

COMPARATIVE EVALUATION OF ANTIBODY POSITIVE TITER BY ELISA AND IFA IN *THEILERIA ANNULATA* VACCINATED CATTLE IN IRAN

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Summary:

An enzyme linked immunosorbent assay (ELISA) was used to evaluate antibody positive titer in vaccinated and non-vaccinated cattle using schizont infected myeloid cells as an antigen. The result was compared with indirect fluorescent antibody level in the same animals. For this study 116 milking cows, 95 vaccinated and 21 non-vaccinated, were bled in order to prepare sera. They were tested with both ELISA and IFA tests. 94 sera had positive antibody titer and 22 sera were negative through ELISA test but, with IFA test, only 89 sera showed positive antibody titer and 27 were negative. Thereby, it was concluded that the sensitivity and specificity of ELISA test in comparison with IFA test was 95.5 % and 66.6 % respectively. This study generally indicated that ELISA could be an effective test for sero-epidemiological investigations of bovine tropical theileriosis, and it is considered to be valid as an additional test to distinguish the vaccinated from the non vaccinated cattle in order to schedule vaccination programs.

KEY WORDS : *Theileria annulata*, ELISA, IFA, vaccination.

Résumé : ÉVALUATION COMPARATIVE PAR ELISA ET IFA DES TAUX D'ANTICORPS DU BÉTAIL VACCINÉ PAR *THEILERIA ANNULATA* EN IRAN

Un test ELISA a été utilisé afin d'évaluer les taux d'anticorps du bétail vacciné ou non, en ayant recours en tant qu'antigène à des cellules myéloïdes infectées par des schizontes. Les résultats ont été comparés à ceux obtenus par immunofluorescence indirecte chez les mêmes animaux. Les sérums de 116 vaches laitières, 95 vaccinées et 21 non vaccinées, ont été examinés. Ils ont été testés par ELISA et IFA : 94 sérums avaient des taux d'anticorps positifs et 22 négatifs en ELISA, pour 89 positifs et 27 négatifs en IFA. Ainsi, les sensibilités et spécificités des tests en ELISA comparées à celles obtenues en IFA ont été de 95,5 et 66,6 % respectivement. Cette étude indique de façon générale que l'ELISA pourrait être un bon test séro-épidémiologique de détection de la theileriose bovine tropicale, et de distinction des animaux vaccinés et non vaccinés pour l'élaboration de programmes de vaccinations.

MOTS CLÉS : *Theileria annulata*, ELISA, IFA, vaccination.

Tropical bovine theileriosis due to *Theileria annulata* (Dshunkowsky and Luhus, 1904) is a fatal and highly important tick-borne disease in Asia, North Africa and Middle East including Iran. The mortality rate of the disease in acute form, in cross and pure-bred cattle in Iran, reaches 40 and 80 % respectively (Hashemi-Fesharki, 1988, 1998).

Different serological tests such as complement fixing, haemagglutination, agar gel precipitation, counter immuno-electrophoresis, capillary tube agglutination, and IFA test were performed for monitoring antibody responses in epidemiological survey of theileriosis, but a reliable test has not yet been established to monitor induced immunity in vaccinated cattle with *Theileria annulata* vaccine (Hooshmand-Rad & Hashemi-Fesharki, 1971; Pipano *et al.*, 1969; Manuja *et al.*, 2001; Kachani *et al.*, 1992). Recently ELISA test has been extensively used for serological diagnosis of parasite diseases and evaluation of immunity in vaccinated

animals (Beniwal *et al.*, 1997; Gubbles *et al.*, 2000; Manuja *et al.*, 2001; Pipano E, 1990; Voler *et al.*, 1976). This paper describes whether the ELISA test comparing to IFAT is capable to monitor the induced immunity in vaccinated animals and could also be considered as an additional test in vaccination schedule.

MATERIALS AND METHODS

ANTIGEN

Theileria annulata schizont infected myeloid cells were grown in modified Stoker medium (Hashemi-Fesharki, 1988). Then the infected cells at low sub-passages and at the beginning of stationary phase were harvested, centrifuged and washed twice at 1,600 rpm for a period of 15 min (Hooshmand-Rad & Hashemi-Fesharki, 1971). The resulting pellet was re-suspended in PBS with pH: 7.2 at the final concentration level of 25×10^6 cells per ml. The cells were sonicated (28,000 w) for five min in ice and centrifuged 10 min at 10,000 g by washing the pellet four times with PBS (Kachani *et al.*, 1992). The collected supernatant

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as an antigen was analyzed in ELISA test and its protein concentration was determined by Lowry's method (1951).

ANIMALS

Totally 116 Holstein Frisian milking cows, 12 months old, were chosen in three different farms located around the Institute. The animals were carefully examined in order to ensure that they had no signs of any infectious diseases. They were divided into two groups: the first group consisted of 95 animals receiving the *Theileria annulata* vaccine, and the second group of 21 animals, considered as controls. Prior to vaccination and 60 post-vaccination-day both groups were bled in order to prepare sera which were frozen at -20°C until use.

PREPARATION OF RABBIT ANTI-COW- γG -HRP CONJUGATE

Rabbit anti-cow immunoglobulin was conjugated with peroxidase using sodium periodate 12 mM (Wilson & Nakane, 1978). The concentration level of γG was twice higher than peroxidase. This complex was passed through Sephadex G-25 and sodium borohydrate was used for reduction, then samples were dialyzed against 0.01 M PBS.

OPTIMIZING ANTIGEN CONCENTRATION AND CONJUGATE DILUTION

The optimal antigen and conjugate concentrations levels were determined by checkerboard titration using doubling dilutions of the antigen and known positive/negative control sera.

ENZYME LINKED IMMUNOSORBENT ASSAY (ELISA)

The test was performed with some modifications as described by Kachani *et al.* (1992, 1996). Different dilutions of antigen at concentration level of 10, 20, 40 and 8 $\mu\text{g}/\text{ml}$ were prepared with 0.05 M carbonate/bicarbonate buffer pH 9.6. The wells of polystyrene micro titer plate (Nunc) were filled with 100 μl of diluted antigens. They were incubated overnight at 4°C and washed three times with PBS + Tween 20, and shaken to be dried. To block non-specific banding, PBS containing Tween 20 + 0.5 % gelatin, instead of dried milk, was added to each well of the coated plates and incubated. Then, they washed again three times with PBS + Tween 20.

Pooled standard positive/negative sera at a dilution level of 1/100 with PBS + Tween 20 + 0.1 % gelatin were added to each well and incubated for 1.5 h at 37°C . They were washed and dried as before. The conjugate diluted at level of 1/1,000, 1/2,000 and 1/4,000 were added to each well and the plates were incubated at 37°C for a period of 1.5 h. The wells were washed and

dried again, then orthophenylene diamine (1 mg/ml, in phosphate/citrate buffer PH: 5.5 containing 0.01 % hydrogen peroxide) was added to each well and incubated at room temperature for 10 min. The enzyme reaction was stopped with 2 M sulfuric acid solution and the intensity of development of yellow color reaction was measured with spectrophotometer at 492 nm. The controls (PBS without antigen and known positive/negative reference sera) were also evaluated.

IMMUNOFLUORESCENT ASSAY (IFAT)

This was performed, with a minor modification, as described by Burrige (1971). *Theileria annulata* antigen was fixed on slides and incubated with the sera. The slides were washed and rabbit anti-cow γG -FITC conjugate was added to each slide then they were evaluated by immunofluorescent microscope.

INTRA- AND INTER-ASSAY

Reproducibility of the ELISA test was analyzed with intra- and inter-assay as follows: two samples within different plates were analyzed for four times. The formula used for calculating the results was $\mu = \Sigma xi/n$ and $\% \text{ CV} = 100 \delta/\gamma$.

SENSITIVITY AND SPECIFICITY OF THE TESTS

To calculate sensitivity and specificity of these two tests, the following formula were used:

$$\text{Sensitivity} = (\text{Tp}/\text{Tp} + \text{Fn}) 100$$

$$\text{Specificity} = (\text{Tn}/\text{Tn} + \text{Fp}) 100$$

RESULTS

Checker board assay

Total protein of the antigen measured by Lowry's method (1951) was 2.2 mg/ml. The optimum concentration level of antigen and optimum dilution level of conjugate were 40 $\mu\text{g}/\text{ml}$ and 1/2,000 respectively.

Assessment of antibody levels by ELISA

94 out of 116 sera through ELISA test were positive and the rest showed no positive titer. The cut off point for positive sera, determined by the mean OD of standard positive sera, was 1.257 (Fig. 1).

Comparison of antibody positive titer between ELISA and IFA tests

Antibody titers of sera using ELISA and IFA tests were compared and the correlation factor was 0.88, which could be in acceptable range (Fig. 2).

Intra and inter assay

The intra and inter assay calculation showed (Table I) that their percentage of coefficient of variation were determined as 7.25 % and 8.25 % respectively.

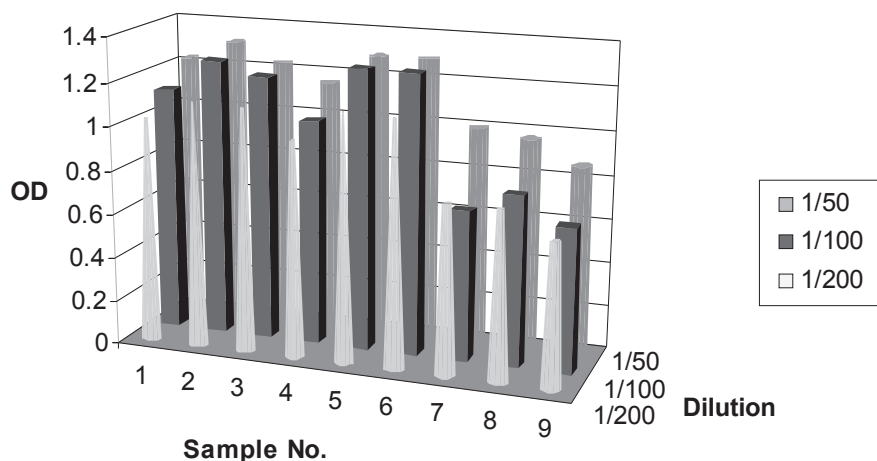


Fig. 1. – Cut off point for the standard positive and negative sera.
 Cut off = Mean (OD positive sera) + 2 Standard deviation
 Cut off = 1.257
 Optimum sera for cutoff = 1/100
 Sample No 1 to 6 = Positive sera
 Sample No 7 to 9 = Negative sera
 OD = Optical density

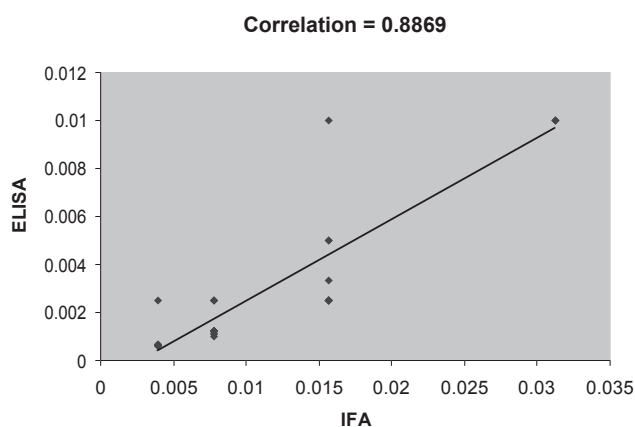


Fig. 2. – Correlation curve between IFA and ELISA.

NO/OD	1	2	3	4	μ	δ	% CV
Intra assay	1.332	1.314	1.121	1.282	1.282	0.093	7.25
Inter assay	1.273	1.278	1.028	1.267	1.211	0.1	8.25

μ = Mean

δ = Standard deviation

CV = Coefficient of variation.

Table I. – Determination of intra and inter assay for showing the reproducibility of ELISA.

Sensitivity and specificity of ELISA

The sensitivity and specificity of ELISA on comparison with IFAT were 95.5 % and 66.6 % respectively.

DISCUSSION

The availability and quality of effective antigen and technical reproducibility are important features to be considered in the development of successfully serodiagnostic tests. In this study, the

antigen used to perform ELISA and IFA tests was prepared from schizont infected myeloid cells, then harvested at low sub-passages and at stationary phase. So, it has been more immunogenic in comparison to the antigens prepared by other scientists (personal experiments). This particular antigen could also be stored at -70°C and successfully used for a period of one year. Gubbels *et al.* also showed that the antigen remained at least one year. Moreover, in this study, gelatin instead of milk has been more effective to block non-specific bindings.

Though different serological tests such as complement fixing, immuno-precipitation, capillary agglutination, haemagglutination, and IFA were used in cattle following infection with *Theileria annulata* (Pipano 1977; Pipano & Cahana 1969; Kachani *et al.*, 1992; Hooshmand-Rad *et al.*, 1971). It is considered that ELISA test is preferable in comparison with other tests, since it is less subject to operator error and operator stress, thus eliminating the subjective bias inherent in appraisal of fluorescence by eye in IFA test. In addition, the ELISA test is more specific, quantitative, less expensive and a greater number of serum samples can be assessed with it in a shorter time (Kachani *et al.*, 1996; Voler *et al.*, 1976).

Our study also indicated that ELISA is more sensitive and specific than IFA, and it could be a test of choice in seromonitoring of vaccinated animals. Limited cross-reaction was found only with *Theileria parva* antisera (Manuja *et al.*, 2001) but not with other *Theileria* or *Babesia* species. This cross-reaction was not significant, and at lower dilution than 1/64 would disappear.

Finally, it should be emphasized that although, fortunately, the tissue culture vaccine of *T. annulata* has proved to be effective in order to protect cross-bred and pure bred cattle against tropical theileriosis (Hashemi-Fesharki, 1988, 1998), but there are some contraversies about the duration of protective immunity induced by the vaccine; Hashemi-Fesharki, 1990; Zhany, 1990;

Zablotsky, 1990; Pipano, 1990), and there is no accurate test yet available to indicate the revaccination date of cattle with *Theileria annulata* vaccine. Moreover, the protective immunity of *Theileria annulata* usually related to cell mediated and humoral immunity, but it is necessary to emphasize that cell-mediated immunity has a more effective role to protect vaccinated animals. Conversely, humoral immunity has only a partial role when the schizont infected cells degenerate and schizonts become free and able to invade the healthy leucocytes and erythrocytes. This hypothesis is not completely acceptable because it happens that the animal with highly positive titer of antibody will die due to acute *Theileria*; in contrast, the animal with low positive titer of antibody will recover from the disease (personal experiments). Furthermore, it is needed to add that the positivity of antibody titer could only indicate that the vaccinated or infected animal had been exposed to mild or acute theileriosis. Therefore, it could be suggested that ELISA test together with delayed hypersensitivity theilerin test (1998) are the tests of choice to monitor the duration of vaccine protective immunity against *T. annulata* infections and help us to arrange an accurate schedule for vaccination program in our country.

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