

Epidemiological studies on tropical theileriosis (*Theileria annulata* infection of cattle) in Kurdistan Region, Iraq

Adel T. Mohammad Al-Saeed · Lokman Taib Omer ·
Jassim Abdo · Gholamreza Habibi · Diaeldin A. Salih ·
Ulrike Seitzer · Jabbar Ahmed

Received: 20 October 2009 / Accepted: 23 October 2009 / Published online: 13 November 2009
© Springer-Verlag 2009

Abstract In an ad hoc survey conducted during 2006, the epidemiology of tropical theileriosis in Kurdistan Region, Iraq, was addressed. For this purpose, a total of 299 blood samples were collected from female cattle older than 1 year reared under open system management in Duhok ($n=99$), Sulaimanyia ($n=100$) and Erbil ($n=100$) governorates. The samples were subjected to TaSP indirect ELISA as well as polymerase chain reaction (PCR) and nested PCR assays. The results indicated that the seroprevalence was 77.9%, and PCR reported an infection rate of 68.9% in the Kurdistan Region of Iraq. The implication of the results in the epidemiology of tropical theileriosis in the region is discussed with emphasis on comparisons between the two tests used and recommendations for the future work are outlined.

Introduction

Livestock are a key resource for the production of milk and meat in tropical and subtropical regions of the world. One of the major factors preventing improvement of the productivity of livestock is that many of the animals are suffering from diseases which reduce the production and growth of the diseased animals. In addition to the problems associated with management and financial shortages, livestock in Iraq, including the Kurdistan Region, suffers from two major groups of diseases present with different epidemiological, economic, and regulatory implications namely, epidemic diseases such as Foot and Mouth disease and endemic diseases such as tick-borne diseases (TBDs), particularly theileriosis, babesiosis, and anaplasmosis which have considerable economic importance locally and regionally (Ahmed et al. 2002).

Theileriosis caused by *Theileria* parasites infect a vast number of wild and domestic animals and are transmitted trans-stadially by various members of tick vectors of the family *Ixodidae*. *Theileria annulata* causes tropical theileriosis, which is transmitted by ticks of the genus *Hyalomma* (Uilenberg 1981). The disease occurs in southern Europe and extends to southern Russia, the Middle East, Central Asia, China, India, northern Africa and Sudan, Eritrea, and Mauritania where an overall number of 250 million cattle are estimated to be at risk (McCosker 1979).

In the Kurdistan Region, Iraq, little is known about the epidemiology of tropical theileriosis, the reasons probably being due to the lack of reliable and accurate diagnostic methods. Traditionally, the diagnosis of *Theileria* parasites is based on the clinical symptoms and demonstration of morphological features of piroplasms inside red blood cells in stained blood smears (Latif et al. 1977; Muslih et al. 1988). However, this method is reliable for the detection of

Adel T. Mohammad Al-Saeed and Lokman Taib Omer contributed equally to this work.

A. T. Mohammad Al-Saeed · L. T. Omer
College of Veterinary Medicine, Duhok University,
Duhok, Kurdistan Region, Iraq

J. Abdo · D. A. Salih · U. Seitzer · J. Ahmed (✉)
Division of Veterinary Infection Biology and Immunology,
Research Center Borstel,
Parkallee 22,
23845 Borstel, Germany
e-mail: jahmed@fz-borstel.de

G. Habibi
Department of Protozoology and Production of Protozoal
Vaccines, Razi State Serum and Vaccine Research Institute,
P.O. Box 3197619751, Karaj, IR, Iran

acute cases and has limited value for chronic cases. In addition, high expertise is needed to differentiate among various pathogenic and non-pathogenic piroplasm species. The indirect fluorescent antibody test (IFAT) based on *T. annulata* schizont antigen has been described (Burrige and Kimber 1973); however, it is tedious to carry out for large sample sizes, is subjective in interpretation, and has the major drawback of observed cross-reactions between *Theileria* species (Kiltz et al. 1986). ELISA assays have advantages over IFAT in that they are less laborious, easy to perform, and a large number of samples can be tested in quite a short time. In addition, the use of recombinantly expressed specific antigens reduces cross-reaction problems. Several ELISAs based on recombinant proteins have been developed for detection of *Theileria* infection (reviewed in Bakheit et al. 2007). Recently, *T. annulata* surface protein (TaSP) has been characterized (Schnittger et al. 2002), and its application in indirect ELISA has been documented (Bakheit et al. 2004) and validated (Salih et al. 2005). However, since antibodies can remain circulating for some time after the parasite has been cleared from the animal, serological assays do not always provide information about the actual presence of the parasite. The development of molecular biology has made accurate tools available for the direct detection of parasite DNA. The first polymerase chain reaction (PCR) application for *T. annulata* diagnosis in the bovine host was based on the *Tams1* gene (d'Oliveira et al. 1995). Reverse line blot to detect and differentiate all known *Theileria* and *Babesia* species on the basis of their differences in 18S subunit rRNA gene sequences was also developed (Gubbels et al. 1999).

In the present study, the recombinant ELISA assay based on TaSP was applied for the detection of parasite-specific antibodies in bovine sera collected from different provinces in Kurdistan Region, Iraq. Moreover, PCR and nested PCR techniques were applied to determine the infection rate of *T. annulata* among these cattle.

Materials and methods

Study area

The survey was conducted during the period from April to June 2006, in Duhok, Sulaimanyia, and Erbil governorates of Iraq. A total of 299 blood samples were collected from indigenous apparently healthy cattle. The age of the cattle was more than 1 year, and all of them were females and reared under open system management. One hundred blood samples each were collected from Duhok and Sulaimanyia governorates, while 99 blood samples were collected from Erbil governorate. Blood was collected in plain vacutainer tubes and sera were separated by centrifugation at 1500 rpm

for 10 min. Each serum sample was collected using a sterile Pasteur pipette in an Eppendorf tube, labeled indicating locality, date, and animal number and then stored at -20°C until used.

Serology

Indirect ELISA was performed with TaSP as described by Bakheit et al. (2004). The samples were applied in duplicate in each plate which contained one positive and one negative reference serum sample each applied into four wells of the plate. The median optical density (OD) values of the control sera as well as the mean OD of the duplicate test sera were calculated, and the results were expressed as a percentage positive value of the reference positive control.

DNA extraction and nested PCR for molecular detection of *T. annulata*

DNA was extracted from whole blood samples using genomic DNA purification Kit (Promega, USA) following the protocol of the manufacturer, and the concentration of DNA was measured by spectrophotometry.

Species-specific primers for *T. annulata* were used for detection of infection based on *Tams1* encoding gene of *T. annulata* (accession number Z48739) as described by Habibi et al. (2007). The primers Tams1 (external) 5'ATGTTGTC AGGACCACCCTCAAG, Tams2 (internal) 5'TTAAAGGA AGTAAAGGACTGATGAGAAGACG, Tams3 (internal) 5'CGAGACCTACTACGATGAAG, and Tams4 (external) 5'GATAAGTTGTTACGAACATGG were synthesized by Cinnagen Company, Shahrak Ekbatan, Tehran, Iran. The first PCR was performed using the external Tams1 and Tams4 primers, while the second PCR was carried out using the internal Tams2 and Tams3 primers. For detection of the PCR product, an aliquot of 5 μl of PCR product was subjected to electrophoresis on a 1.5% agarose gel in a Tris-acetic acid-EDTA buffer at 90 V for 1 h and then visualized under UV light after staining with ethidium bromide. Only those samples that were negative in the first round PCR were subjected to a second round nested PCR.

For verification of the PCR product, seven DNA positive samples from the nested PCR were selected for sequencing. Generated pure DNA fragments of the PCR product were sent to the Cinnagen Company, Shahrak Ekbatan, Tehran, Iran, for sequencing. Compilation, editing, and assembly of sequences were performed with the EditSeq and SeqMan analysis program components of Lasergene software package for windows (DNASTAR, Madison, WI, USA). BLAST searches were performed at the NCBI website (<http://www.ncbi.nlm.nih.gov>) and TIGR institute website (<http://www.tigrblast.tigr.org>).

Analysis of the sequences was performed using the BioEdit program alignment and identity matrix function, which shows the proportion of identical residues between all of the sequences in the alignment.

Statistical analysis

The differences between different parameters were evaluated by using a computerized database structure (SPSS program). The Chi-square test (χ^2) was used to test the null hypothesis for significant difference between the disease in different governorates and techniques. Probability values less than 0.05 were considered significant.

Results

Serology

Using TaSP indirect ELISA, it was found that the seroprevalence of *T. annulata* was 77.9%, and the distribution of seropositivity in the three governorates is shown in Table 1. It is shown that the rate of seropositivity of *T. annulata* in Erbil was 75.8%, Duhok 88%, and Sulaimanyia 70%, respectively. Statistical analysis revealed that there is a significant difference at a level of $P < 0.05$ between the governorates of Kurdistan Region.

Molecular detection of infection

Agarose gel electrophoretic analysis of PCR amplification for detection of *T. annulata* in amplified DNA samples obtained from cattle of the three governorates of Kurdistan Region revealed products of 870 bp in case of using the Tams1 and Tams4 primers (first round PCR) and products of 466 bp in case of using the Tams2 and Tams3 primers (nested PCR) as shown in Fig. 1.

The infection rate of *T. annulata* in all samples combining the first and second round nested PCR results (only samples negative in the first round were subjected to a second nested PCR) was 68.6% (205/299). A total of 191 DNA samples were found to be negative for *T. annulata* by a single PCR assay using Tams1 and Tams4 primers; 97 (50.7%) of these samples were found to be positive by

nested PCR assay using Tams2 and Tams3 primers (Table 1).

The infection rate of theileriosis among cattle for each governorate was 62.6% in Erbil, 69% in Duhok, and 74% in Sulaimanyia (Table 1). Statistical analysis showed that a significant difference in rates of infection at a level of $P < 0.05$ between the three governorates of Kurdistan Region existed.

Results of sequence alignment and evaluation

A total of seven samples (two from Sulaimanyia, three from Duhok, and two from Erbil) were sequenced and submitted to GenBank (accession number FJ159695, GU130189-GU130194). Analysis using the BioEdit program showed clearly that the sequences differed in between and inter-provinces. The level of difference between the two samples from Sulaimanyia was 0.0042, between the two samples from Erbil 0.0172, and between the three samples from Duhok 0.0155–0.0260. The differences between the three provinces showed the least for Sulaimanyia and Duhok (0.0042–0.0260), and the highest difference between Erbil and Duhok (0.0725–0.1073).

Discussion

The Kurdistan Region is located within the northern region of Iraq, and the climatic conditions are similar to the Mediterranean area in which rainfall occurs in winter and moderate rain in autumn and spring and no rainfall in the summer season. With respect to the climate, the region is defined as having cold winters, hot summers, and neutral springs and autumns with a wide range of temperatures. The mean temperature in summer is about 40°C and in winter less than 0°C. This type of climate with seasonal fluctuation provides a very suitable environment for development and spread of Ixodidae ticks which have the capacity to transmit tropical theileriosis. The intensity of *Theileria* infection in cattle and infestation levels in ticks are influenced by many factors such as seasonal variation, breeding, and management systems in any region.

Table 1 Distribution of *Theileria annulata* infection in Erbil, Duhok, and Sulaimanyia during 2006 as determined by ELISA and PCR

| Governorate | Number | ELISA | 1 st round PCR | 2 nd round (nested) PCR | 1 st +2 nd round PCR |
|-------------|--------|-------------|---------------------------|------------------------------------|--|
| Erbil | 99 | 75 (75.8%) | 33 (33.3%) | 29 (29.3%) | 62 (62.6%) |
| Dohuk | 100 | 88 (88%) | 36 (36%) | 33 (33%) | 69 (69%) |
| Sulaimanyia | 100 | 70 (70%) | 39 (39%) | 35 (35%) | 74 (74%) |
| Total | 299 | 233 (77.9%) | 108 (36.1%) | 97 (32.4%) | 205 (68.6%) |

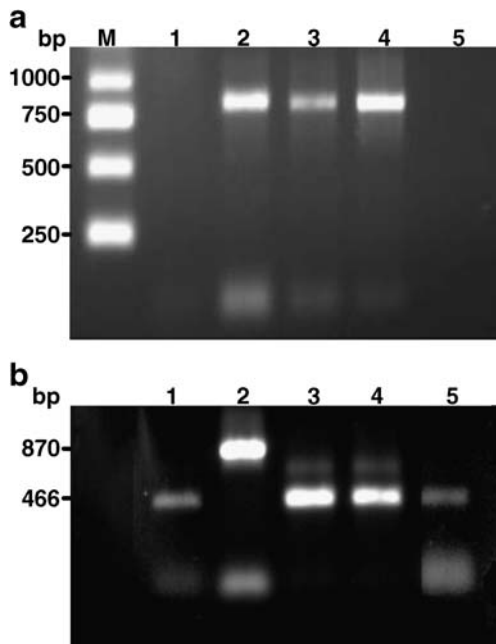


Fig. 1 Agarose gel electrophoresis of the first and second round PCR for detection of *Tams1* gene. **a** Agarose gel electrophoresis analysis of first round PCR amplification for detection of *T. annulata* in four extracted DNA samples by using *Tams1* and *Tams4* primers. Lanes *M* marker, *1* negative control, *2–4*, one positive sample each from Sulaimanyia, Duhok, and Erbil governorates (870 bp), *5* negative sample. **b** Agarose gel electrophoresis analysis of the second round nested PCR amplification for detection of *T. annulata* in DNA samples by using *Tams2* and *Tams3* primers. Lanes *1* positive control for nested PCR (466 bp), *2* positive control for first round PCR (870 bp), *3–5* positive samples in nested PCR (466 bp)

The system of breeding in the Kurdistan Region of Iraq is small herds of cattle moved from the villages to the pasture in the daytime for grazing that are brought back to the villages in the evening. The grazing season extends from March to November, and the animals are kept indoors during the winter season. The shelter of cattle during the indoor time is characterized by poor hygienic conditions, and they are kept together with other animals like sheep and goats. These factors could be the explanation why the Kurdistan Region is an endemic area for many TBDs and other livestock diseases. Regarding TBDs and in particular, infection with *T. annulata*, irregular tick control is practiced by some owners using acaricides, and there is no available vaccine against tropical theileriosis.

The results of the present study revealed a high infection rate among cattle in the three governorates, with 62.6%, 69%, and 74% positive cases among cattle in Erbil, Duhok, and Sulaimanyia governorates, respectively. The statistical analysis revealed a significant difference at a level of $P < 0.05$ between the three governorates of Kurdistan region. The differences in the infection rate with *T. annulata* from area to area may be affected by many factors like climatic condition, susceptibility of breeds, distribution of vector,

system of breeding, vaccination, and strategy of prophylactic and treatment methods. The expectation of presence of this disease in Kurdistan region was pointed out by Omer et al. (2007) in a study on identification of different tick species in the Duhok governorate of Kurdistan region. They observed that *Hyalomma anatolicum anatolicum* and *Hyalomma marginatum marginatum* were the most dominant species infesting cattle and demonstrated that the chance of bovine tropical theileriosis existing in this area may be high. Thus, the high infection rate with *T. annulata* among cattle in this area as shown in this study correlates with the high distribution of *Hyalomma* ticks infesting cattle.

Compared with investigations performed in eastern Turkey, where 37% of cattle were found positive for *T. annulata* when examined by PCR (Dumanli et al. 2005), the results of the present study showed a very high rate of infection with *T. annulata* (68.6%) using PCR in cattle from Erbil, Duhok, and Sulaimanyia governorates of Kurdistan region. An explanation for this high number is found in the transportation of local breed calves and cows from the South and middle parts of Iraq, where the disease is endemic, to the Kurdistan Region for breeding or slaughtering. Hence, 30% of slaughtered cattle in Sulaimanyia governorate were positive for *T. annulata* (Ali Hussein Hassan, personal communication). This animal movement is thought to play a major role for the spread of the disease into the northern part of the country.

Concerning the use of PCR for detection of infection, the application of nested PCR is recommended for determination of more accurate percentages of the infection rate with *T. annulata*. Nested PCR proved to be much more sensitive when compared with single PCR technique for detection of *T. annulata*, indicating that this method is suitable for detection of disease in animals surviving piroplasm infection or carrier animals harboring a very low parasite level.

Concerning the application of PCR and ELISA, a difference in results was seen with 77.9% of cattle positive by ELISA and 68.6% by PCR. In PCR-based tests for direct detection of the parasite, the genetic diversity of organisms according to geographic distribution might have an influence (d'Oliveira et al. 1995). The genetic and antigenic diversity of *T. annulata* has also been reported for the *Tams1* gene used in this study (Gubbels et al. 2000; Katzer et al. 2002). Therefore, organisms from different regions or countries may differ enough in sequence to produce false negative results, and samples should thus be tested for genetic and antigenic diversity in the study region. Since the sequencing results showed some diversity between the different samples collected in the three regions under investigation, this could be a cause for false negative results in this study. With respect to ELISA, the infection rate with *T. annulata* was higher by examination with this

method. The reason may be that the serologic test detected antibodies in animals including carriers and those which recovered from the disease but still harbored the antibodies against the parasite in high titer. This means that serologic tests do not depend on the presence of parasites at the time of collection of blood samples. Thus, these results revealed that the ELISA test can be used to sensitively detect antibodies in the sera of examined animals. The ELISA has the added benefit of being suitable for field application due to its relative simplicity in handling and inexpensiveness compared with PCR-based techniques. In another study, Salih et al. (2007) compared two serological tests (ELISA and IFAT) and microscopic examination for detection of *T. annulata* in cattle. Their results led to the recommendation of ELISA for epidemiological studies and microscopic examination as the convenient test for day-to-day diagnosis of clinical cases in the field.

Based on the findings of this study, further investigations are recommended for detection of *T. annulata* and other pathogens in vector ticks by using PCR technique with a focus on seasonal variation. Further epidemiological surveys are also recommended to determine the prevalence of theileriosis among small ruminants in all governorates of the Kurdistan Region. In addition, it should be attempted to perform phylogenetic analysis studies of different strains and isolates in future studies to determine the phylogenetic tree of the isolates in the region. Assessment and monitoring of the economic impact caused by tropical theileriosis among cattle in the Kurdistan Region is also a subject for future work. Lastly, the apparently high prevalence of tropical theileriosis needs great efforts for planning strategic programs of treatment and vaccination to control or at least minimize the rate of infection.

References

- Ahmed JS, Yin H, Schnittger L, Jongejan F (2002) Ticks and tick-borne diseases in Asia with special emphasis on China. *Parasitol Res* 88:51–55
- Bakheit MA, Schnittger L, Salih DA, Boguslawski K, Beyer D, Fadl M, Ahmed JS (2004) Application of recombinant *Theileria annulata* surface protein in an indirect ELISA for the diagnosis of tropical theileriosis. *Parasitol Res* 92(4):299–302
- Bakheit MA, Seitzer U, Mbatia PA, Ahmed JS (2007) Serological diagnostic tools for the major tick-borne protozoan diseases of livestock. *Parassitologia* 49(S1):53–62
- Burridge MJ, Kimber CD (1973) Duration of serological response to the indirect fluorescent antibody test of cattle recovered from *Theileria parva* infection. *Res Vet Sci* 14:270–271
- d'Oliveira C, van der Weide M, Habela MA, Jacquet P, Jongejan F (1995) Detection of *Theileria annulata* in blood samples of carrier cattle by PCR. *J Clin Microbiol* 13:2665–2669
- Dumanli N, Aktas M, Cetinkaya B, Cakmak A, Koroglu E, Saki CE, Erdogmus Z, Nalbantoglu S, Ongor H, Simsek S, Karahan M, Altay K (2005) Prevalence and distribution of tropical theileriosis in eastern Turkey. *Vet Parasitol* 4:9–15
- Gubbels JM, de Vos AP, van der Weide M, Viseras J, Schouls LM, de Vries E, Jongejan F (1999) Simultaneous detection of bovine *Theileria* and *Babesia* species by reverse line blot hybridization. *J Clin Microbiol* 37:1782–1789
- Gubbels JM, Katzer F, Hide G, Jongejan F, Shiels B (2000) Generation of a mosaic pattern of diversity in the major merozoites piroplasm surface antigen of *Theileria annulata*. *Mol Biochem Parasitol* 110:23–32
- Habibi GR, Esmail-Nia K, Bozorgi S, Najjar E, Hashemi-Fesharki R, Bordbar N (2007) PCR-based detection of *Theileria annulata* infection and molecular characterization of Tams I *T. annulata* vaccine strain. *Arch Razi Inst* 62:83–89
- Katzer F, McKellar S, Ferguson MAJ, d'Oliveira C, Shiels BR (2002) A role for tertiary structure in the generation of antigenic diversity and molecular association of the Tams I polypeptide in *Theileria annulata*. *Mol Biochem Parasitol* 122:55–67
- Kiltz HH, Uilenberg G, Franssen FFJ, Perié NM (1986) *Theileria orientalis* occurs in Central Africa. *Res Vet Sci* 40:197–200
- Latif BM, Hawa NJ, Bakir FA (1977) Incidence of malignant Theileriosis (*Theileria hirci*) of sheep in Iraq. *Iraqi J Vet Med* 1:29–37
- McCosker PI (1979) Global aspects of the management and control of ticks of veterinary importance. *Recent Adv Acarol* 11:45–53
- Muslih NJ, Zangana IK, Arsalan SH (1988) Incidence of various clinical diseases in sheep and goats in north Iraq (Mosul). *Int J Anim Sci* 3:157–163
- Omer LT, Kadir MA, Seitzer U, Ahmed JS (2007) A survey of ticks (Acari: Ixodidae) on cattle, sheep and goats in the Duhok Governorate, Iraq. *Parasitol Res* 101:S179–S181
- Salih DA, Ahmed JS, Bakheit MA, Ali EB, EL Hussein AM, Hassan SM, Shariff OE, Fadl M, Jongejan F (2005) Validation of the indirect TaSP enzyme-linked immunosorbent assay for diagnosis of *Theileria annulata* infection in cattle. *Parasitol Res* 97(4):302–308
- Salih DA, Hassan SM, EL Hussein AM (2007) Comparisons among two serological tests and microscopic examination for the detection of *Theileria annulata* cattle in northern Sudan. *Prev Vet Med* 81:323–326
- Schnittger L, Katzer F, Biermann R, Shayan P, Boguslawski K, McKellar S, Beyer D, Shiels BR, Ahmed JS (2002) Characterization of a polymorphic *Theileria annulata* surface protein (TaSP) closely related to PIM of *Theileria parva*: implications for use in diagnostic tests and subunit vaccines. *Mol Biochem Parasitol* 120:247–256
- Uilenberg G (1981) *Theileria* species of domestic livestock. In: Irvin AD, Cunningham MP, Young AS (eds) *Advances in the control of theileriosis*. Martinus Nijhoff Publishers, The Hague, Boston, London, pp 4–37