

Detection of theileriosis in cattle and buffaloes by polymerase chain reaction

V. R. Kundave · A. K. Patel · P. V. Patel ·
J. J. Hasnani · C. G. Joshi

Received: 2 July 2013 / Accepted: 29 October 2013 / Published online: 19 November 2013
© Indian Society for Parasitology 2013

Abstract Bovine tropical theileriosis caused by *Theileria annulata* is a tick-borne disease of great economic importance in tropical and subtropical regions of the world. The present study was undertaken to detect theileriosis in cattle and buffaloes by polymerase chain reaction (PCR). The diagnosis of theileriosis is usually carried out by blood smear staining technique, which is not sufficiently sensitive to detect the piroplasms in the carrier animals. In this study, a total of 116 samples were collected from infected as well as apparently healthy cattle and buffaloes. Screening of blood smears by Giemsa staining detected 15 samples (12.93 %) positive for *Theileria* piroplasms out of 116 samples. However, the PCR based screening using the specific primers from the major merozoite-piroplasm surface antigen sequence of *T. annulata* (Tams1) gene detected 74 samples (63.79 %) positive for *T. annulata* which included 59 samples found negative by Giemsa staining. Our study suggests that the PCR based screening is more sensitive and accurate method for diagnosis of tropical theileriosis in cattle and buffaloes.

Keywords *Theileria annulata* · PCR · Sensitive · Cattle · Buffalo

Introduction

In tropical countries like India, ticks are major havoc to the livestock since they cause damage to the health, and also act as a vector for the transmission of haemoprotozoan diseases such as theileriosis, babesiosis and anaplasmosis. The exotic (*Bos taurus*) and crossbred cattle, are known to be the highly susceptible group, while the indigenous cattle possess inherent resistance to disease (El Hussein et al. 1991). Cross-breeding programmes in India, was an initiative taken towards the genetic improvement of the livestock, but its dark side was the decreased resistance to ticks and tick-borne diseases (Uilenberg et al. 1982). Brown (1997) reported a loss of US \$ 800 million per annum due to tropical theileriosis at global level. The annual cost of *T. annulata* infection in India was estimated to be 384.3 million US\$ (Minjauw and McLeod 2003). Loss of productivity and mortality in theileriosis causes great economic loss (Campbell et al. 1999; Razmi et al. 2003). *Theileria* are small round, ovoid, irregular or bacilliform shaped parasites, which are placed in the Phylum Apicomplexa, Sub-class Piroplasmorina, Order Piroplasmiorina and Family Theileriidae. Clinically inapparent to rapidly fatal symptoms have been recorded in theileriosis (Darghouth et al. 1996).

The diagnosis of theileriosis in acute cases is mainly relied upon clinical findings and microscopic examination of stained thin blood smears. However the native and treated cattle turn out to be long standing carriers, with only a few number of infested erythrocytes, thus posing difficulty in the demonstration of parasite in blood smear (Nayel et al. 2012). Such long standing carriers act as the major contributors of infection through the ticks. Transport of carrier cattle to non-endemic areas can lead to disease outbreak (Bilgic et al. 2013). Hence, detection of

V. R. Kundave (✉) · P. V. Patel · J. J. Hasnani
Department of Veterinary Parasitology, College of Veterinary
Science and Animal Husbandry, Anand Agricultural University
(AAU), Anand 388 001, Gujarat, India
e-mail: kun.vet@gmail.com

A. K. Patel · C. G. Joshi
Department of Animal Biotechnology, College of Veterinary
Science and Animal Husbandry, Anand Agricultural University
(AAU), Anand 388001, Gujarat, India

piroplasms in carrier animals becomes more challenging. The schizonts and piroplasms lack a clear morphological distinction between the different *Theileria* species and thus complicates the species differentiation on blood slides (Dolan et al. 1984). Moreover smear method is associated with false negative results and has low sensitivity in detecting the carrier cattle (Nayel et al. 2012).

The advent of molecular diagnostic tests like polymerase chain reaction (PCR) has paved way to efficient diagnosis than the conventional techniques (Collins et al. 2002). Based on the studies performed on a wide range of parasites it has been concluded that PCR is more sensitive than the conventional techniques (Bishop et al. 1992; Tahar et al. 1997). Bekker et al. (2002) developed PCR, reverse line blot assay and DNA probes for the identification of *Theileria* species. Four diagnostic tests which includes, blood and lymphnode biopsy smear examination and PCR of blood and lymphnode biopsy sample were developed for the detection of *T. annulata*. Among them the PCR assay was found to be sensitive and accurate than the microscopic examination (Dehkordi et al. 2012). PCR helps to arrive at an accurate diagnosis of theileriosis (Zaemi et al. 2011). The objective of the study was to assess the presence and accurately diagnose theileriosis in cattle and buffaloes by PCR.

Materials and methods

Collection of blood samples

A total of 91 blood samples from cross bred cow and 25 samples from buffaloes were collected from animals showing clinical signs suggestive of theileriosis like anorexia, pyrexia, drop in milk production, tick infestation, lymphnode enlargement, pale mucous membrane, suspended rumination, bilateral nasal discharge, lacrimation etc., and also from apparently healthy ones. The blood smears were prepared from blood collected from jugular vein according to the method described by Soulsby (1982). In order to carry out the polymerase chain reaction blood samples, 3 ml each were collected in EDTA coated vacutainers.

Giemsa staining

Thin blood smears were prepared and stained using Giemsa stain. The parasites were identified according to the characters described by Soulsby (1982).

Isolation of DNA from blood samples

Genomic DNA was extracted from 200 µl of the whole blood using a DNA extraction kit (HiPura, Himedia)

according to the manufacturer's instructions. The isolated DNA was quantified spectrophotometrically and run on 0.8 % agarose gel. Aliquots of extracted DNA were stored at -20°C until further use.

Polymerase chain reaction

The primers were designed based on the coding sequence of the major merozoite surface antigen (Tams 1 gene) of *T. annulata*. The forward primer sequence is 5'-CCAG GACCACCTCAAGTTC-3' and the reverse primer sequence is 5'-GCATCTAGTTCCTTGGCGGA-3'. The PCR reaction was performed in a total volume of 15 µl containing 30 ng of template DNA, 7.5 µl of 2× Master mix (Fermentas), 0.5 µl of each forward and reverse primer (10 pmol/µl), 5.5 µl of nuclease free water. Reactions were initiated at 95 °C for 5 min, followed by 37 cycles of 95 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s and a final elongation step at 72 °C for 5 min, with a final hold at 4 °C in a thermal cycler (Veriti). A negative control (sterile water), and a positive control DNA from a *T. annulata*, were included in each amplification run.

Agarose gel electrophoresis

Amplified samples were analyzed by electrophoresis (120 V/208 mA) in 1.5 % agarose gel. Positive and negative controls were run along with each series of amplifications. The gel was stained with ethidium bromide (0.5 µg/ml) and viewed on a UV transilluminator (Gel Doc, Syngene).

Statistical analysis

Chi square test was done to test the association between blood smear examination and PCR.

Results

Blood smear examination

The stained blood films revealed the presence of *Theileria* piroplasms, forms with diameter of 0.5–1.5 micrometer (Fig. 1) in 15 samples (12.93 %) of cattle.

Polymerase chain reaction

The PCR based screening using the specific primers from the major merozoite-piroplasm surface antigen sequence of *Theileria annulata* (Tams1) gene detected 74 samples (63.79 %) positive for *T. annulata* which included 59 samples found negative by Giemsa staining. Out of 74

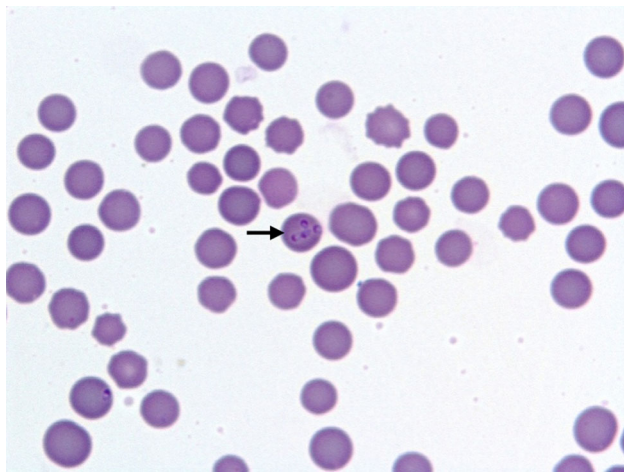


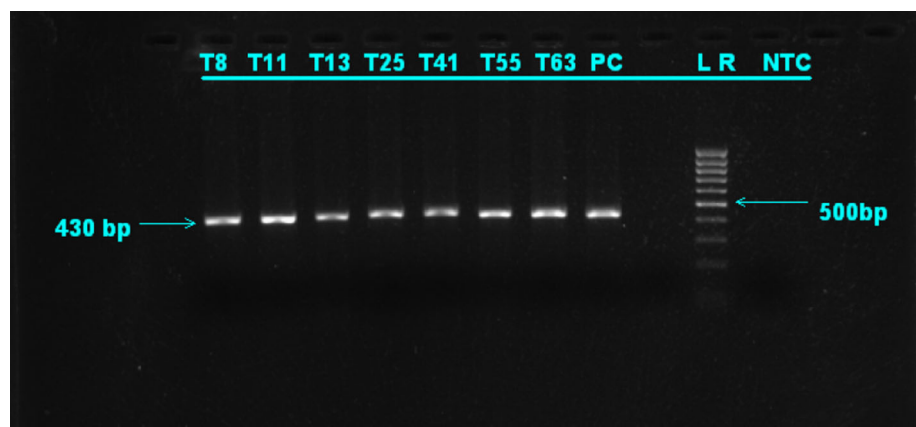
Fig. 1 Piroplasmic forms of *T. annulata* in a microscopic field by Giemsa staining method

Table 1 The age wise prevalence in cattle by PCR

Age	Number of animal screened	Total positive by PCR	Total number of negative	Total positive percent
<1 year	7	1	6	14.28
1–2 year	8	6	2	75.00
2–5 year	8	3	5	37.50
>5 year	68	56	12	82.35
Total	91	66	25	72.52

samples that were positive by PCR, 66 were of cattle and 8 samples were of buffaloes. The age wise prevalence in cattle when screened by PCR is given in Table 1. Highest prevalence was recorded in cattle above 5 years of age (82.35 %) and the lowest prevalence was recorded in calves less than 1 year of age (14.28 %). A desired product size of 430 bp was obtained in *T. annulata* positive samples (Fig. 2). The 2 × 2 contingency table for blood smear examination and PCR is shown in Table 2.

Fig. 2 PCR products run on 1.5 % agarose gel positive samples showing amplified product of 430 bp. Lane 1 Sample 8 (positive), lane 2 sample 11 (positive), lane 3 sample 13 (positive), Lane 4 sample 25 (positive), lane 5 sample 41 (positive), lane 6 sample 55 (positive), Lane 7 sample 63 (positive), lane 8 positive control, lane 10 DNA ladder (mass ruler low range), Lane 11 negative control



Statistical analysis

The observed value of Chi Square was 8.76. Since this value is more than 3.841, the value of Chi square is at 5 % level of significance, it is significant and therefore, it gives sufficient grounds to accept that PCR is more efficient in diagnosis of theileriosis compared to microscopic examination.

Discussion

Tropical theileriosis often leads to a severe and fatal outcome. Theileriosis infection in cattle is characterized by clinical signs like anorexia, emaciation, depressed rumination, lacrimation, corneal opacity, nasal discharge, diarrhea, terminal dyspnoea, frothy nasal discharge (Fukasawa 2003). The clinical signs and microscopic examination are usually relied upon for the diagnosis of piroplasmic infection since it is easy and less expensive. However, the lack of adequate sensitivity and accuracy of staining method may lead to false diagnosis. Though serological tests are being used for detection of latent infection, chances of false positive and negative results are likely to occur (Saeid et al. 2013). The PCR method is more accurate in comparison with Immunosorbent assays (ELISA), Immunofluorescent Antibody Test (IFAT) and Indirect Haemagglutination Assay (IHA) as well as microscopic detection of piroplasmic forms (Gubbles et al. 2000).

The figure obtained in the current study is similar to the findings of Hoghooghi et al. (2011) who showed the prevalence of theileriosis to be 6.25 % (10 positive cases out of 160 samples) by the staining method. The prevalence of *Theileria* infection by PCR assay was found to be 70 % (21 out of 30) while by Giemsa staining method was 30 % (9 out of 30) in a study conducted by Mahmmod et al. (2010); Roy et al. (2000) also reported that the number of positive cases of theileriosis out of 50 blood

Table 2 2 × 2 Contingency table for blood smear and PCR

	Blood smear examination		Total
	Positive	Negative	
PCR			
Positive	15 ^a	59 ^b	74
Negative	0 ^c	42 ^d	42
Total	15	101	116

^a True positive^b False positive^c False negative^d True negativeSensitivity of PCR = $a/a + c \times 100 = 100 \%$ Specificity of PCR = $d/b + d \times 100 = 41.58 \%$

samples of native cattle by PCR and smear method were 22 (44 %) and 8 (16 %), respectively. Saeid et al. (2013) found that 68 of 150 (45.33 %) of the carrier cattle were positive by PCR. The results obtained in our study indicates that PCR is more efficient in detecting theileriosis than the conventional staining technique and is in agreement with previous studies (Mahmmod et al. 2010; Roy et al. 2000; Hooghoghi et al. 2011) and Saeid et al. (2013). Bilgic et al. (2013) developed a multiplex PCR assay for simultaneous detection of *T. annulata*, *Babesia bovis*, *Anaplasma marginale* and found this to be an effective diagnostic tool.

The primers used in our study were designed to amplify the Tams1 gene encoding the 30 kDa major *T. annulata* merozoite surface antigen. Kirvar et al. (2000) had used the 30 kDa merozoite surface protein gene as detective target sequence by designing specific primer to amplify only *T. annulata*, which is comparable to the target used in our study. Detection of *T. annulata* was carried out using primers specific for Tams1 gene and the PCR assay found to be sensitive and specific (Haibibi et al. 2007). Mahmoud et al. (2011) studied, the efficiency of two primer sets, primer set one (N516/N517) and primer set two (Tams1F/Tspm1R) to amplify the 30 kDa major merozoite surface antigen gene for the diagnosis of *T. annulata* infection in cattle, and concluded that primer set Tams1F/Tspm1R is the first choice when diagnosis of *T. annulata* is concerned. This justifies the reason for using primers specific for the Tams1 gene in our study, and also shows the efficacy of PCR technique for confirmational diagnosis of theileriosis.

Ananda et al. (2009) recorded a higher prevalence of *T. annulata* in cattle of 4–6 years of age, which is in agreement with the present study in which highest prevalence was recorded in cattle above 5 years of age. The physiological factors like oestrus, pregnancy and lactation leads to temporary suppression in immunity which in turn leads to the increased rate of disease occurrence in adult

cattle as suggested by (Durrani 2003). Antibodies to sporozoites, schizonts and piroplasms have been recorded in the colostrum of immune cows and the serum of their calves which protects the calves against theileriosis could be the reason for low prevalence in calves less than a year in our study (Morzaria et al. 1988). The resistance of young animals to *T. annulata* as compared to adults reported in the present study was also in accordance with findings of Utech and Wharton (1982) who reported that young cows were more resistant than older cows. The age-related resistance in young cattle to most tick-borne protozoan and rickettsial diseases has been reported by Bailey (1955) and Dumanli et al. (2005).

In our study the Giemsa stained blood smears had shown false negative in visual examination under light microscope, which shows low sensitivity of this test. It may be due to several reasons like the visual mistakes made during the examination of slides, very low parasitaemia, destruction of piroplasmic forms in red blood cells due to hemolysis, the thickness, dirtiness or unsuitable blood smear staining (Hoghooghi et al. 2011). Moreover, the microscopic detection of piroplasms in samples that were negative by PCR tests was not possible. This fact, confirms the superiority of PCR over blood smear examination. Statistical comparison of blood smear and PCR had shown significant difference at 5 % level. Considering blood smear examination as the gold standard assay, the sensitivity of PCR method was found to be 100 %. The present study findings are in agreement with Azizi et al. (2008) who reported that sensitivity and accuracy of PCR in detection *T. annulata* was superior to the blood smear examination. Sanchez et al. (1999) verified the utility of this technique in epidemiological studies and compared it with conventional diagnostic techniques and reported that PCR was more sensitive for detection of *T. annulata* infections.

Drug therapy causes drop in the parasitaemia and hinders accurate diagnosis (Ahmed and Mehlhorn 1999; Glass 2001). In such cases, PCR assays can be used to detect low parasitaemias in the blood of carrier cattle. Durrani (2003) found that overall comparative efficacy of PCR test was highest 31.6 %, followed by microscopic lymph node smear examination 8.25 %, and microscopic blood smear examination 6 %, in diagnosis of field challenges of theileriosis. Thus it is confirmed that PCR test is more sensitive in detecting low grade of infections in carrier animals and hence is more suitable for epidemiological surveys as compared with microscopic blood and lymph node smear examination. Similar observations were made by Christine (1995) who suggested that carriers are important contributors to the infection within ticks.

The difficulties faced in detection and differentiation of *Theileria* piroplasms by conventional method is overcome by the molecular methods like PCR. The high efficacy and

sensitivity of PCR makes it an attractive tool in diagnosing the tick-borne infections which is in accordance with Olivier et al. (1999) and (Aktas et al. 2006). Hence, our study clearly proves that PCR can be used for accurate diagnosis of theileriosis, and can also be used to detect the carrier animals, which serve as a potential source of infection to the healthier groups through the infected ticks.

References

- Ahmed JS, Mehlhorn H (1999) Review: the cellular basis of the immunity to and immunopathogenesis of tropical theileriosis. *Parasitol Res* 85:539–549
- Aktas M, Altay K, Dumanli N (2006) A molecular survey of bovine theileria parasites among apparently healthy cattle and with a note on the distribution of ticks in eastern Turkey. *Vet Parasitol* 138:179–185
- Ananda KJ, D'Souza PE, Puttalahamma GC (2009) Prevalence of haemoprotzoan diseases in crossbred cattle in Bangalore North. *Vet World* 2(1):15–16
- Azizi H, Shiran B, Farzaneh Dehkordi A, Salehi F, Taghadosi C (2008) Detection of *Theileria annulata* by PCR and its comparison with smear comparison with smear method in native carrier cows. *Vet Parasitol* 99:249–259
- Bailey WS (1955) Veterinary parasite problems. *Public Health Rep* 70:976–982
- Bekker CP, De Vos S, Taoufik A, Sparagano OA (2002) *Anaplasma* and *Ehrlichia* species in ruminants and detection of *Ehrlichia ruminantium* in *Amblyomma variegatum* ticks by reverse line blot hybridization. *Vet Microbiol* 89:223–238
- Bilgic BH, Karagenc T, Simuunza M, Shiels B, Tait A, Eren H, Weir W (2013) Development of multiplex PCR assay for simultaneous detection of *Theileria annulata*, *Babesia bovis* and *Anaplasma marginale* in cattle. *Exp Parasitol* 133:222–229
- Bishop R, Sohanpal B, Kariuki DP, Young AS, Nene V, Baylis H, Allsopp BA, Spooner PR, Dolan TT, Morzaria SP (1992) Detection of a carrier state in *Theileria parva*-infected cattle by the polymerase chain reaction. *Parasitol* 104:215–232
- Brown CGD (1997) Dynamics and impact of tick borne diseases of cattle. *Trop Anim Health Prod* 29:15–35. doi:10.1007/BF02632905
- Campbell JDM, Spooner RI (1999) Macrophages behaving badly: infected cells and subversion of immune responses to *Theileria annulata*. *Parasitol Today* 15(1):10–15
- Christine O (1995) Detection of *Theileria annulata* in blood samples of carrier cattle by PCR test. *J Clin Microbiol* 77:266–269
- Collins NE, Allsopp MT, Allsopp BA (2002) Molecular diagnosis of theileriosis and heartwater in bovines in Africa. *Trans R Soc Trop Med Hyg* 96(Suppl 1):S217–S224
- Darghouth MEA, Bouattour A, Ben Miled L, Sassi L (1996) Diagnosis of *Theileria annulata* infection of cattle in tunisia: comparison of serology and blood smears. *Vet Res* 27:613–627
- Dehkordi FS, Parsaei P, Saberian S, Moshkelani S, Hajshafei P, Hoseini SR, Babaei M, Ghorbani MN (2012) Prevalence study of *Theileria annulata* by comparison of four diagnostic techniques in southwest Iran. *Bulg J Vet Med* 15:123–130
- Dolan TT, Teale AJ, Stagg DA, Kemp SJ, Cowan KM, Young AS, Grocock CM, Leitch BL, Spooner RL, Brown CG (1984) A histocompatibility barrier to immunization against east coast fever using *Theileria parva*-infected lymphoblastoid cell lines. *Parasite Immunol* 6:243–250
- 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* 127:9–15
- Durrani AZ (2003) Epidemiology, serodiagnosis and chemoprophylaxis of theileriosis in cattle. University of Veterinary and Animal Sciences, Lahore
- El Hussein AM, Mohamed SA, Osman AK, Osman OM (1991) A preliminary survey of blood parasites and brucellosis in dairy cattle in northern state. *Sudan J Vet Res* 10:51–56
- Fukasawa M, Kikuchi T, Konashi S, Nishida N, Yamagishi T (2003) Assessment of criteria for improvement in *Theileria orientalis sergenti* infection tolerance. *Anim Sci J* 74:67–72
- Glass EJ (2001) The balance between protective immunity and pathogenesis in tropical theileriosis: what we need to know to design effective vaccines for the future. *Res Vet Sci* 70:71–75
- Gubbles MJ, Hong Y, Weide MVD, Qi B, Nijman IJ, Guangyuan L, Jongejan F (2000) Molecular characterization of the *Theileria buffeli/orientalis* group. *Int J Parasitol* 30:943–952
- Habibi GR, Esmail NK, Bozorgi S, Najjar E, Hashemi FR, Bordbar N (2007) PCR-based detection of *Theileria* infection and molecular characterization of Tams 1 *T. annulata* vaccine strain. *Arch Razi Inst* 62:83–89
- Hoghooghi-Rad N, Ghaemi P, Shayan P, Eckert B, Sadr-Shirazi N (2011) Detection of native carrier cattle infected with *Theileria annulata* by semi-nested PCR and smear method in Golestan Province of Iran. *World Appl Sci J* 12(3):317–323
- Kirvar E, Ilhan T, Katzer F, Hooshmand-rad P, Zwegarth E, Gerstenberg C, Phipps P, Brown CG (2000) Detection of in cattle and vector ticks by PCR. *Parasitol* 120(3):245–254
- Mahmmod YS, El-Balkemy FA, Yuan ZG, El-Mekki MF, Monazie AM, Zhu XQ (2010) Field evaluation of PCR assays for the diagnosis of tropical theileriosis in cattle and water buffaloes in Egypt. *J Ani Vet Adv* 9(4):696–699
- Mahmoud R, Ellah Abd, Amira AT, Hosary AL (2011) Comparison of primer sets for amplification of 30 kDa merozoite surface antigen of bovine theileriasis. *J Ani Vet Adv* 10(12):1607–1609
- Minjauw B, McLeod A (2003) Tick-borne diseases and poverty. The impact of ticks and tick-borne diseases on the livelihood and marginal livestock owners in India and Eastern and Southern Africa Research report, DFID Animal Health Programme, Centre Of Tropical Veterinary Medicine, University of Edinburgh
- Morzaria SP, Musoke AJ, Latif AA (1988) Recognition of theileria parva antigens by field sera from Rusinga, Kenya (Abstract). *The Kenya Vet* 12(2):88–90
- Nayel M, El-Dakhly KM, Aboulaila M, Elsify A, Hassan H, Ibrahim E, Salama A, Yanai T (2012) The use of different diagnostic tools for *Babesia* and *Theileria* parasites in cattle in Menofia, Egypt. *Parasitol Res* 111:1019–1024
- Olivier AE, Gubbels MJ, Guido R, Alexander P, Jongejan F (1999) Proceedings of the integrated molecular diagnosis of *Theileria* and *Babesia* Species of cattle in Italy. *Stvm*, 5th Biennial Conf. Society For Tropical Veterinary Medicine
- Razmi GR, Hossini M, Aslani MR (2003) Identification of tick vectors of ovine theileriosis in an endemic region of Iran. *Vet Parasitol* 116:1–6
- Roy KC, Ray D, Bansal GC, Singh RK (2000) Detection of *Theileria annulata* carrier cattle by PCR. *Indian J Exp Biol* 38:283–284
- Saeid R, Fard N, Khalili M, Ghalekhani N (2013) Detection of *Theileria annulata* in blood samples of native cattle by PCR and smear method in southeast of Iran. *JOPD*. doi:10.1007/s12639-013-0333-2
- Sanchez MJ, Viseras J, Adroher FJ, Garcia Fernandez P (1999) Nested polymerase chain reaction for detection of *Theileria annulata* and

- comparison with conventional diagnostic techniques: its use in epidemiology studies. *Parasitol Res* 85:243–245
- Soulsby EJJ (1982) Helminths, arthropods and protozoa of domesticated animals, 7th edn. Baillier Tindall and Cassel Ltd, London
- Tahar R, Ringwald P, Basco LK (1997) Diagnosis of *Plasmodium malariae* infection by the polymerase chain reaction. *Trans R Soc Trop Med Hyg* 91:410–411
- Uilenberg G, Perie NM, Lawrence JA, de Vos AJ, Paling RW, Spanjer AA (1982) Causal agents of bovine theileriosis in southern Africa. *Trop Anim Health Prod* 14:127–140
- Utech KB, Wharton RH (1982) Breeding for resistance to *Boophilus microplus* in Australian illawarra shorthorn and Brahman x Australian illawarra shorthorn cattle. *AVJ* 58:41–46
- Zaemi M, Haddadzadeh H, Khazraiiinia P, Kazemi B, Bandehpour M (2011) Identification of different *Theileria* species (*T. lestoquardi*, *T. ovis*, and *T. annulata*) in naturally infected sheep using nested PCR-RFLP. *Parasitol Res* 108:837–843