Parasitol Res (2005) 97: 281–286
DOI 10.1007/s00436-005-1434-3

ORIGINAL PAPER

Parviz Shayan · Sadegh Rahbari

Simultaneous differentiation between *Theileria* spp. and *Babesia* spp. on stained blood smear using PCR

Received: 9 May 2005 / Accepted: 13 June 2005 / Published online: 9 July 2005

Abstract The tick-borne diseases of livestock constitute a complex of several diseases with different etiological agents. *Theileriosis* and *babesiosis* belong to this complex and are severe and often fatal protozoan tick-borne diseases of ruminants worldwide. This results in high economical losses yearly in Iran. The most common diagnostic method for the identification of piroplasms in Iran is Giemsa staining of blood smear, which is unspecific, accompanied by some technical problems and, in some cases, impossible, due to the carriers. In contrast, immunostaining is more specific and can only be performed with suitably prepared blood smears, but cannot be used also for the carriers. The most specific method for the differential diagnosis of piroplasms is the method of polymerase chain reaction. We extracted DNA from different sources of blood samples, including from already stained blood smears. The extracted DNA was subsequently amplified using specific primers derived from Theileria heat shock protein hsp70, *Theileria lestoquardi ms1-2* gene, Babesia rhoptry protein gene and piroplasms hyper variable region V4 of *18S rRNA* gene. The results show that it is possible to detect piroplasms in already stained blood smears as well enabling a simpler method to be developed for the collection of the samples. Furthermore, it is possible to analyse the already stained and registered blood smears from the patients with unclear differential diagnosis, e.g. in the carriers. In addition, the results revealed that using a primer designed from the hyper variable region V4 of 18S rRNA, it is possible to detect and differentiate simultaneously the genera *Theileria* and *Babesia* in DNA samples isolated from already stained blood smears.

P. Shayan (✉) · S. Rahbari
Pathobiology Group of Faculty of Vetrinary Medicine,
University of Tehran,
Tehran, Iran
E-mail: pshayan@ut.ac.ir

Introduction

The tick-borne diseases of livestock constitute a complex of several diseases with different etiological agents, such as protozoa, rickettsia, bacteria and viruses. The only common feature between these diseases is that they can all be transmitted by ticks. *Theileriosis* and *Babesiosis* belong to this complex and cause diseases in the livestock with high morbidity and mortality thereby resulting in high economical losses worldwide (Barnett 1974a, 1974b; Mehlhorn and Schein 1984; Mehlhorn et al. 1994; Ahmed et al. 2002).

For the long time it was assumed that *Theileria lestoquardi* is the only pathogenic parasite in small ruminants (Luo and Yin 1997). Recently, however, a previously unidentified parasite has been described as a species of *Theileria*, which is pathogenic for small ruminants as well, causing fatal diseases of small ruminants so read over North China (Luo and Yin 1997; Schnittger et al. 2000b, 2000c; Bai et al. 2002).

Interestingly, reviews of tick-borne diseases have made people increasingly aware of this public health problem. *Babesia* Divergens, the main agent of the bovine babesiosis in Europe is not only a cause of significant loss to the cattle industry, but can also infect immunocompromised humans, causing medical emergencies characterized by rapid fulmination and parasitemia that may exceed 70% (Zintle et al. 2003). Recently, a *Babesia*-like organism (WA1) has been detected and characterised as morphologically identical to *Babesia* microti, but biologically and genetically distinct, and more closely related to a known canine pathogen (*Babesia gibsoni*) and to the *Theileria* species than to some members of the genus *Babesia* (Thomford et al. 1994; Persing et al. 1995).

The Giemsa stained of blood smear is the common method for the identification and characterisation of these piroplasms in Iran, which accompanied with some technical problems cause false morphological diagnosis, and in some cases needs special diagnostic knowledge. In

certain cases, serological methods such as the immune fluorescence antibody test (IFAT) or immunoperoxidase test have also been applied (Jianxus and Hung 1997; Leemans et al. 1997; Shayan et al. 1999).

One problem discussed in protozoan infection is the determination and characterization of the transmitter agent. Since many analyses were performed with the salivary gland smear using Methyl-green-puronin-staining method, or Feulgen-staining method, the transfer vector remains unanswered in some cases. Uilenberg (1997) stated that *T. lestoquardi* transmit by Hyalomma anatolicum anatolicum, while some other investigators believe that it is transmitted by Repicephalus bursa or probably also by Repicephalus sanguinius (Dschunkowsky and Urodschevich 1924; Ramzi et al. 2003). Considering the complicated preparation of the samples and their transport to specialized laboratories, we describe an easy method for the preparation of samples involving minimal space, and not requiring special cold storage. Furthermore, we show that the same sample can be used first for Giemsa-staining and than as a source for the extraction of DNA for further genetical analysis. In addition, we show that this material is suitable for the simultaneous detection and differentiation of genera *Theileria* and *Babesia*.

Materials and methods

Materials

We obtained 30 peripheral blood samples from sheep with suspicion of theileriosis or babesiosis. Ten of them were prepared with EDTA and ten samples were fixed with ethanol (1 ml blood/3 ml absolute ethanol), and ten unstained or Giemsa stained blood smears. All tissues had been obtained with consent given according to institutional guidelines.

DNA extraction

DNA extraction using phenol/chloroform

In the case of more than 200 µl blood, erythrocytes were first lysed in 0.155 M NH_4Cl, 0.01 M $KHCO_3$ and 0.1 mM EDTA for 10 min washed twice with PBS at 1000 *g* and the pellet was resolved in 200 µl of 10 mM NaCl, 20 mM Tris–HCl pH 8.0 and 1 mM EDTA. Then 20 µl proteinase K (10 mg/ml) was added and the sample incubated for 10 min at 55°C to digest the proteins. After addition of equal volume of Tris–HCl pH 8.0 saturated phenol, samples were gently vortexed and centrifuged at 12,000 rpm for 15 min. Upper liquid phase was transferred to the clear tube. The last step was repeated once with phenol/chloroform/Isoamylalcohol (25/24/1) to eliminate proteins and once with chloroform/Isoamylalcohol (24/1) to remove rest phenol in the solution. Finally, 6 M Na-acetat (1/10 volume of the sample) and ethanol 96% (2.5 volume of the sample) were added and the samples were incubated for 20 min at −70°C or overnight at −20°C. DNA was than precipitated at 12,000 rpm and, after washing with 70% ethanol, the pellet was dissolved in TE-buffer (10 mM Tris–HCl, 0.1 mM EDTA pH 8.0).

DNA extraction using TriPure isolation reagent

DNA extraction was performed according to the manufacturer's instruction (Roche, Germany). Briefly, 5–10 × 10^6 blood cells were homogenized with 1 ml TriPure Isolation reagent. After addition of 0.5 ml Isopropanol, the suspension was first incubated for 10 min at room temperature, then centrifuged for 10 min at 12,000 *g* at 2–8°C. A 300 µl 96% ethanol was added to the lower and intermediate phases. DNA was precipitated after incubation for 2–3 min at room temperature by centrifugation at 2000 *g* at 2–8°C. The pellet was then washed twice with 0.1 M sodium citrate in 10% ethanol and subsequently dissolved in TE-buffer.

DNA extraction using MBST-kit

In contrast to the above mentioned two methods, this method is based on the specific binding of the DNA to the carrier. Therefore, neither phenol/chloroform nor DNA precipitation was used. For > 200 µl blood, erythrocytes were first lysed in blood samples using Erys-Lysing-Buffer. DNA was extracted using a DNA isolation kit (MBST, Germany/Iran) according to the manufacturer's instructions. Briefly, cells were first lysed in 180 µl lysis buffer and the proteins were degraded with 20 µl proteinase K for 10 min at 55°C. After addition of 360 µl Bindings buffer and incubation for 10 min at 70°C, 270 µl ethanol (100%) was added to the solution and after vortexing, the complete volume was transferred to the MBST-column. The MBST-column was first centrifuged, then washed twice with 500 µl washing-buffer. Finally, DNA was eluted from the carrier with Elution buffer.

DNA extraction from ethanol fixed blood

Ethanol fixed blood was first centrifuged for 20 min at 13,000 rpm at 8°C and then air dried by converting of tube. The pellet material was dissolved in lysis buffer (obtained from Phenol/chloroform method or from MBST kit) using proteinase K for various time intervals, until the solution was homogenized. Finally, DNA was extracted according to the protocol of phenol/chloroform method or the MBST manufacturer's instructions.

DNA extraction from blood smears

Each blood smear was cleaned in separate vessels by a short passage in acetone and ethanol. Approximately, half the blood smear before and after Giemsa staining

was dissolved in 100 µl saturated phenol or 100 µl Tri-Pure reagent or 100 µl Lysis buffer of the MBST kit. DNA was extracted as described above and dissolved in 100 µl TE buffer.

Polymerase chain reaction and seminnested PCR

Approximately 100 to 500 ng DNA, or 5–10 µl DNA solution in the case of extraction from blood smear, was used for the PCR analysis. The PCR was performed on 100 µl total volume including one time PCR buffer, 2.5 U Taq Polymerase (Cina gene, Iran), 2 µl of each primer (20 mM, MWG, Germany), 200 µM of each dATP, dTTP, dCTP and dGTP (Fermenta) and 1.5 mM $MgCl_2$ in automated Thermocycler (Eppendorf, Germany) with the following program: 5 min incubation at 95°C to denature double strand DNA, 35–38 cycles of 45 s at 54–58°C (annealing step), 45 s, at 72°C (extension step) and 45 s at 94°C (denaturing step). Finally, PCR was completed with the additional extention step for 10 min. The PCR products were analysed on 1.8% Agarose gel in 0.5 times TBE buffer and visualized using Ethidium bromide and UV-eluminator.

To control the specificity of the PCR products from the 18s rRNA, seminested PCR technique was used, in which the additional primer is designated within the hyper variable region of the V4 region of *18s rRNA* gene. The primers are listed in the Table 1.

Seminested PCR was performed with the PCR product isolated from agarose gel using the MBST-Kit according to the manufacturer's instructions. Briefly, the DNA bands were cut from the gel under UV control and dissolved in the binding buffer at 60°C. The dissolved agarose was transferred into the MBST-column. After washing, the bound DNA was eluted using 100 µl TE-buffer. A 1–5 µl of the eluted DNA was amplified with the primers P8/P9, P8/P10 and P8/P11 separately.

Results and discussion

The specific PCR products could be amplified corresponding by with the DNA extracted by the three methods.

DNA was extracted from different sources of blood samples from suspected sheep infected with *Theileria* or *Babesia*.using the phenol/chloroform method, Tripure reagent and MBST-Kit. DNA extraction using the Tripure reagent must be modified in some cases such as ethanol fixed blood samples due to the absence of some reagents (Lysis buffer and Proteinase K). While the cell count was more than 10^6, DNA could be extracted and analysed by PCR with all the methods used. DNA isolation from the cells under 10^6 was not effective with the phenol/chloroform method and Tripure reagent, but it was clearly detectable on agarose gel by MBST-Kit. The possible explanation for this is that DNA isolation by MBST kit requires neither the phenol and chloroform

Table 1 The sequences for primers used in PCR from heat shock protein (hsp^{70}), *T. lestoquardi ms1-2* gene, Babesia rhoptry protein gene and hyper variable region V of *18S rRNA* gene and primers for seminesdted PCR from *Theileria* spp. and *Babesia* spp

PCR-product	Nucleotide sequences	Publication references and gene bank code	Name of primer	No.
275 bp	5′ TGTCAAGGAGGCCTCAAATTA 3′	Heat shock protein 70 (hsp^{70}) (Shayan et al. 1998; Schnittger et al. 2000a)	*T. annulata* sense and *T. annulata*-antisense	P1
	5′ TTTGACTTTGAATAGGCTGCC 3′	AY271268 NCBI		P2
669 bp	5′ GTTACTCTCACTTCATGTGAG 3′	*T. lestoquardi ms1-2* gene	*T. lestoquardi*-sense	P3
	5′ GGAGAACTTGTCGACAGCTGG 3′	AJ006448 NCBI	*T. lestoquardi*-antisense	P4
239 bp	5′ CAGGATTGCTTTCGCAACAAAG 3′	Rhoptry protein gene Dalrymple et al. 1993)	*Babesia*-sense	P5
	5′ CCTTGACATAACCGGCGAGG 3′	M91176 NCBI	*Babesia*-antisense	P6
426–430 bp (Theil.)	5′ CACAGGGAGGTAGTGACAAG 3′	Hyper variable region V4 of 18S rRNA (Schnittger et al. 2004)	18S rRNA gene sense	P7
389–402 bp Bab.)	5′ AAGAATTTCACCTCTGACAG 3′	AJ006446 NCBI	*18S rRNA* gene antisense	P8
235 bp	5′ ATTGCTTGTGTCCCTCCG 3′	AY260176 NCBI	Seminested primer for *T. lestoquardi*	P9
181 bp	5′ TGCGCGCGGCCTTTGCGT 3′	AY260178 NCBI	For *B. ovis*	P10
179 bp	5′ GCTTGCTTTTTGTTACTTTG 3′	AY260179 NCBI	For *B. motasi*	P11

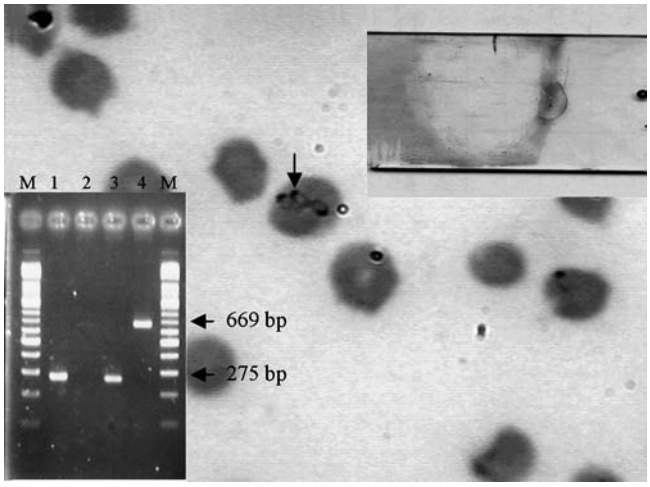

Fig. 1 DNA was extracted from *T. lestoquardi* infected blood smear and amplified in PCR using primer derived from heat shock protein 70 (hsp^{70}) and *T. lestoquardi ms1-2* gene (*Insert upper right*: the same blood smear after DNA isolation, *Insert lower left*: *M* 100 bp Marker, *lane 1* positive control with heat shock protein specific primer, *lane 2* negative control, *lane 3* amplified DNA from blood smear with heat shock protein specific primer, *lane 4* amplified DNA from the same blood smear with *T. lestoquardi* ms1-2 gene)

extraction step nor the ethanol precipitation step and is grounded on the selective binding of nucleic acids to a silica based membrane in the presence of chaotropic salts.

DNA was extracted from Giemsa stained and unstained blood smears from infected and uninfected animals. The extracted DNA was resolved in 100 µl TE buffer then 5 µl of isolated DNA was amplified either with the Primers derived from the heat shock protein of Theileria annulata (hsp^{70}) or with *T. lestoquardi ms1-2* gene or with Babesia rhoptry protein gene (Figs. 1, 2). As a positive control for PCR analysis, DNA from *Theileria annulata*, *T. lestoquardi* infected cell lines and from the erythrocytes of an experimental sheep infected with *Babesia ovis* was used. In some cases, for PCR analysis, the isolated DNA with the Phenol/Chloroform and Tripure methods must be extracted again with Chloroform/Isoamylalcohol (24/1) and/or precipitated with ethanol to avoid the phenol and/or high salt contamination.

The localization of heat shock protein 70 (hsp^{70}) in the mitochondria of Theileia macroschizonts has been shown by Schnittger et al. (2000a). Amplification of mitochondrial *hsp*^{70} gene with the template DNA from infected blood smear revealed that not only the genomic DNA of parasites but also the DNA from other parasite organels could be detectable by MBST-Kit (Fig. 1). The DNA extracted from infected blood smear with *T. lestoquardi*, *Theileria annulata*, *B. ovis* and *Babesia motasi* could be amplified with the common primer pair derived from hyper variable region V4 of 18S rRNA (P7 and P8) (Table 1, Fig. 3). Interestingly, the *Theilerial* hypervariable region V4 of 18S rRNA consists of more nucleotides than in *Babesia* spp. (Schnittger et al. 2004). Therefore, the application of the mentioned primer pair (P7 and P8) could easily and simultaneously differentiate between *Theileria* spp. and *Babesia* spp. The PCR product of *Theileria* spp. and *Babesia* spp. is 426–430 and 389–402 bp, respectively. The difference of ca. 30 bp in the length of the PCR product is easily determinable in 1.8% agarose gel (Fig. 2). Additional primers (P9 for T. lestoquardi, P10 for *B. ovis* and P11 for *B. motasi*) (Schnittger et al. 2004) were designed from the variable region V4 as a sense primer to show that PCR products were species specific (Fig. 3). Seminested PCR of the PCR products has been done with primer P8 as an antisense primer and P9, P10 or P11 as a sense primer. The results showed that the PCR products with primers P7 and P8 were amplifiable with the above mentioned primers and produced the calculated DNA length derived from the data bank. Seminested PCR of the PCR products of *B. ovis* with the primers P8/P11 and *B. motasi* with the primers P8/P10 were amplifiable as well (Fig. 3). It is most probably due to the partial similarity between *B. ovis* and *B. motasi* hypervariable region V4 of *18S rRNA* genes specially with the 3′-ends of the primers. Interestingly, additional repeatable smaller DNA band occurred by seminested PCR with the PCR product of *B. motasi* with the primers P8/P11, its source remaining unknown (Fig. 3).

Control and management of livestock health could be understood as the two sides of a gold coin for a successful and healthy economy in stock-farming. Here, the control of tick-borne diseases plays a prominent role. One of the most important diseases in small ruminant is the infection with protozoan parasites, *Theileria* and

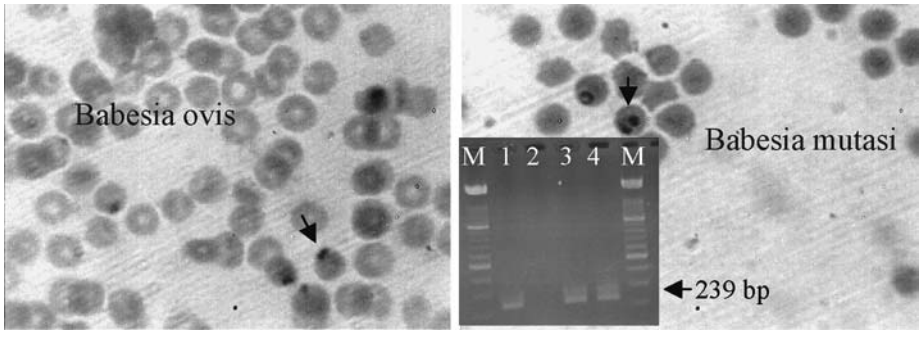

Fig. 2 Extracted DNA from *B. ovis* infected blood smear (*right*) and *B. motasi left*) analysed using primer drived from Rhoptry protein gene. *Insert*: *M* 100 bp marker, *1* positive control, *2* negative control, *3* amplified DNA from *B. ovis* infected blood smear with primers P5/P6, *4* amplified DNA from *B. motasi* infected blood smear with primers P5/P6

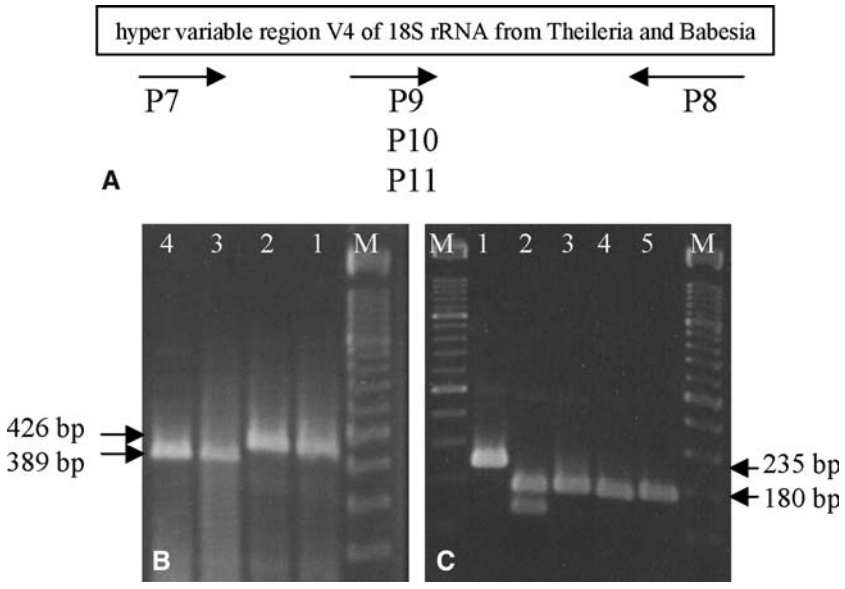

Fig. 3 Extracted DNA from infected blood smear with *T. listoquardi*, *B. ovis*and *B. motasi* analysed with the primers drived from hyper variable region V4 of 18S rRNA. **a** chematicaly demonstration of the partial gene from hyper variable region V4 of 18S rRNA and localization of the different primers for PCR and seminested PCR. **b** Amplification of DNA from blood smear infected with *T. annulata* (*lane 1*), *T. lestoquardi* (*lane 2*), *B. ovis lane 3*) and *B. motasi lane 4*) using primer P7/P8, *M* 100 bp marker. **c** Seminested PCR with the PCR products from B: *Lane 1 T. lestoquardi* PCR product amplified with primer P9/P8, *lane 2 and 3 B. motasi* PCR product amplified with primers P11/P8 and P10/P8, respectively, *Lane 4 and 5 B. ovis* PCR product amplified with primers P10/P8 and P11/P8, respectively

Babesia, which cause annually high economical losses in Iran. Furthermore, reviews of tick-borne diseases has been increasingly recognized worldwide as highlighting this public health problem. *Babesia* Divergens, the main agent of the bovine babesiosis in Europe is not only a cause of significant loss to the cattle industry, but can also infect immunocompromised humans, causing medical emergencies characterised by rapid fulmination and parasitemia that may exceed 70% (Zintle et al. 2003). *B. ovis* was also described as a pathogenic agent in humans (Rios et al. 2003). Taken together, these piroplasms are not only important in the animal health but also in public health. Taking a high number blood samples, cold storage and sending probes to the laboratory are time consuming costly and problematic.

Our results suggest that it is possible to develop new strategies on the basis of blood smear for the collection of samples and sending them to specialized laboratories without the problems of cooling and high transport cost. Furthermore, our results revealed that it is possible to extract the piroplasm's DNA from the already stained blood sample and analyse the DNA using PCR. Therefore, in cases of doubt old registered blood smear can be reanalysed with the described method.

Schnittger et al. (2004) recently described a reverse line blotting method for the simultaneous detection and differentiation of *Theileria* and *Babesia* parasites infecting small ruminants. At present in Iran this method is unpracticable due to the high cost. But our results showed that a common primer derived from hyper variable region V4 of 18S rRNA can be used for simultaneous differentiation of *Theileria* from *Babesia* by PCR on the 1.8% agarose gel in the carrier animals.

Acknowledgment This work was supported by Grand no. 215/777 of university of Tehran and by the Investigating Unit Molecular Biological System Transfer (Iran).

References

Ahmed J, Yin H, Schnittger L, Jongejan F (2002) Ticks and tick-borne diseases in Asia withspecial emphasis on China. Parasitol Res 88:S51–S55

Bai Q, Liu G, Liu D, Ren J, Li X (2002) Isolation and preliminary characterization of a large *Babesia* sp. From sheep and goats in the eastern part of Gansu province, China. Parasitol Res 88:16–21

Barnett SF (1974a) Economical aspects of protozoal tick-borne diseases in livestock in parts of the world other than Britain. Bull Off Int Epiz 81(1–2):183–196

Barnett SF (1974b) Economical aspects of tick-borne disease control in Britain. Bull Off Int Epiz 81(1–2):167–182

Dschunkowsky E, Urodschevich V (1924) Theileriosis in goats, sheep and cattle with description of *Theileria* hirci from Serbia. Parasitology 16:107–110

Jianxus L, Hong Y (1997) Theileriosis of sheep and goats in china. Trop Anim Health Peod 29(Suppl 9):8S–10S

Leemans I, Hooshmand-Rad P, Uggla A (1997) The indirect fluorescent antibody test based on schizont antigen for study of the sheep parasite *Theileria lestoquardi*. Vet Parasitol 69:9–18

Luo J, Yin H (1997) Theileriosis of sheep and goats in China. Trop Anim Health Prod 29:8S–10S

Mehlhorn H, Schein E (1984) The piroplasms: life cycle and sexual stages. Adv Parasitol 23:37–103

Mehlhorn H, Schein E, Ahmed JS (1994) *Theileria*. In: Kreier JP (ed) Parasitic protozoa, vol 7. Academic Press, San Diego, pp 217–304

Persing DH, Herwaldt BL, Glaser C, Lane RS, Thomford JW, Mathiesen D, Krause PJ, Phillip DF, Conrad PA (1995)

Infection with a *Babesia*-like organism in northern California. N Engl J Med 2 332(5):298–303

Ramzi GR, Hosseini M, Aslani MR (2003) Identification of tick vectors of ovine theileriosis in an endemic region of Iran. Vet Parasitol 116:1–6

Rios L, Gonzalo A, Blair S (2003) Serological and parasitological study and report of the first case of human babesiosis in Colombia. Rev Soc Bras Med Trop 36(4):493–498. Epub 2003 Aug 13

Schnittger L, Shayan P, Biermann R, Mehlhorn H, Gerdes J, Ahmed JS (2000a) Molecular genetic characterization and subcellular localization of *Theileria annulata* mitochondrial heat-shok protein 70. Parasitol Res 86:444–452

Schnittger L, Yin H, Jianxun L, Ludwig W, Shayan P, Rahbari S, Voss-Holtmann A, Ahmed JS (2000b) Ribosomal small-subunit RNA gene-sequence analysis of *Theileria lestoquardi* and a *Theileria* species highly pathogenic for small ruminants in China. Parasitol Res 86(5):352–358

Schnittger L, Yin H, Jianxun L, Ludwig W, Shayan P, Rahbari S, Voss-Holtmann A, Ahmed JS (2000c) Phylogenetic analysis by rRNA comparison of the highly pathogenic sheep-infecting parasites *Theileria lestoquardi* and a *Theileria* species identified in China. Ann N Y Acad Sci 916:271–275

Schnittger L, Yin H, Qi B, Gubbels MJ, Beyer D, Niemann S, Jongejan F, Ahmed JS (2004) Simultaneous detection and differentiation of *Theileria* and *Babesia* parasites infecting small ruminants by reverse line blotting. Parasitol Res 92(3):189–196

Shayan P, Biermann R, Schein E, Gerdes J, Ahmed JS (1998) Detection and differentiation of *Theileria annulata* and *Theileria* parva using macroschizont-derived DNA probes. An N Y Acad Sci 29:88–95

Shayan P, Gerlach G, Huegel F-U, Kay G, Ahmed JS (1999) Proliferation-associated nuclear protein Ki-67 in the bovine system: partial characterisation and its application for the determination of the proliferation of *Theileria*-infected bovine lymphoblastoid cells. Parasitol Res 85(8–9):613–620

Thomford JW, Conrad PA, Telford SR, Mathiesen D, Bowman BH, Spielman A, Eberhard ML, Herwaldt BL, Quick RE, Persing DH (1994) Cultivation and phylogenetic characterization of a newly recognized human pathogenic protozoan. J Infect Dis 169(5):1050–1056

Uilenberg G (1997) General review of tick-borne diseases of sheep and goats worldwide. Parasitology 39:161–165

Zintle A, Mulcahy G, Skerrett HE, Taylor SM, Gray JS(2003) *Babesia* divergens, Bovine blood parasite of veterinary and zoonotic importance. Clin Microbiol Rev 16(4):622–636