

Detection of Antibodies to *Ehrlichia risticii* in Horses

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Received: 01.04.1997

Abstract: In this study, presence of antibodies to *Ehrlichia risticii* in the sera of 100 throughbreed horses brought to Turkey Jockey Club Stud from different regions was investigated by indirect fluorescent antibody (IFA), western immunoblotting, indirect enzyme linked immunosorbent assay (ELISA) and competitive ELISA (CELISA) using monoclonal antibody.

Ten sera gave various degrees of fluorescence on IFA test. The sera giving fluorescence were sent to University of Maryland and the test was repeated. The results were similar. Five sera from those positives were also tested on western immunoblotting. Amongst those, only one serum reacted with one of the 9 surface antigens of *E. risticii*. This serum reacted with an antigen on 38 kD region on the first test and with 28 kD region on the second. Therefore, the reaction obtained by this serum on western immunoblotting was considered non-specific as the result were inconsistent. No antibody to *E. risticii* was detected in any of 100 horse sera by CELISA and indirect ELISA.

In conclusion, 10 sera of 100 horse sera gave fluorescence on IFA test for *E. risticii*. However, these were negative on western immunoblotting, indirect ELISA and CELISA using monoclonal.

Key Words: *Ehrlichia risticii*, horses, IFA, CELISA, ELISA, western immunoblotting.

Atlarda *Ehrlichia risticii* Antikorlarının Araştırılması

Özet: Bu çalışmada Türkiye Jokey Klubü pansiyon hasarına çeşitli bölgelerden gelen 100 damızlık kısırakda *Ehrlichia risticii*'ye karşı antikorların varlığı araştırılmıştır. İndirekt Floresans Antikor (IFA), western immunoblotting, monoklonal antikor kullanılarak yapılan kompetetive ELISA (CELISA) ve indirekt ELISA teknikleri kullanılmıştır.

İndirekt Floresans Antikor testi sonucunda 10 serumda değişen derecelerde atipik floresans saptanmıştır. Floresans veren serumlar Maryland Üniversitesi'ne gönderilmiş ve aynı test ile aynı sonuç alınmıştır. Daha kuvvetli floresans veren 5 seruma western immunoblot testi iki kez uygulanmıştır. Testlerin sonucunda 4 adet serumun *E.risticii*'nin başlıca 9 antijenik komponenti ile hiç bir reaksiyon göstermediği, bir serumun ise ilk testte 38 kD, ikinci tekrarda 28 kD antijeni ile kuvvetli bir reaksiyon gösterdiği saptanmıştır. Sadece bir antijenik komponente karşı görülen bu reaksiyonun spesifik olmadığı düşünülmüştür. CELISA ve indirekt ELISA yöntemleri ile işlenen tüm serumların *E.risticii*'ye karşı antikor taşımadığı saptanmıştır.

Sonuç olarak AME yönünden test edilen 100 serumun 10'un da IFA tekniği ile floresans görülmüş ancak western immunoblot, CELISA ve indirekt ELISA ile negatif oldukları saptanmıştır.

Anahtar Sözcükler: *Ehrlichia risticii*, At, IFA, ELISA, CELISA, western immunoblotting.

Introduction

Equine monocytic ehrlichiosis (EME) was first recognized in horses as a disease syndrome in 1979 near to Potomac River in Maryland (1). The causative agent of the disease was discovered and named as *Ehrlichia risticii* in 1984 (2, 3). It has been reported that the organism is

highly heterogenous, differing in their antigenic, genomic and pathogenic characteristics. Different isolates have nine major surface proteins which differs in molecular weights (4).

At present, the disease has been reported in horses serologically in many regions of the United states,

Canada, France, Denmark, the Netherlands and in Italy (5, 6, 7). These studies have indicated that the fatality rates is around 20-30% which is economically important (4, 8). The EME cases generally seen in summer especially in June and August. This indicates that there is a seasonal difference in the occurrence of the disease (1, 9, 10).

The transmission route(s) of the disease is obscure at present (4, 11). Other ehrlichial diseases are transmitted by hemotophagous insects have led investigators to suggest arthropod transmission. However, to date, no arthropod has been found to harbor the ehrlichial agent (2, 6, 10, 11). Although some studies (3, 12) have indicated that direct transmission of *E.risticii* between same - stall horses appears not to occur. There is growing evidence that an oral route of transmission may contribute to spread of the disease (13, 14). In one study, four healthy ponies were given *Ehrlichia* infected mouse monocyte tissue culture cells in gelatin capsules and the infection has occurred in 2 ponies after the oral administration (14), suggesting the oral transmission of the disease. Therefore, one might make a speculation that the agent can be taken orally through helminths (13, 14).

The seroprevalence studies in other animals showed that antibodies to *E.risticii* in cats (6, 15), swine and in one goat (15) have been detected. The agent has been isolated from the dogs (4).

Affected horses may have any of the following clinical signs in any combination: Leucopenia, mono or biphasic fever, selective anorexia, depression, mild to profuse diarrhoea, laminitis and mild to severe colic. But, in some cases only one symptom can be seen or the disease is asymptomatic (1, 9, 10, 16, 17).

Serological test such as IFA, ELISA, CELISA and western immunoblotting are widely used for detecting antibodies to *E.risticii* by many investigators (2, 8, 18, 19). The ELISA antibody titers were correlated with the indirect fluorescent antibody (IFA) test titers (1). Competitive enzyme linked immunosorbent assay (CELISA) using a monoclonal antibody, and western immunoblotting are more sensitive than IFA and indirect ELISA (18, 19).

In Turkey, the situation of EME is not clear at present. Therefore, studies on the presence of EME should be performed. The reason for that is there is big traffic of horse industry because of the racings. Horses from other countries in which the EME has been reported, comes to Turkey for racing. This traffic might be the source of the infection. For this reason, the aim of this study is to investigate the presence of EME in Turkey especially in racing horses.

Materials and Method

Animals and collection of the sera: Throughbred English horses from different localities which were kept in Turkey Jokey club for breeding reason were used in this study. They were all female and clinically healthy. This sampling was based on 95% confidence limit with 10% accuracy (20).

Antigen and, positive and negative sera: The test antigen and, positive and negative control sera were kindly supplied from Professor S.K. Dutta (University of Maryland College Park, Virginia, Maryland Regional College of Veterinary Medicine, USA).

Serological analysis: The IFA, indirect ELISA, CELISA using monoclonal antibody and western immunoblotting tests were used for detecting antibodies to *E. risticii*. The IFA tests were used for detecting antibodies to *E. risticii*. The IFA test was performed at the Department of Microbiology in Veterinary Faculty of Istanbul.

Indirect fluorescent antibody test: Serum samples were diluted 1 : 40 and 1 : 80 with 2% casein buffer (w/v) solution (casein buffer, pH 7.5 - 8.0) and screening of samples was performed at that dilution. Negative control serum was diluted 1 in 20 and positive control serum was 1 in 40. The multiwell slides were taken from storage and allowed to warm to 22°C. 25 µl volume of each diluted sample was pipetted onto the previously prepared and fixed multiwell slides. Slides were incubated at 37°C for 30 minutes in humidified chamber. Slides were washed in Sorenson's phosphate buffer solution (S-PBS, 0.1 M, pH 7.2) for 5 minutes and then washed in distilled water for 5 minutes then were air-dried. 10 µl of the secondary antibody fluorescein-labeled rabbit anti-horse IgG (Sigma, F 7759), was applied and slides were incubated at 37°C for 20 minutes in a humidified chamber. Slides were then washed in Sorenson's phosphate buffer solution and distilled water as previously mentioned. Slides were air-dried and examined by using of a UV microscope (2).

Sera giving fluorescence on IFA tests were sent to University of Maryland and analysed by IFA, indirect ELISA, CELISA using monoclonal antibody and western immunoblotting by the method as described by others (2, 8, 18, 19).

Results

Indirect fluorescent antibody test: Fluorescence were detected in slides by 10 horses sera at a dilution of 1 in 40 and 1 in 80. This was compared with the positive and

Table 1. The results of indirect fluorescent antibody test. Only the positive sera included.

Serum no	1/40	1/80
2	+	+
4	+	+
5	+	+
6	2.5+	+
11	+	+
12	2+	+
13	+	+
15	+	+
17	+	+
24	+	+

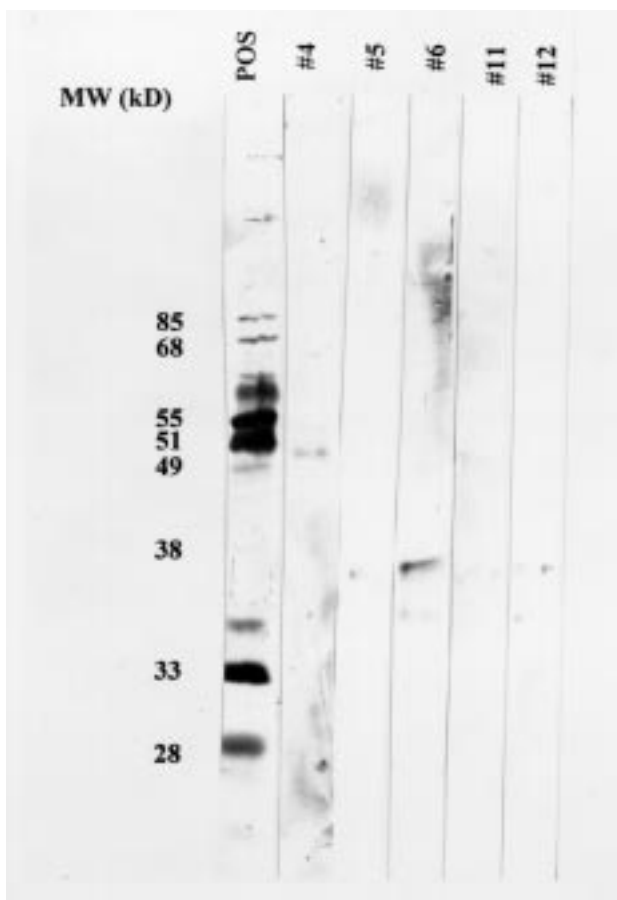


Figure 1. The results of western immunoblotting test. Numbers on the left hand side indicates the molecular weight markers. Number on top indicates the horse sera tested (4, 5, 6, 11, 12). POS=positivie control serum.

negative sera and was different from the positive sera. Therefore, it was concluded as atypical fluorescence. The fluorescence staining was evaluated by numbering from 1 to 5 (Table 1). The results were confirmed in Maryland University using the same test.

Western immunoblotting: The positive sera (4, 5, 6, 11 and 12) which was detected by IFA test were also used on western immunoblotting. Only one sera (6) reacted with one of the nine major surface antigens (85, 68, 55, 51, 49, 38, 28 kD) of *E. risticii* at a region of 38 kD. The test was repeated and the same sera has reacted with an antigen on the 28 kD region. This means that the serum (6) giving reaction with *E. risticii* antigens on western Immunoblotting reacted with different antigens on each test. Therefore, the reaction was considered nonspecific rather than a recognition of a specific determinants of *E. risticii* antigens (Fig. 1).

CELISA using monoclonal antibody and indirect ELISA: All sera were negative on CELISA and ELISA tests. It was concluded no sera had antibody to *E. risticii*.

Discussion

EME has been reported in the USA and Europe (5, 6, 7). The seroprevalence of the disease in the USA ranges from 7.5% to 76% depending on the region (9, 13, 17, 21, 22). In France, the disease has been diagnosed in an 8 years old mare with symptoms. The serological tests were used on diagnosis. The blood taken from this mare was given to mice and a pony intravenously for an experimental infection and the disease has occurred in both (23). The disease has also been diagnosed serologically by IFA test in the Netherlands in a 4-year - old mare, born and reared in North Brabant (Netherlands), showing colic and anorexia (24). There is no report of EME in Turkey at present. In this study, antibodies to *E. risticii* were not detected in 100 horse sera by using IFA, indirect ELISA, CELISA using monoclonal antibody and western immunoblotting.

Studies on EME (1, 2, 11, 18) have indicated that the indirect fluorescent antibody test is very sensitive and specific for the diagnosis. However, the difference in fluorescent staining in slides makes the interpretation difficult. Therefore, in the present study the positive sera showing atypical fluorescence from IFA tests were send to Maryland University for confirmation. Same results were obtained. In order to confirm the IFA results, other test, western immunoblotting, CELISA using monoclonal antibody, and indirect ELISA were also used. This was suggested by others (2, 18, 19). The bands on western immunoblotting were different. This difference might be because of the strain variation in Turkey and America. Because, American isolate was used on western immunoblotting as an antigen.

Antibodies were not detected in 100 healtly horse

sera in the present study. The number of the sera analysed may not be sufficient to say that there is no EME in Turkey. More sera should be analysed by taking the blood from horses showing the symptoms of EME.

Transmission of EME in horses has not been well established (4). However, the age, sex and breed of the host and vectors and environmental factors such as climate and watery regions are the important factors which

have been suggested by several authors (3, 12, 13). These factors should be taken into account in controlling EME if there is an epidemic.

Acknowledgement

This study is funded by the research foundation of Istanbul University. We would like to thank to University of Istanbul for funding.

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