

## Immunokinetics of *Theileria equi* specific antibodies: a comparison in serial and single dilution ELISA antibody end titres

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**Abstract:** *Theileria equi*, a haemoprotozoan of equids, is responsible for endemic infection/latency in tropical/subtropical parts of the world. The present study investigated the immunokinetics of *T. equi* infection in experimental animals and the diagnostic applicability of single dilution ELISA vis-à-vis conventional serial dilution ELISA. Sequential serum samples were collected up to 90 days postinfection (PI) from 4 donkeys experimentally infected with *T. equi*. Immunokinetics of *T. equi* antibodies end titre were determined by serial and single dilution ELISA. A significant rise in initial ELISA antibody end titre (1:800 in serial dilution ELISA and from 1:600 to 1:650 in single dilution ELISA) was observed from day 9 PI in all 4 donkeys, which plateaued between days 66 and 73 PI (end antibody titre varied between 1:6400 to 1:25,600 and 1:6050 to 1:22,100). The efficacy of these ELISAs was also determined by testing 256 field serum samples at 1:200 dilution, collected from apparently healthy animals. Sample analysis revealed overall seroprevalence of 91.01% in the horse population. The frequency distribution of antibody titre of these field samples by single dilution ELISA exhibited that most of the positive horses had high levels of specific antibodies. This study has emphasised the usefulness of single dilution ELISA as a diagnostic assay in determining the end antibody titre by testing it at a single dilution of 1:200.

**Key words:** *Babesia equi*, ELISA, equine piroplasmiasis, immunokinetics, *Theileria equi*

### 1. Introduction

Equine piroplasmiasis is an economically important disease of equids, and sporadic outbreaks are not uncommon (1,2). Equine piroplasmiasis is an acute, subacute, or chronic tick-borne disease of Equidae, caused by an intraerythrocytic protozoan, *Theileria equi* or *Babesia caballi*. The disease is characterised by fever (sometimes of intermittent nature), anaemia, icterus, and hepatomegaly and splenomegaly. A significant segment of the equine population has carrier status to this infection, due to which the draughtability of these animals gets lowered and poor farmers suffer economically. Indian-bred horses and mules are considered to be preimmune carriers of the *T. equi* infection (3). Malhotra et al. (4) recorded 50.1% prevalence of the disease among the horse population. Later, Kumar et al. (5,6) observed 47.6% incidence by performing enzyme-linked immunosorbent assay (ELISA) on horse and donkey samples.

Microscopic demonstration of the intraerythrocytic parasite is still the most reliable, simple, economical, and sustainable method for confirming diagnosis of equine babesiosis, but is not suitable for detection of carrier

animals (7,8). Therefore, serological tests such as the indirect fluorescent antibody test (9) and ELISA (5,6,10–12) are the preferred and recommended official tests by World Organisation for Animal Health (OIE) regulations. The complement fixation test has inherent disadvantages of giving false positive results and a low sensitivity for detecting latent infections (5,13–15).

Many versions of ELISA have also been standardised for detecting *T. equi* antibodies in carrier donkeys. Among these, Dot-ELISA (5), serial dilution ELISA, and single dilution ELISA (1,6) are the most important. Serial dilution ELISA is more time-consuming and requires large quantities of antigen, conjugate, or other reagents as compared to single dilution ELISA when the end titre of the sample is to be determined (16,17). In conventional serial dilution ELISA the end titre of a sample is known by a serial 2-fold dilution of the sample until the optical density (OD) becomes negative, but contrarily in single dilution ELISA, the sample is tested and diluted to only 1 particular dilution and the OD value is used in a regression equation to determine the end antibody titre. This study investigated the immunokinetics of antibody titre in

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donkeys experimentally infected with *T. equi* parasite and validated the application of single dilution ELISA as a diagnostic assay.

## 2. Materials and methods

### 2.1. Animals and serum samples

Four indigenous nondescript donkeys (1–1.5 years old) were used in the present study, which were obtained from the local area of Hisar, India. Their *T. equi* free status was ascertained by examining blood smears for the absence of the *T. equi* parasite on 3 consecutive days and serologically by Dot-ELISA (5). These donkeys were maintained in a tick-free animal housing facility of the All India Coordinated Research Project (AICRP) on Blood Protista, CCS Haryana Agricultural University, Hisar, India. These donkeys were infected by inoculating *T. equi* infected blood (0.5 mL kg<sup>-1</sup> body weight) showing >70% parasitaemia. Sequential serum samples were collected from these donkeys up to 90 days postinfection (PI) for the study of immunokinetics of *T. equi* antibodies in ELISAs.

### 2.2. *Theileria equi* parasite antigen

Whole merozoite native antigen of *T. equi* was prepared as described by Kumar et al. (6). The total protein concentration was estimated as per the method of Lowry et al. (18) and stored at 4 °C for further use in ELISAs. The antigen was diluted to obtain a working protein concentration of 18.5 µg/mL, which gave optimum results in ELISA.

### 2.3. Reference serum

A *T. equi* latently infected donkey had been maintained in the animal house of the laboratory and its serum was used as a reference known positive serum in ELISA, whereas the serum collected from a neonate foal (before colostrum feeding and whose mother also tested negative for *T. equi* antibodies) was used as a reference known negative serum sample in ELISA. *Babesia caballi* serum was obtained from the United States Department of Agriculture. Reference positive sera for *Babesia bigemina* (cattle), *Trypanosoma evansi* (horse), and *Theileria annulata* (cattle) were obtained from laboratories in India that were working on the diagnostics of these parasites.

## 2.4. ELISA

### 2.4.1. Serial dilution ELISA

The optimum dilutions of whole merozoite *T. equi* antigen, conjugate (rabbit antihorse γ globulin (IgG) HRP, Sigma Aldrich Co., USA) were determined by checker board titration method. The optimum dilution considered was the highest dilution of antigen/conjugate that gave a maximum contrast in terms of OD between known positive and known negative serum dilution. The method as described already by Kumar et al. (6) was followed. The serum samples were tested at 1:100 dilutions and titrated

onward up to 1:51,200. Any set of samples showing OD > [average + 3(standard deviation)] of the negative well was recorded as positive. The highest dilution of the serum sample showing a positive reaction was considered as the titre.

### 2.4.2. Single dilution ELISA

The standardisation procedure for single dilution ELISA was described by Kumar et al. (6). This method is based on derivation of the regression equation between the log<sub>10</sub> end point titre [y] and the positive/negative ratio [x]. Briefly, the log<sub>10</sub> end point antibody titre of 24 known positives and 3 known negatives (as above) were determined and average OD values of known positive sera at 1:50, 1:100, 1:200, 1:400, and 1:800 dilutions were divided by corresponding average OD values of known negative sera to work out the positive/negative ratio (P/N). The coefficient of correlation (r) was calculated (19) between the log<sub>10</sub> end titre and the P/N ratio. The r values were calculated with respect to each mean P/N ratio at a dilution of 1:50 to 1:800. The respective serum dilution showing the highest value of r was chosen for derivation of the regression equation ( $y = a + bx$ , where  $y = \log_{10}$  antibody titre of the test serum,  $x =$  the P/N ratio of the test serum at the chosen dilution,  $a =$  the constant, and  $b =$  the regression coefficient). We obtained the highest value of r (0.938) at a serum dilution of 1:200 and the derived regression equation was  $y = 2.05 + 0.51x$ . The serum samples were tested at this single dilution (1:200) and the P/N value as  $x$  was used to calculate the log<sub>10</sub> end point titre as  $y$ .

### 2.5. Unknown field sera

The field horse samples (256) were collected from different parts of Haryana State, India. These horses belonged to farmers who were using these animals as packs or for carts. The samples were tested at 1:200 dilutions in serial and single dilution ELISA. The end antibody titre of these samples in single dilution ELISA was obtained by substituting the value of  $x$  (P/N ratio) in the regression equation (as above) and the results were compared.

### 2.6. Statistical calculations

The chi-square test was applied for comparison of log<sub>10</sub> end antibody titres, at a 5% ( $P < 0.05$ ) level of significance, obtained in serial dilution and single dilution ELISA on serum samples collected from 4 donkeys experimentally infected with the *T. equi* parasite.

## 3. Results

### 3.1. Immunokinetics by ELISA

In all 4 experimental donkeys, parasitaemia by blood smear examination was observed from day 3 PI onward. Although the parasitaemia varied from animal to animal, the parasite could be detected in blood smear throughout the experimental period of 90 days. A significant rise in

initial ELISA antibody end titre (1:800 in serial dilution ELISA and 1:600 to 1:650 in single dilution ELISA) was observed from day 9 PI in all 4 donkeys experimentally infected with the *T. equi* parasite. The highest end antibody titre by serial and single dilution ELISA was observed between days 66 and 73 PI and the end antibody titre varied between 1:6400 and 1:25,600 and between 1:6050 and 1:22,100, respectively. The highest antibody end titre plateau and no decline were observed during the experimental period (Figure 1). No cross-reaction was observed in ELISA with reference positive serum for *B. caballi*, *B. bigemina*, *T. evansi*, or *T. annulata*.

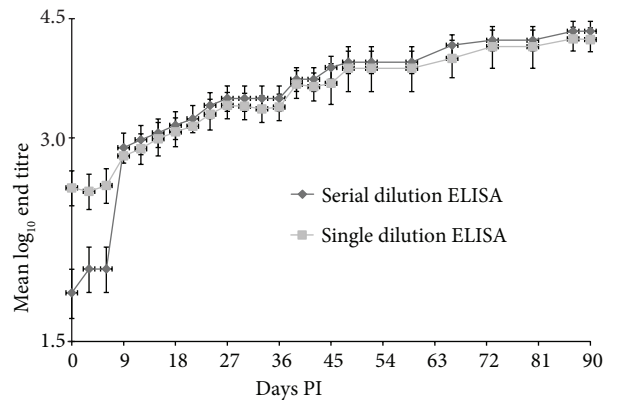
The chi-square test analysis on  $\log_{10}$  antibody end titres as observed by serial and single dilution ELISA on serum samples collected from 4 infected donkeys (days 0–90 PI) indicated nonsignificant ( $P < 0.05$ ) difference (Figure 2) in the  $\log_{10}$  end antibody titres obtained in these ELISAs. Furthermore, a correlation coefficient between serial and single dilution titres was also very high ( $r = 0.963$ ).

### 3.2. Unknown field sera

Serosurveillance for presence of *T. equi* antibodies by single dilution ELISA on 256 samples collected from the field revealed an overall prevalence of 91.01% in the equine population. The frequency distribution of antibody titre of these field samples by single dilution ELISA exhibited that most of the positive equids had high levels of specific antibodies. The results obtained by these 2 ELISA were comparable (Table) and only 3 negative samples detected in serial dilution ELISA were recorded as weakly positive in single dilution ELISA at a dilution of 1:200 to 1:1600.

## 4. Discussion

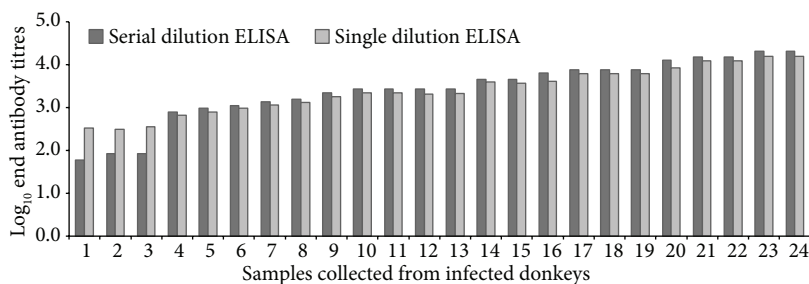
Application of conventional serial dilution for detection of antibodies against *T. equi* parasite is now well established (6,10,12, 20). The diagnostic specificity and sensitivity of single dilution ELISA for detection of *T. equi* antibodies in equine serum has previously been reported (6). This study was aimed to determine the immunokinetics of *T. equi* antibody response in experimentally infected donkeys so as to ascertain the diagnostic sensitivity of single dilution ELISA vis-à-vis routinely used serial dilution ELISA. The end antibody titre was successfully



**Figure 1.** Immunokinetics of *Theileria equi* infection in experimental donkeys by serial dilution and single dilution ELISA.

determined by both ELISA and a rise in antibody titre was initially observed from day 9 PI onward. This observation is in close conformity with earlier reports by Kumar et al. (5), Knowles et al. (10), Weiland (21), and Kuttler et al. (22). The antibody titre plateau after days 66–73 PI with no further rise was recorded. These ELISAs were detecting the presence of specific IgG antibodies, which are long lasting, as compared to IgM (detected in complement fixation test) (23), and are predominant in *T. equi* latently infected equids due to the persistence of the parasite in the circulation (1,24). Kumar et al. (5) and Knowles et al. (10) detected the specific *T. equi* antibodies in experimentally infected horses and donkeys up to week 115 and day 90 PI, respectively.

There was nonconformity in 1.17% field samples unknown by these ELISAs (3 samples were negative in serial dilution ELISA and detected positive with single dilution ELISA), which have very low antibody titres (1:200 to 1:1600). Single dilution ELISA considered the P/N ratio instead of the OD value (serial dilution ELISA) for ascertaining the status of unknown samples (positive or negative). The OD value of a particular sample depends largely on assay conditions and time of incubation (particularly in the colour development step), whereas the P/N ratio is more stable (6). These factors influence the



**Figure 2.** Chi-square analysis and comparison of end antibody titre observed in *Theileria equi* infected experimental donkeys as obtained in ELISAs.

**Table.** Frequency distribution of *Theileria equi* antibodies titre as detected by serial dilution and single dilution ELISA on unknown equine sera collected from the field.

Single dilution ELISA		Serial dilution ELISA	
Reciprocal antibody titre based on OD value at 1:200	Number of samples	Tested at 1:200	Number of samples
Up to 200 (-)	23	<1:200 (-)	26
200–1600 (+)	67	>1:200 (+)	230
1601–4800 (++)	27		
>4801 (+++)	139		
Total negative	23		26
Total positive	233		230

status of samples having low titres and may be a plausible explanation for the high sensitivity of single dilution ELISA on these samples.

This study successfully applied and determined the end antibody titre by single dilution ELISA on serum samples collected from experimental and field animals and obtained high correlation with serial dilution ELISA (Figure 2). This study has emphasised the usefulness of single dilution ELISA as an efficient diagnostic assay on

unknown field samples and in determining end antibody titre, which would aid in devising a *T. equi* containment strategy on an organised equine farm.

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#### References

- Kumar, S., Kumar, R.: Diagnosis of *Babesia equi* infection: an update on the methods available. Perspectives in Agriculture, Veterinary Science, Nutrition and Natural Resources, 2007; 2: 1–14.
- USDA: Equine piroplasmosis domestic pathways assessment 2011: pathways assessment for the spread of the causative agents of equine piroplasmosis from the movement of a horse from quarantined premises within the contiguous United States. USDA: Fort Collins, CO, USA. 2011.
- Kumar, S., Sugimoto, C., Igarashi, I.: Equine babesiosis in Asia. In: Asian Parasitology, Vol. 4 – Toxoplasmosis and Babesiosis in Asia. AAA Committee – The Federation of Asian Parasitologists, Department of Infection and Host Defense, Chiba University Graduate School of Medicine, Inohana, Chiba, Japan. 2005, 187–195.
- Malhotra, D.V., Gautam, O.P., Banerjee, D.P.: Prevalence of latent cases of *Babesia equi* infection in some parts of North-West India as measured by capillary tube agglutination test. Equine Vet. J., 1978; 10: 24–26.
- Kumar, S., Malhotra, D.V., Dhar, S.: Serodiagnosis of *Babesia equi* infection – a comparison of Dot-ELISA, complement fixation test and capillary tube agglutination test. Vet. Parasitol., 1997; 69: 171–176.
- Kumar, S., Kumar, Y., Malhotra, D.V., Dhar, S., Nichani, A.K.: Standardisation and comparison of serial dilution and single dilution enzyme linked immunosorbent assay (ELISA) using different antigenic preparations of the *Babesia (Theileria) equi* parasite. Vet. Res., 2003; 34: 71–83.
- Mahoney, D.F., Saal, J.R.: Bovine babesiosis: Thick blood films for the detection of parasitaemia. Aust. Vet. J., 1961; 37: 44–47.
- Friedhoff, K., Bose, R.: Recent developments in diagnostics of some tick-borne diseases. In: Uilenberg, G., Permin, A., Hansen, J.W. Eds. Use of Applicable Biotechnological Methods for Diagnosing Haemoparasites. Proceedings of the Expert Consultation, Merida, Mexico, 4–6 October 1993. FAO, Rome, Italy. 1994, 46–57.
- Madden, P.A., Holbrook, A.A.: Equine piroplasmosis: indirect fluorescent antibody test for *Babesia caballi*. Am. J. Vet. Res., 1968; 29: 117–123.
- Knowles, D.P. Jr, Kappmeyer, L.S., Stiller, D., Hennager, S.G., Perryman, L.E.: Antibody to a recombinant merozoite protein epitope identified horses infected with *Babesia equi*. J. Clin. Microbiol., 1992; 30: 3122–3126.
- Ikadai, H., Xuan, X., Igarashi, I., Tanaka, S., Kanemaru, T., Nagasawa, H., Fujisaki, K., Suzuki, N., Mikami, T.: Cloning and expression of a 48-kilodalton *Babesia caballi* merozoite rhoptry protein and potential use of the recombinant antigen in an enzyme-linked immunosorbent assay. J. Clin. Microbiol., 1999; 37: 3475–3480.
- Hirata, H., Ikadai, H., Yokoyama, N., Xuan, X., Fujisaki, K., Suzuki, N., Mikami, T., Igarashi, I.: Cloning of a truncated *Babesia equi* gene encoding an 82-kilodalton protein and its potential use in an enzyme-linked immunosorbent assay. J. Clin. Microbiol., 2002; 40: 1470–1474.

13. Brüning, A.: Equine piroplasmosis an update on diagnosis, treatment and prevention. *Br. Vet. J.*, 1996; 152: 139–151.
14. Knowles, D.P., Perryman, L.E., Goff, W.L., Miller, C.D., Harrington, R.D., Gorham, J.R.: A monoclonal antibody defines a geographically conserved surface protein epitope of *Babesia equi* merozoites. *Infect. Immun.*, 1991; 59: 2412–2417.
15. McGuire, T.C., Van Hoosier, G.L., Henson, J.B.: The complement-fixation reaction in equine infectious anaemia demonstration of inhibition by IgG(T). *J. Immunol.*, 1971; 107: 1738–1744.
16. Khatri, N., Nichani, A.K., Sharma, R.D., Khatri, M., Malhotra, D.V.: Effect of vaccination on young calves born during winter season in the field with the *Theileria annulata* (Hisar) cell culture vaccine. *Vet. Res. Commun.*, 2001; 25: 179–188.
17. Manuja, A., Nichani, A.K., Kumar, R., Sharma, R.D., Kumar, B.: Single dilution ELISAs using piroplasm, cellular schizont and soluble schizont antigens for the detection of antibodies against *Theileria annulata*. *Vet. Res.*, 2001; 32: 165–173.
18. Lowry, O.H., Rosebrough, N.J., Farr, A., Randall, R.J.: Protein estimation with the Folin phenol reagent. *J. Biol. Chem.*, 1951; 193: 265–275.
19. Snedecor, G.W., Cochran, W.G.: *Statistical Methods*. 8th edn., Iowa University Press, Ames, IA, USA. 1989.
20. Xuan, X., Chahan, B., Huang, X., Yokoyama, N., Makala, L.H., Igarashi, I., Fujisaki, K., Maruyama, S., Sakai, T., Mikami, T.: Diagnosis of equine piroplasmosis in Xinjiang province of China by the enzyme-linked immunosorbent assays using recombinant antigens. *Vet. Parasitol.*, 2002; 108: 179–182.
21. Weiland, G.: Species-specific serodiagnosis of equine piroplasmosis infections by means of complement fixation test, immunofluorescence and enzyme-linked immunosorbent assay. *Vet. Parasitol.*, 1986; 20: 43–48.
22. Kuttler, K.L., Goff, W.L., Gipson, C.A., Blackburn, B.O.: Serologic response of *Babesia equi*-infected horses as measured by complement-fixation and indirect fluorescent antibody tests. *Vet. Parasitol.*, 1988; 26: 199–05.
23. Mayer, M.M.: The complement system. *Sci. Am.*, 1973; 229: 54–66.
24. De Waal, D.T., van Heerden, T.: Equine babesiosis. In: Coetzer, J.A., Thomson, G.R., Tustin, R.C. Eds. *Infectious Diseases of Livestock with Special Reference to Southern Africa*. Oxford University Press, Oxford. 1994; 295–304.