera.

N. caninum is associated with abortion and reproductive loss in cows worldwide. Hence researchers have developed a number of diagnostic tests to investigate the epidemiology of infection and disease. The different type of ELISA techniques are mostly used world-wide for the diagnosis of *Neospora* infection in cattle [18,19]. Despite the high specificity and sensitivity of microplate ELISA tests, the use of these methods requires an ELISA reader which is an expensive instrument which is not available in many underdeveloped areas of the world. Moreover, ELISA procedure is time consuming, requires technical skills and experience to run. In contrast, the DDIA requires only non-expensive commercially available materials and reagents with long half-lives and the test can be assessed visually [20]. Serological investigation for several infection agents was recently accomplished using a dipstick colloidal dye immunoassay [21-26]. The dipstick dye is a variation of labeled immunoassay that uses nitrocellulose membrane as a support and colloidal dye as the label for the rapid reaction of Ag and Ab. In this study, a DDIA strip test for the detection of *N. caninum* infection in cattle was developed. We successfully applied the blue color colloidal dye particles to label rabbit anti-bovine IgG to detect IgG antibodies against *N. caninum*. Comparing the developed DDIA with commercial ELISA kit, there was no significant difference between the two tests, but the DDIA was faster and much easier to perform than the commercial ELISA kit and also more suitable for use in the field. The dye antibody suspension, with dye concentration at A (λmax) = 15 giving the highest color intensity without back-

ground was chosen. It is suggested that at a lower dye concentration, only little amount of immuno-protein were dyed which resulted in a lower color intensity on the strip. On the other hand, at a higher dye concentration the epitopes of the antibody might be shielded by dye molecules which cause antibody inactivation. Also, in the higher dye concentrations, the aggregation of dyed-antibody may occur, which could result in background of NC membrane during the assay process [28]. It is also observed that higher color intensities were obtained when dyeing was conducted with a longer incubation time. The DDIA which utilize rabbit anti bovine IgG-colloidal dye conjugates as a visualizing agent may also be used for detection of specific antibodies in other infectious diseases with specific detectable antigens. The DDIA was also associated with other advantages such as easy conjugate preparation and test performance. Where costs are an issue, and in the absence of more elaborated diagnostic tests in many developing countries, simple tests such as DDIA could still deliver reliable information that could monitor individuals and/or herds infected with *N. caninum*. To our best knowledge this is the first report of DDIA for the detection of *N. caninum*.

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