# Development of an ELISA technique for the detection of *Babesia ovis* and serological survey of the parasite in Khouzestan province, southern Iran

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## Summary

To develop an ELISA technique for the detection of antibodies against *Babesia ovis*, the infected erythrocytes were lysed and the supernatant soluble antigen, after sonication and ultracentrifugation of the lysate was used as antigen. Optimal dilution of the antigen was determined by checkerboard titrations, using positive and negative control sera. A correlation of 85% was observed between the results of the developed ELISA and IFA techniques. To study the seroprevalence of *Babesia ovis* in Khouzestan province, south of Iran, a total of 1000 sheep sera were collected from different areas of the province and tested against *Babesia ovis* using the ELISA technique developed. The results showed an average seroprevalence of 47.5% in the province. Our results indicated a significant increase of the seroprevalence by advancement of age of the animals. There was no significant difference between the seroprevalence of female and male sheep.

**Key words:** *Babesia ovis*, ELISA, Seroprevalence

## Introduction

*Babesia ovis* is a small *Babesia*, being 1–2.5 $\mu$m in length. The majority of the organisms occurring at the margin of the red cells, are round (Soulsby, 1986). *Babesia ovis* is distributed over a wide range of geographical regions, having been recorded in Africa, Asia and southern Europe (Ristic, 1981). The acute infections with *Babesia spp* are characterized by a systemic inflammatory response, as evidenced by hyperthermia, anemia with concomitant hemoglobinuria and apathy. Accurate diagnosis of *Babesia ovis* infection is essential for disease control and for epidemiological studies (Patricia *et al*., 2001). Several serodiagnostic tests have so far been described among which, the indirect immunofluorescence assay (IFA) is the most widely-used technique. The enzyme-linked immunosorbent assay (ELISA) has been described as a very sensitive test (Waltisbuhl *et al*., 1987) and holds the greatest promise for the future (Bose, 1995). A sero-prevalence of 42.1% (Sevinc, 1996) and as high as 88.9% (Yeruham *et al*., 1995) has been reported for *Babesia ovis* infection from different geographical areas. The sole study performed in Iran by IFA, has indicated a seroprevalence of 36% in the country (Tavassoli and Rahbari, 1998). Because of the limited provincial samples studied in the previous survey, the present work was undertaken to develop an ELISA technique for detecting antibodies against *Babesia ovis* in sheep and to study the seroprevalence of the parasite in Khouzestan province, southern Iran. To the best of our knowledge, this is the first report regarding application of ELISA to study the seroprevalence of the parasite in Iran.

## Materials and Methods

### Preparation of *Babesia ovis* ELISA antigen

Antigen preparation was based on the technique described by Waltisbuhl *et al*., (1987) for *Babesia bovis*. A 4-month-old, hemoparasite-free lamb was splenectomized and inoculated intravenously with *Babesia ovis* infected blood. The animal was daily monitored for the presence of parasites by microscopic examination of Giemsa-stained blood smears. When a 5% parasitemia was observed, the infected blood was collected in the presence of EDTA as anticoagulant. The blood was washed three times in five volumes of phosphate buffered saline (PBS). The packed red cells were lysed in distilled water at 4°C and the parasites were pelleted by centrifugation at 12000 g for 30 min. The pellet was washed three times in PBS by resuspension and centrifugation at 4°C and then, resuspended in one to two volumes of PBS. The parasites were sonicated in appropriate volumes, using a medium power for 60–90 sec. The sonicated material was ultracentrifuged at 105000 g for 60 min at 4°C and the supernatant was retained. The supernatant was mixed with an equal volume of glycerol and stored in 2–5 ml aliquots at -70°C.

### *Babesia ovis* IFA antigen

Infected blood of splenectomized lamb was used as IFA antigen too. A part of infected blood, collected and washed for ELISA antigen preparation, was resuspended in PBS at the original volume of blood. Three 5-$\mu l$ spots of blood were placed on clean glass slides and air dried. The slides were wrapped in aluminum foil and stored at -70°C.

### Sera

To develop the ELISA test, a positive control serum, from an experimentally *Babesia ovis* infected sheep (Tavassoli and Rahbari, 1998) and five negative control sera, from newborn lambs, before colostrum ingestion, were used. The seroprevalence survey was performed on a total of 1000 sera, collected from ten cities of Khouzestan province and their suburbs. Based on a cluster random sampling (Thrusfield, 1995), the sample size for each location was calculated as regard to their sheep population.

### ELISA procedure

The method used was earlier described by Waltisbuhl *et al*., (1987) with some modifications. Different dilutions of ELISA antigen in coating buffer (0.1 M carbonate buffer pH = 9.6) were prepared and used for coating (100 $\mu l$/well) of ELISA plates (Greiner company, Germany). The plates were incubated at 4°C overnight. Thereafter, antigen-coated plates were blocked by 100 $\mu l$/well of 10% bovine serum albumin (BSA) in coating buffer, for three hrs at room temperature and washed once with 300 $\mu l$/well of washing buffer (PBST: PBS pH = 7.2 containing 0.1% Tween 20). One hundred $\mu l$ of sheep sera, diluted 1:100 in PBST containing 10% BSA were added in duplicate wells of plates and incubated for one hr at room temperature. After three washes, 100 $\mu l$ of a peroxidase conjugated anti-sheep IgG (Sigma Chemicals, USA), diluted 1:1400 in PBST containing 10% BSA was added to each well. The plates were then incubated for one hr at room temperature and washed three times, as mentioned previously. Subsequently, 100 $\mu l$ of ABTs peroxidase substrate (Roche, Germany) was added to each well. The substrate reaction was allowed to continue for 15 min, after which it was stopped by adding 100 $\mu l$/well of 1% SDS. The absorbance was read at 405 nm and the cut-off point for positive sera was set at 1.5× average value of five negative control sera. To determine the validity of the developed ELISA, 50 ELISA-positive and 50 ELISA-negative sera were tested by IFA.

### IFA procedure

Microscope glass slides, spotted with infected blood, were fixed in cold solution of 70% acetone and 30% methanol for five min at room temperature. They were then blocked with 1% BSA in PBS, overnight at room temperature. After blocking, the slides were gently rinsed twice with PBST, 15 min

each time and once with PBS. Ten $\mu l$ volumes of the test sera, diluted 1:40 in 1% BSA in PBS were added on the blood spots and the slides were incubated for 30 min at 37°C, in a humid chamber. The slides were washed as before and probed with a commercial fluorescein isothiocyanate-conjugated rabbit anti-sheep IgG (Sigma Chemicals, USA), at a dilution of 1:200 in 1% BSA in PBS. After the incubation time and washing, as mentioned above, the slides were dried, mounted in 10% buffered glycerin and examined by a fluorescent microscope.

## Results

### Standardization of ELISA

Checkerboard titration of ELISA antigen showed a 1:300 dilution, as the optimal dilution of the antigen for being used in ELISA. The mean absorbance value of negative control sera was 0.150 ± 0.01, resulting in a calculated cut-off value of 0.225. Based on the testing of 50 ELISA-positive and 50 ELISA-negative sera by IFA, a correlation of 85% was observed between the results of two tests.

### Seroprevalence of the *Babesia ovis* infection in Khouzestan province

The results of the serological survey by ELISA are summarized in Table 1, 2 and 3. Of the 1000 tested sera, 475 (47.5%) were found to be positive and 525 (52.5%) were negative. The highest seroprevalence rate (58.76%) was found in Ahvaz and the lowest (32.55%) in Behbahan. All other regions had a prevalence of >30% (Table 1). The prevalence of infection had a significant ($\chi^2$ test; $P<0.05$) increase with respect to the age of the animals. The rate of infection in sheep aged 6–12 months, 12–24 months and older than two years was 33.78, 44.36 and 59.45%, respectively (Table 2). The prevalence of *Babesia ovis* infection in

**Table 1: The prevalence of *Babesia ovis* infection in different regions**

| Location | No. animals tested | Seropositive | | Seronegative | |
|---|---|---|---|---|---|
| | | No. | % | No. | % |
| Ahvaz | 194 | 114 | 58.76 | 80 | 41.24 |
| Susangerd | 98 | 46 | 46.93 | 52 | 53.07 |
| Behbahan | 43 | 14 | 32.55 | 29 | 67.45 |
| Shush | 113 | 49 | 43.36 | 64 | 56.65 |
| Izeh | 121 | 55 | 45.45 | 66 | 54.55 |
| Ramhormoz | 90 | 50 | 55.55 | 40 | 44.45 |
| Mahshahr | 60 | 34 | 56.66 | 26 | 43.34 |
| Andimeshk | 68 | 24 | 35.29 | 44 | 64.71 |
| Dezful | 138 | 52 | 37.68 | 86 | 62.32 |
| Masjed soleiman | 75 | 37 | 49.33 | 38 | 50.67 |
| Total | 1000 | 475 | 47.5 | 525 | 52.5 |

**Table 2: The prevalence of *Babesia ovis* infection according to age group**

| Location | No. sera 6–12 months | No. pos. (%) | No. sera 12–24 months | No. pos. (%) | No. sera >24 months | No. pos. (%) |
|---|---|---|---|---|---|---|
| Ahvaz | 34 | 15 (44.11) | 87 | 51 (58.62) | 73 | 48 (65.75) |
| Susangerd | 16 | 4 (25) | 46 | 20 (43.47) | 36 | 22 (61.11) |
| Shush | 15 | 2 (13.33) | 16 | 4 (25) | 12 | 27 (47.36) |
| Andimeshk | 23 | 3 (13.04) | 29 | 8 (27.58) | 16 | 8 (66.66) |
| Izeh | 20 | 2 (10) | 52 | 15 (28.84) | 49 | 38 (77.55) |
| Mahshahr | 25 | 15 (60) | 19 | 10 (52.63) | 16 | 9 (56.25) |
| Ramhormoz | 18 | 8 (44.44) | 35 | 22 (62.85) | 37 | 20 (54.05) |
| Andimeshk | 23 | 3 (13.04) | 29 | 8 (27.58) | 16 | 13 (81.25) |
| Dezful | 31 | 13 (41.93) | 63 | 21 (33.33) | 44 | 18 (40.9) |
| Masjed soleiman | 20 | 6 (41.93) | 25 | 15 (60) | 30 | 16 (53.33) |
| Total | 222 | 75 (33.78) | 408 | 181 (44.36) | 370 | 220 (59.45) |

**Table 3: The distribution of *Babesia ovis* infection according to the age and sex groups**

| Locations | No. (6–12) months sera | | | | No. (12–24) months sera | | | | No. (>24) months sera | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Male | | Female | | Male | | Female | | Male | | Female | |
| | pos. | % | pos. | % | pos. | % | pos. | % | pos. | % | pos. | % |
| Ahvaz | 6 | 42.85 | 9 | 45 | 17 | 60.72 | 34 | 57.62 | 12 | 66.66 | 36 | 65.45 |
| Susangerd | 2 | 28.57 | 2 | 22.22 | 7 | 41.17 | 13 | 44.82 | 7 | 63.63 | 15 | 60 |
| Behbahan | 1 | 14.28 | 1 | 12.5 | 1 | 25 | 3 | 25 | 2 | 50 | 6 | 75 |
| Shush | 3 | 27.27 | 3 | 33.33 | 3 | 33.33 | 13 | 48.14 | 9 | 52.24 | 18 | 45 |
| Izeh | 1 | 90.09 | 1 | 11.11 | 4 | 30.76 | 11 | 28.20 | 11 | 78.57 | 27 | 77.14 |
| Ramhormoz | 6 | 42.89 | 2 | 50 | 5 | 71.42 | 17 | 60.71 | 9 | 56.25 | 11 | 52.83 |
| Mahshahr | 11 | 57.89 | 4 | 66.66 | 2 | 50 | 8 | 53.33 | 3 | 75 | 6 | 50 |
| Andimeshk | 1 | 14.28 | 2 | 12.5 | 2 | 25 | 6 | 28.57 | 5 | 83.33 | 8 | 80 |
| Dezful | 6 | 50 | 7 | 36.84 | 9 | 3.5 | 12 | 30.76 | 3 | 25 | 15 | 46.87 |
| Masjed soleiman | 2 | 33.33 | 4 | 28.57 | 4 | 66.66 | 11 | 57.89 | 4 | 36.36 | 12 | 63.15 |
| Total | 39 | 36.11 | 35 | 30.7 | 54 | 45 | 128 | 44.44 | 65 | 57.52 | 154 | 59.92 |

males and females is shown in Table 3. The results indicated the seropositivity of 46.33% in 341 males and 48.10% in 659 females. The distribution of seropositivity according to sex was not statistically significant.

## Discussion

To understand the epidemiological status of parasites and for their control and monitoring, development and use of serological tests are very important. Among the serological tests, ELISA can be a very sensitive and efficient test. In contrast to many other tests, including IFA, by ELISA an objective reading is obtained, the data are amenable to computer evaluation and a large number of samples can be handled (Bose, 1995). Here, we described for the first time, the development of an ELISA technique with soluble antigens of *Babesia ovis*. Previous seroepidemiological studies of *Babesia ovis* infection by ELISA (Duzgun *et al*., 1991; Aktas *et al*., 2001; Emre *et al*., 2001) have been performed with *Babesia bovis* native or recombinant antigens. In this work, ELISA antigen was prepared as described earlier by Waltisbuhl *et al*., (1987) for *Babesia bovis*. An important step of our study was to find a suitable blocking solution. So far, different blockers have been used for other *Babesia spp.* Purnell *et al*., (1976), Barry *et al*., (1982) and Woodford *et al*., (1990) suggested 20% soya milk as the best blocker for *Babesia bigemina* antigen. Waltisbuhl *et al*., (1987), Bose *et al*., (1990) and Duzgun *et al*., (1991) used 2% horse serum in PBS, 0.5% gelatin in carbonate buffer pH = 9.6, and 2% skim milk powder in 0.1 M carbonate buffer pH = 9.6, as the blocking agents for *Babesia bovis*, respectively. The present investigation, concluded that a 10% BSA in 0.1 M carbonate buffer pH = 9.6 was the best blocker for *Babesia ovis* antigen. To obtain the highest difference between positive and negative control sera, sera and conjugate dilution buffer did also contained 10% BSA. A high percentage of correlation was observed between the results obtained from the developed ELISA and IFA techniques. Therefore, we used the ELISA in our serological survey.

The average seroprevalence found in the studied area was 47.5% that was higher than that reported by Tavassoli and Rahbari (1998). They found a seroprevalence of 39.33% among 181 sheep, tested by IFA. The reported prevalence rates of *Babesia ovis* antibodies in other geographical were different. Clmak *et al*., (1991) found that 72% of 141 sheep which have been tested by IFA were seropositive in the Samsun area of

Turkey. Habela *et al*., (1990) found an IFA seropositivity of 57.7%, by testing 97 sheep from Extramadura, southern Spain. Yeruham *et al*., (1995) found a positive serological response in 88.9% of the 523 ewes in Israel and Papadopoulus *et al*., (1996) reported an IFA seroprevalence of 52.1% in 720 sheep from Macedonia. By ELISA, Sevinc (1996) found a positive serum reaction in 42.1% of sheep tested in Konya area of Turkey and Dumanli *et al*., (1997) found a seroprevalence of 45% in 19 flocks of Elazig area in the same country. In another study performed in Turkey, Aktas *et al*., (2001) showed an ELISA seropositivity of 55.9% in 220 Malatia sheep. The distribution of the parasite is correlated with the distribution of tick vector species. Our results indicated a significant increase in seropositivity rate by increasing the age of the animals. This finding was in agreement with the studies of Dumanli *et al*., (1997) and Aktas *et al*., (2001) but in contrast with the reports of Tavassoli and Rahbari (1998) and Razmi *et al*., (2002). This discrepancy may be attributed to the kind of tests which were used. ELISA is a very sensitive and specific test and it is possible to detect antibodies shortly after infection (Waltisbuhl *et al*., 1987; Habela *et al*., 1990). In our study, we did not find a significant difference between female and male sheep seropositivity. Similar results have been reported by Tavassoli and Rahbari (1998), Aktas *et al*., (2001), Duzgun *et al*., (1991) and Razmi *et al*., (2002).

In conclusion, the present study reports the development of an ELISA test for serological survey of *Babesia ovis* infection in sheep population. It also indicates a widespread distribution of *Babesia ovis* infection in all regions of Khouzestan province and the need for establishing control strategies for ovine babesiosis in the province.

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