Parasitological and molecular detection of bovine babesiosis in endemic areas of Karnataka state

Article *in* The Indian journal of animal sciences · April 2017

CITATIONS
0

READS
165

1 author:

P.P. Sengupta
Indian Council of Agricultural Research
52 PUBLICATIONS **185** CITATIONS

SEE PROFILE

Indian Journal of Animal Sciences **87** (4): 422–426, April 2017/Article

Parasitological and molecular detection of bovine babesiosis in endemic areas of Karnataka state

K U VIJAYAKUMAR[1], G C PUTTALAKSHMAMMA[2], PLACID E D'SOUZA[3], P P SENGUPTA[4], B M CHANDRANAIK[5] and C RENUKAPRASAD[6]

Veterinary College, Hebbal, Bengaluru, Karnataka 560 024 India

Received: 21 July 2016; Accepted: 12 September 2016

ABSTRACT

A study was conducted to compare the specificity and sensitivity of parasitological methods (Giemsa and Fluorescent acridine orange staining) and PCR to detect the carrier status, subclinical and clinical babesiosis in cattle from endemic regions of Karnataka state in South India. Out of 148 cattle screened, 3 (2.03%), 7 (4.73%) and 16 (10.81%) were found positive for *B. bigemina* infection by Giemsa's staining, acridine orange staining and PCR, respectively. The conserved primers for *B. bigemina* derived from *SpeI-AvaI* restriction fragment were used in PCR which amplified a fragment of 278 bp in the positive cattle blood and was 83.26% sensitive and 91.16% specific for detection of babesiosis in cattle.

Key words: Acridine orange staining, Bovine babesiosis, *Babesia bigemina*, Giemsa, Fluorescent, PCR

Bovine babesiosis an important disease entity in livestock health and production. The diagnosis of ruminant piroplasmosis in acute cases is routinely done by microscopic examination of Giemsa stained blood smears prepared from the suspected cases based on typical clinical symptoms (Gupta *et al.* 2005). The carrier and subclinical infections of babesiosis are difficult to detect because of the low number of parasites in circulation and lack of sensitivity and specificity of conventional microscopic and serological techniques (Sharma *et al.* 2013, Sharma *et al.* 2016). Hence, early diagnosis and subsequent treatment of these infections in carrier animals is important to overcome the economic losses as well as to prevent transmission. Molecular detection based on nucleic acid identification and their amplification are the most sensitive and reliable techniques to accurately detect carrier and sub clinical infections (Bose *et al.*1995). Therefore, a study was undertaken to compare the specificity and sensitivity of PCR along with routine parasitological methods (Giemsa and acridine orange staining) to ascertain the status of carrier, clinical and subclinical babesiosis in cattle in endemic regions of Karnataka state in South India. Haemolymph test is a good technique for detection of kinete stages in the adult female ticks infected with *Babesia.* Early detection of babesia infection in ticks is critical to take up control measures as well as to prevent transmission by vectors from carrier animals.

Present address: [1]Veterinary Officer, Veterinary Dispensary Hatti(A), Koppal,Department of Animal Husbandry and Veterinary Services, Government of Karnataka. [2]Associate Professor (puttalakshmamma @gmail.com); [3]Professor and Head, Department of Veterinary Parasitology. [4]Principal Scientist, NIVEDI, Bengaluru.[5]Scientist, Centre for Animal Disease Diagnostic Laboratory, IAH&VB, Bengaluru.[6]Vice-chancellor, KVAFSU, Bidar.

MATERIALS AND METHODS

Blood samples (148) were collected in 10 ml EDTA vacutainers from cattle in endemic regions viz. Bangalore Rural, Chikkaballapur and Kolar districts of Karnataka state, South India. Two thin blood smears were prepared; out of two, one smear was used for Giemsa staining and another for acridine orange staining.

Giemsa staining: Thin blood smears were immediately air dried and fixed in methanol for one minute and labelled.The smear was stained immediately with Giemsa stain (Himedia) diluted at the ratio of 1:5 with phosphate buffer saline of pH 6.8–7.2 for better differential staining of blood. Acridine orange (AO) stain (0.01%) was prepared by adding 20 mg AO powder to 190 ml sodium acetate buffer. Stock solution of the sodium acetate buffer was prepared by adding 13.6 g of sodium acetate to 100 ml of distilled water and 90 ml of 1N HCI. The final pH was adjusted to 3.5 by adding 1N HCl. The blood smears were stained with AO stain as per the methodology followed by Ravindran *et al.* (2007a). Briefly, the methanol fixed blood smears were flooded with 0.01% AO stain and allowed for 2 min and then washed slowly in tap water. The smears were mounted with a cover slip and examined when moist, under a fluorescent microscope. A drop of glycerol saline (1:1) was applied over the cover slip before examination.

The ticks were also collected from the animal body for identification. Haemolymph smears were prepared as described by Toni *et al.* (2012). The legs of the tick were amputed using BP blade at the distal joint/coxa-tibial joint after surface sterilization with 70% ethanol. After the legs were cut, gentle pressure was applied to the ticks dorsum for the haemolymph to ooze out of the legs onto the slide and tick was gently moved out of the circle allowing the haemolymph to spread on the slide. Since the drop was too small to make a smear, it was allowed to dry after spreading. Haemolymph smears were labelled and fixed in methanol for 1 min. Stained with 10% Giemsa stain for 30 min. The smears were examined under oil immersion for kinetes.

The blood sample was stored at 4°C for DNA extraction. The genomic DNA was extracted from the 200 µl of cattle blood using the "QIAamp DNA minikit" of Qiagen, Germany as per standard protocol with slight modifications. The extracted DNA was stored at –20°C till further use. The genus and species specific PCR was done by targeting *SpeI-AvaI* restriction fragment of *B. bigemina*. The published *B. bigemina* specific primers (Figueroa *et al.* 1992) were synthesized by Eurofins Biotech, Bengaluru. The nucleotide sequences of primers are Forward 5'-CAT CTAATT TCT CTC CATACC CCTCC-3'; Reverse 5'-CCT CGG CTT CAA CTC TGA TGC CAA AG-3'. The amplification reactions were carried out with a reaction mixture volume of 25 µl containing 12.5 µl of PCR master mix, Primer (20 pm) 1 µl each, 5 µl template DNA and 5.5 µl nuclease free water and the reaction mixture was pre-incubated at 95°C for 5 min for initial denaturation of DNA. This was followed by denaturation for 1 min at 95°C, annealing for 1 min at 60°C for 35 cycles and extension for 1.5 min at 73°C with final extension at 73°C for 15 min. The DNA from the blood sample of cattle showing clinical symptoms of babesiosis and which were positive for *B. bigemina* by Giemsa's and acridine orange blood smear examination was used as a known positive control. DNA from one month old healthy calf blood was used as negative control and sample without template DNA was used as a no template control (NTC).The amplified DNA fragments were analyzed after electrophoresis on 1% agarose gel.

The six positive PCR products (two from each district) were purified by using QIA quick gel extraction kit as per the manufacturer protocol.The PCR products were sequenced at Amnion Biosciences, Bangalore. The nucleotide sequences were aligned and checked for homology using Basic Local Alignment Search Tool (BLAST) (http://www.ncbi.nlm.nih.govt/BLAST). The phylogenetic analysis and pair wise distance analysis of the nucleotide sequences of present study in relation to nucleotide sequences available in the GenBank was done with MEGA 6 software.

RESULTS AND DISCUSSION

Blood samples (148) from 34 clinical/suspected cattle and 114 healthy/tick infested cattle in 11 endemic taluks of three districts were screened by Giemsa staining, fluorescent staining and by polymerase chain reaction. The results of comparison of three diagnostic techniques for detection of bovine babesiosis are tabulated in Table 1. The overall prevalence of infection by Giemsa staining, fluorescent staining and PCR methods was 2.03, 4.73 and 10.81% respectively.

The *B. bigemina* organisms in Giemsa stained thin blood smears were characteristically pear shaped and in pairs forming an acute angle in the red blood cells (Fig. 1). Giemsa staining method detected only 18.75% (3/16) of clinical cases of *B. bigemina* infection. Atypical forms of *Babesia bovis* like organisms observed in Giemsa stained blood smear examination as single pear shaped trophozoites and amorphous shaped organisms (Fig. 2). Muraleedharan *et al.* (1984) found two cases of babesiosis in buffaloes due to *Babesia bovis* from Mysore and Mandya districts of Karnataka by Giemsa stained blood smears examination. Seshadri *et al*. (1985) reported 2.33% prevalence of *B. bigemina* and 0.18% of *B. bovis* infection in cattle in 8 districts of Karnataka based on morphology of the parasite in Giemsa stained blood smear. Pandey and Mishra (1984) also observed and reported morphological variations in shape, size and arrangements including single piriform,

Table 1. Prevalence of *Babesia bigemina* infection in cattle in three endemic districts of Karnataka state based on parasitological and molecular methods

Diagnostic method	No. examined			No. found positive			% Positive		Overall % positive
	Clinical/ suspected	Healthy/ Tick infested	Total	Clinical/ suspected	Healthy/ Tick infested	Total	Clinical/ suspected	Healthy /Tick infested	
Giemsa stained blood smear examination	34	114	148	3	0	3	8.82	0	2.03
Fluorescent (AO) stained blood smear examination	34	114	148	7	0	7	20.59	0	4.73
PCR	34	114	148	11	5	16	32.35	0	10.81

Clinical, Giemsa positive with clinical signs; Subclinical, Giemsa negative with clinical signs; Carrier, Giemsa negative with no clinical signs (Healthy).

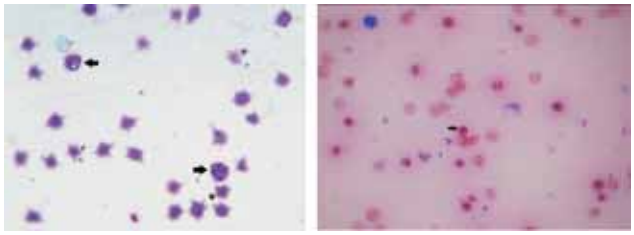


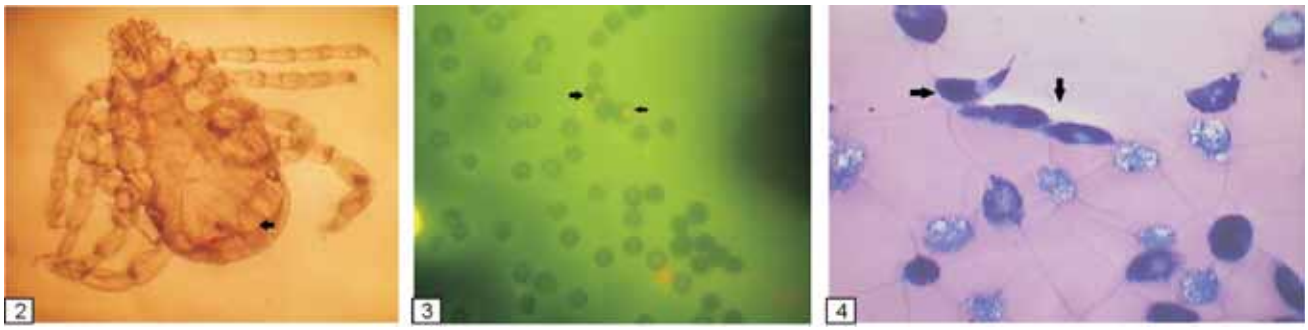
Fig. 1. *Babesia bigemina* in Giemsa stained blood smear (Paired at acute angle – (1000×).

single or paired round forms, elongated or oval or amorphous forms.

The prevalence of *B. bigemina* infection in cattle (2.03%) in the present study is in accordance with previous studies reported by Setty *et al*. (1985) (2.32%), Seshadri *et al*. (1985) (2.33%) and Harish *et al.* (2006) (1.74%) with little variation. This indicated that *B. bigemina* infection is endemic in these areas since 1985 and the status of *B. bigemina* infection remained unchanged.This could be due to carrier status of the recovered animals from acute infection, creating a potential source of infection to healthy susceptible population (Callow 1984). Whereas, the higher prevalence of *B. bigemina* infections of 12.12% was reported from Bangalore north region by Ananda *et al.* (2009) and 12.5% by Krishnamurthy *et al*. (2014) from Shimoga region when compared to present study could be due to collection of more blood samples from the clinically suspected animals during monsoon months and the environmental conditions favourable for survival of ticks and hence the occurrence of the disease is more (Roy *et al*. 2004 and Corson *et al*. 2003).

In the present study, 4.73% cattle (7/148) were found positive for *B. bigemina* infection by fluorescent staining (AO) method. The nucleus of the *B. bigemina* organism took a fluorescent green staining while the cytoplasm had a fluorescent light orange colour (Fig. 3). The study finds that acridine orange staining is accurate, simple, rapid, easy and reliable technique for the detection of *B. bigemina* infection and these observations were in accordance with the observations reported by Hansen *et al*. (1970), Ravindran *et al*. (2007), Nair *et al*. (2011) and Bal *et al.* (2012). Bose *et al*. (1995) also reported that fluorescent staining (AO) method is more sensitive (10^{-7} i.e. one parasite per 10^7 erythrocytes) compared to Giemsa staining technique (10^{-5}) in the detection of *Babesia* spp.

Ticks were identified as *Rhipicephalus* (*Boophilus*) *annulatu* sticks as per the morphological characters described by Walker (1994) which were the classical vectors for *B. bigemina* (Jagannath *et al*. 1979, Aulakh *et al.* 2005). Out of 106 haemolymph smears screened for *Babesia* kinetes by Giemsa staining, only three haemolymph smears (2.83%) showed vermicular structures similar to *Babesia* kinetes with measurements of 21–22µ length × 5–6µ width (Fig. 4). This was the first report of *Babesia* kinetes in the haemolymph smear in Karnataka. These measurements were in accordance with Kamani *et al.* (2011) who reported similar vermicules of *Babesia* species in haemolymph smears of *Amblyomma variegatum* tick in Nigeria.The low prevalence (2.83%) of *Babesia* kinetes in *R. annulatus* in the present study is attributed to low density of parasites in the blood of vertebrate hosts, which is important for the establishment of *Babesia* infection in tick (Riek 1964 and



Figs 2–4. **2.** Microscopic appearance of *Babesia* organisms resembling *Babesia bovis* in Giemsa stained blood smear (1000×) **3.** *Babesia bigemina* in acridine orange (Fluorescent staining) stained blood smear (1000×) **4.** *Babesia* kinete like structures in Giemsa stained Haemolymph smear (Red arrow) (1000×).

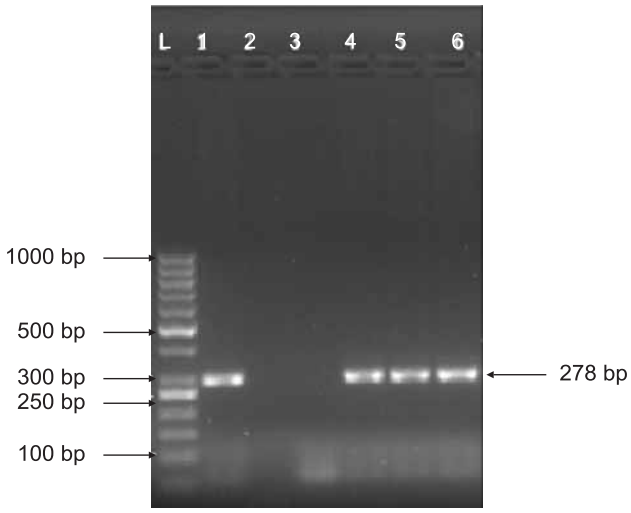


Fig. 5. PCR amplification of 278 bp *B. bigemina* specific fragment. L, 50 bp molecular weight DNA Ladder; L1, Positive Control; L2, Negative Control; L3, No template control (NTC); L4, *B. bigemina* isolate from Bangalore Rural district; L5, *B. bigemina* isolate from Chikkaballapura district; L6, *B. bigemina* isolate from Kolar district.

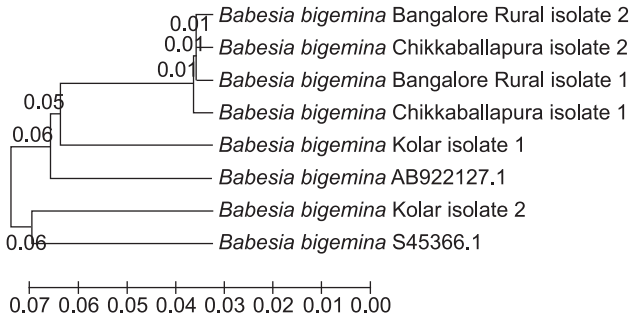


Fig. 6. Phylogenetic analysis of *B. bigemina* isolates.

Mahoney *et al*. 1977) and also due to very low amount of kinetes in female ticks collected from cattle in endemic areas.

The PCR amplification yielded the expected amplification of 278 bp in positive samples (Fig. 5). PCR assay effectively detected *B. bigemina* infection in animals under study revealing 18.75% (3/16) clinical, 50% (8/16) subclinical and 31.25% (5/16) carrier cases of infection. The overall prevalence of infection in three endemic districts of Karnataka by PCR was 10.81% (16/148).

This finding has the strong evidence to show that PCR is more sensitive than parasitological methods (Giemsa and AO) in detecting *B. bigemina* either in clinically infected or apparently healthy animals (carriers). The results of the present study were in accordance with Sharma *et al.* (2013) who reported 5.84% prevalence of *B. bigemina* infection by duplex PCR in dairy animals from Punjab. While with conventional Giemsa stained thin blood smear (GSTBS) examination, they could detect only a very low prevalence of *B. bigemina* (0.48%). Ravindran *et al*. (2006) amplified the template DNA of *B. bigemina* to a sensitivity of 500pg in 14 out of 35 (40%) blood samples collected from Izatnagar (Northern India) and Manipur (Eastern India). They observed only two cases of *B. bigemina* from Izatnagar by microscopic examination of Giemsa stained blood films.

The results of PCR were found to have more specificity (83.26%) and sensitivity (91.16%). The receiver operating characteristic analysis was done to statistically compare the results of three diagnostic methods. The phylogenetic results (Fig. 6) showed that *B. bigemina* isolates obtained during this study were genetically similar among each other and also with the *B. bigemina* isolates published in the GenBank (*B. bigemina* Punjab isolate - Accession number AB922127.1) and *B. bigemina* USA isolate - Accession number S45366.1). The nucleotide sequence analysis results further confirmed the isolates of present study as *B. bigemina.*

The present study concludes that Geimsa's staining is gold standard for detection of Babesiosis in field conditions. Acridine orange staining was more sensitive when compared to Geimsa's staining of blood smear examination. However, the PCR was found more sensitive than other two techniques by detecting low parasitaemia in carrier animals.

REFERENCES

Ananda K J, Placid E D'souza and Puttalakshmamma G C. 2009. Prevalence of haemoprotozoan diseases in crossbred cattle in Bangalore north. *Veterinary World* **2**(1): 15–16.

Aulakh G S, Singla L D, Kaur P and Alka. 2005. Bovine babesiosis due to *Babesia bigemina:* Haematobiochemical and therapeutic studies. *Indian Journal of Animal Sciences* **75**(6): 617–22.

Bal M S, Singla L D, Ashuma, Kumar H, Kaur K and Gupta M P. 2012. Acridine orange staining for diagnosis of haemparasites of cattle and buffaloes. XIth Annual Conference of IAVPHS (December 13–14) at GADVASU, Ludhiana, Punjab.

Bose R, Jorgensen W K, Dalgliesh R J, Friedhoff K T and De Vos A J. 1995. Current state and future trends in diagnosis of babesiosis. *Veterinary Parasitology* **57**: 61–74.

Callow L L. 1984. Piroplasms. *Protozoan and Rickettsial Diseases*. Animal Health in Australia, Australian Bureau of Animal Health, AGPS, Canberra. **5**: 121–160.

Corson M S, Teel P D and Grant W E. 2003. Simulating detection of cattle fever tick (*Boophilus* spp.) infestations in rotational grazing systems. *Ecological Model* **167**: 277–86.

Figueroa J V, Chieves L P, Johnson G S and Buening G M. 1992. Detection of *Babesia bigemina* infected carriers by polymerase chain reaction amplification. *Journal of Clinical Microbiology* **30**(10): 2576–82.

Gupta M P, Singla L D, Singh K B and Sood R M. 2005. Clinical and therapeutic observations in outbreak of babesiosis in crossbred cattle. *Indian Veterinary Journal* **82**: 1103.

Hansen W D, Hunter D T, Richards D E and Allred L. 1970. Acridine orange in the staining of blood parasites. *Parasitology* **56**: 386–87.

Harish B R, Chandranaik B M, Renukaprasad C, Jayakumar S R and Krishnappa G. 2006. Incidence of haemoprotozoan diseases in Karnataka. *Indian Journal of Veterinary Medicine* **26**: 30–31.

Jagannath M S, Muraleedharan K and Hiregoudar L S. 1979. Prevalence of ixodid ticks of cattle at Bangalore. *Indian Journal of Animal Sciences* **49**: 890–94.

Kamani J J, Wander L D and Zirlkallen I U. 2011. Demonstration of vermicules of *Babesia* species in haemolymph smears of *Amblyomma variegatum* in Nigeria. *Journal of Advance in Veterinary Research* **1**: 1–3.

Krishnamurthy C M, Ananda K J and Adeppa J. 2014. Studies on haemoprotozoan infections in livestock of Shimoga region. *Compendium 24th National Congress of Veterinary Parasitology and National Symposium,* Feb 5–7, Mannuthy, Kerala, Abs. No. S1. **29**: 55.

Mahoney D F and Mirre G B. 1977. The selection of larvae of *Boophilus microplus* infected with *Babesia bovis* (syn *B. argentina*). *Research in Veterinary Science* **23**(1): 126–27.

Muraleedharan K, Syedziauddin K, Gopalaswamy T, Muraleedhar and Seshadri S J. 1984. Some observations on clinical cases of *Babesia bovis* in buffaloes. *Indian Veterinary Journal* **61**(1): 76–79.

Nair A S, Ravindran R, Lakshmanan B, Kumar S S, Tresamol P V, Saseendranath M R, Senthilve L K, Rao J R, Tewari A K and Ghosh S. 2011. Haemoprotozoa of cattle in Northern Kerala, India. *Tropical Biomedicine* **28**(1): 68–75.

Pandey N N and Mishra S S. 1984. Morphological variations and intraery throcytic multiplication of *Babesia bigemina* in indigenous cow calves (*Bos indicus). Indian Journal of Veterinary Medicine* **4**(2): 84–86.

Ravindran R, Mishra A K and Rao J R. 2006. Polymerase chain

reaction for the diagnosis of bovine babesiosis. *Journal of Parasitic Diseases* **30**: 142–45.

Ravindran R, Bindu L, Sreekumar C, Lalitha J, Gomathinayagam S and Mishra A K. 2007a. Acridine orange staining for quick detection of blood parasites. *Journal of Veterinary Parasitology* **21**(1): 85–86.

Riek R F. 1964. The life cycle of *Babesia bigemina* in the tick vector *Boophilus microplus. Australian Journal of Agricultural Research* **15**: 802–21.

Roy S, Tiwari A, Galdhar C N, Upadhyay S R, Ratre H K, Sahu S K and Maiti S K. 2004. Seasonal prevalence of haemoprotozoan diseases in cross-bred cattle and buffaloes. *Indian Journal of Veterinary Medicine* **24**: 5–7.

Seshadri S J, Muraleedharan K and Mallikarjun G B. 1985. Haemoprotozoan diseases of bovines, Final report, Part II under co-ordinated animal health coverage programme submitted to UAS, Bangalore.

Setty D R L, Yathiraj S, Ganesh T and Thimmappa R M. 1985. Control of parasite borne diseases in dairy cattle. Final report, Part VI under co-ordinated animal health coverage programme submitted to UAS, Bangalore .

Sharma A, Singla L D, Ashuma, Batth B K, Kaur P, Javed M and Juyal P D. 2013. Molecular prevalence of *Babesia bigemina* and *Trypanosoma evansi* in dairy animals from Punjab, India by duplex PCR: A step forward to detection and management of concurrent latent infections. *Biomed Research International* Article ID 893862. http://dx.doi.org/10.1155/2013/893862.

Sharma A, Singla L D, Ashuma, Batth B K and Kaur P. 2016. Clinicopatho-biochemical alterations associated with subclinical babesiosis in dairy animals. *Journal of Arthropod Borne Diseases* **10**(2): 259–67.

Toni G P, Gabriell E D, Kevin B, Marc C, Dolan P and Robert D G. 2012. Saliva, salivary gland and haemolymph collection from *Ixodess capularis* ticks. *Journal of Visual Experiment* **60**(2): 1–3.

Walker A. 1994. The arthropods of human and domestic animals, 1st Edn, Chapman and Hall, Madras. pp. 34–36.