

A PCR–RFLP method for the simultaneous detection of *Babesia bigemina* and *Theileria annulata* infections in cattle

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Mixed infections due to *Theileria annulata* and *Babesia bigemina* are responsible for widespread morbidity and mortality in cattle and buffalo of the tropics and subtropics. In the present communication, a method for the differentiation of these two common tick-borne infections is described. A polymerase chain reaction (PCR) for the amplification of partial 18S small sub-unit ribosomal DNA fragment from the genomes of *B. bigemina* and *T. annulata* was standardized. The PCR amplified a monomorphic DNA fragment of 564 bp size, which was sequenced and analysed for the restriction enzyme differentiating these two haemoprotozoans in the presence of the host DNA. The enzyme *Cfr131* was found to differentiate the two species.

Keywords: *Babesia bigemina*, cattle, PCR–RFLP, simultaneous detection, *Theileria annulata*.

THEILERIA and *Babesia* species (Apicomplexa, Piroplasmidae) are responsible for tick-borne protozoan diseases in domestic and wild animals¹. The diseases affect cattle and to a lesser extent water buffalo, and are a major constraint to livestock production in the tropics. In India, *Theileria annulata* and *Babesia bigemina* constitute the predominant species responsible for widespread morbidity and mortality in crossbred cattle^{2–8}. Mixed infections are common and if the animals recover from these infections, a long-lasting carrier status occurs with low grade parasitaemia in the affected population. Diagnosis of these low-grade tick-borne protozoan infections is therefore, important for better understanding of the epidemiology and improved management of the diseases.

McLaughlin *et al.*⁹ described a broad-spectrum PCR approach targetted to ribosomal DNA cistron sequences using rDNA amplimers. However, this PCR approach was not tested for differential diagnosis of the infections caused by *T. annulata* and *B. bigemina* in animals. In the present communication, a polymerase chain reaction–restriction fragment length polymorphism (PCR–RFLP) method for differentiation of the two common tick-borne infections caused by *B. bigemina* and *T. annulata* in cattle is described.

Isolation of leucocyte-free, piroplasm-rich erythrocytes was done by CF-11 cellulose chromatography, according to the method of Ray and co-workers¹⁰. Genomic DNA of *B. bigemina* and *T. annulata* was isolated from leucocyte-free, piroplasm-rich erythrocytes following the techniques outlined by Sambrook and associates¹¹. Genomic DNA of infection-free leucocytes was also separated from the blood of a three-day-old bovine calf.

Ribosomal DNA primers described by McLaughlin *et al.*⁹ were custom-synthesized (Bangalore Genei, India). The nucleotide sequences of the primers were as follows:

Forward: 5'-GAG TAA ATT AGA GTG TTC CAA GCA-3'

Reverse: 5'-CGG AAT TAA CAA GAC AAA TC-3'

The PCR was set up in 25 µl reactions. The reaction mixture consisted of 2.5 µl of 10× PCR buffer (Bangalore Genei), 5 µl of 1 mM dNTP mix (Bangalore Genei), 1.5 units of *Taq* DNA polymerase (Bangalore Genei), 1 µl each (40 pmol or 70 ng) of the primers and 2 µl (10 ng/µl) of the template DNA. The volume was made up to 25 µl with autoclaved triple-distilled water.

The cycling conditions described by McLaughlin *et al.*⁹ were followed:

After an initial denaturation step at 94°C for 2 min, 15 cycles of amplification were performed with 1 min denaturation at 94°C, 1 min hybridization at 58°C and 1 min elongation at 72°C. Additional 21 thermal cycles were run with hybridization at 55°C, and a 5 min elongation step at 72°C was used at the end of the amplification programme. Size of the DNA products was estimated by agarose gel electrophoresis on a 2% gel using 60 V power supply for 1.5 h.

B. bigemina (Izatnagar and Wayanad isolates) and *T. annulata* (Parbhani and Izatnagar isolates) were used for amplification. The analytical sensitivity of the PCR for both parasites was determined. The PCR was tested for amplification of the fragment from a control bovine leucocyte DNA (from a three-day-old calf), *Trypanosoma evansi* (camel isolate), and mixed DNA templates (*Babesia*, *Theileria* and bovine leucocyte DNA) to develop this as a method of differentiation of parasites from bovine blood sample.

The nucleotide sequence of PCR-amplified products from *B. bigemina* and *T. annulata* was sequenced. Enzyme selection for digestion experiments was made by analysis of the sequences using Genetool software. The PCR-amplified products were digested with the selected restriction enzyme to determine the differential restriction sites.

The digestion reaction was set up in 20 µl volumes in 500 µl PCR tubes. Five microlitres of the PCR product was used for each digestion. The digestion mixture consisted of 2 µl of the 10× buffer, 5 µl PCR product and 1 µl (10 U) of the restriction enzyme made up to 20 µl

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Table 1. Nucleotide sequence of *Babesia bigemina* rDNA PCR product (EMBL accession no. AJ538183)

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tgagtaaattagagtgtccaagcagacttttgcctgaatacttcagcatggaaataagtagtaggaccttggttctattttgtgttttgagccttggaatggtaaatagg
aacgggtgggggcatcgtatattaactgtcagagggtgaattcttagatttgaagacgaaccactgcgaagcatttccaaggacgttttcattaatcaagaacgaa
agttaggggatcgaagacgatcagataccgtcgtagtcctaaccataaactatgccgactagggttgagggtcgtcattttccgactccttcagcaccttgagagaaa
tcaaagtctttgggttctggggggagtaggtcgaagctcgaactaaaggaattgacggaaggccaccaccaggcgtggagcctgcggcttaattgactcaaca
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gttaa

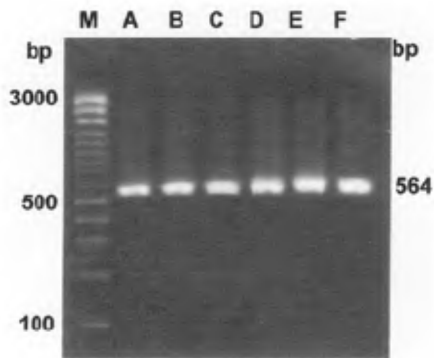
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Table 2. Nucleotide sequence of *Theileria annulata* rDNA PCR product (EMBL accession no. AJ538184)

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tgagtaaattagagtgtccaagcaggctttgcctgaatagtttagcatggaataataagtaggactttggttctattttgtgttttaggtaccaaagtaatggtaata
ggacaggttgggggcatcgtatattaactgtcagagggtgaattcttagatttgaagacgaactactgcgaagcatttccaaggatgttttcattaatcaagaacga
aagttaggggatcgaagacgatcagataccgtcgtagtcctaaccataaactatgccgactagagattggagggtcgtcagttttacgactccttcagcaccttgagag
aatcaaaagtctttgggttctggggggagtaggtcgaagggtgaaactaaaggaattgacggaaggccaccaccaggcgtggagcctgcggcttaattgactc
aacacggggaaactcaccagggtccagacaaaggaaggattgacagattgatagctcttcttgattcttgggtgggtgcatggccgttcttagttgggtgagtgatt
gtcttgtaa

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**Figure 1.** PCR assay with rDNA primers. Lane M, GeneRuler™ 100 bp DNA ladder plus; lane A, Izatnagar 1 isolate of *Babesia bigemina*; lane B, Izatnagar 2 isolate of *B. bigemina*; lane C, Wayanad 1 isolate of *B. bigemina*; lane D, Wayanad 2 isolate of *B. bigemina*; lane E, *Theileria annulata*, and lane F, Bovine leucocyte DNA.

with autoclaved triple-distilled water. The digestion mix was incubated at 37°C overnight. The restriction enzyme analysis of the PCR-amplified DNA fragments was performed by electrophoresis on the ethidium bromide-stained 1.5% agarose gel.

The method was field-tested using thirty-five blood samples from calves (above six months old) collected by ear-prick method into 1.8 ml heparinized Eppendorf tubes. From this, 2.5 µl of the blood was used for DNA isolation utilizing a whole blood genomic DNA isolation kit (Bangalore Genei) with suitable modifications. To a volume of 2.5 µl of heparinized whole blood, 9 µl of solution A (supplied as 5× solution and diluted five times just before use) was added and mixed well. It was left for 10 min and then centrifuged at 5000 rpm on a tabletop centrifuge (Bangalore Genei) at room temperature (RT). After removing the supernatant, the volume was replaced with solution A again and centrifuged to obtain a small white pellet. To the pellet, 5 µl of solution B was added and resuspended well. After 5 min, 7.5 µl of ethanol was added to precipitate the DNA and centrifuged at 10,000 rpm for

5 min at RT. The precipitate was dissolved in 10 µl of autoclaved distilled water and used as template. PCR was performed in 25 µl reaction volume. The products were visualized and confirmed as described earlier. Ten micro-litres of the PCR product was subjected to restriction enzyme digestion with the restriction enzyme identified, differentiating the two parasites in a mixed template situation in the presence of bovine leucocyte DNA.

The rDNA primers amplified a ~564 bp product from both *B. bigemina* and *T. annulata* genomic templates. The products were, however, similar in size from different isolates of the same species. The leucocyte DNA also yielded a ~564 bp product (Figure 1). At the same time, no amplification was observed with *T. evansi* when used as DNA template. The analytical sensitivity of the PCR assay was found to be 150 pg for *B. bigemina* and 10 pg for *T. annulata*. The PCR products of *B. bigemina* (EMBL accession no. AJ538183) and *T. annulata* (EMBL accession no. AJ538184) were sequenced for their nucleotide data (Tables 1 and 2).

The enzyme *Cfr*131 produced a pattern, which could clearly differentiate the two species (Figure 2). The enzyme digestion of *B. bigemina*-specific product yielded 393, 101 and 66 bp sized fragments, while *T. annulata* PCR product revealed two fragments of 463 and 101 bp size. Similarly, digestion experiments with amplicons from leucocyte DNA yielded fragments of different sizes. Restriction digestion experiments with mixed templates, however, produced distinct products for the two species, as described earlier, facilitating easy differentiation of these two parasites (Figure 3).

When the PCR-RFLP method was field-tested also for its ability to differentiate *B. bigemina* and *T. annulata* from heparinized blood samples, *Cfr*131 revealed the restricted products of predicted sizes (Figure 4). The PCR-RFLP method was successfully used for the identification of *B. bigemina* in 19 calves out of a total of 35 animals, while one of them had mixed infection of *B. bigemina* and *T. annulata*.

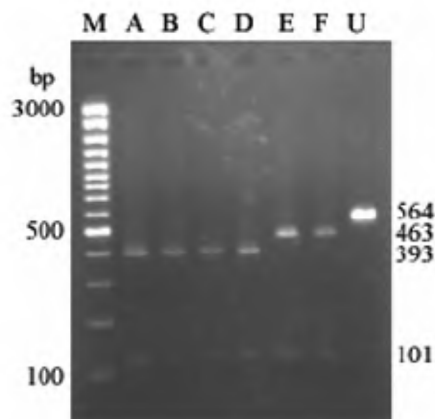


Figure 2. RFLP of rDNA-PCR products with *Cfr131*. Lane M, Gene Ruler™ 100 bp DNA ladder plus; lane A, Izatnagar 1 isolate of *B. bigemina*; lane B, Izatnagar 2 isolate of *B. bigemina*; lane C, Wayanad 1 isolate of *B. bigemina*; lane D, Wayanad 2 isolate of *B. bigemina*; lane E, Parbhani isolate of *T. annulata*; lane F, Izatnagar isolate of *T. annulata*, and lane U, Uncut PCR product.

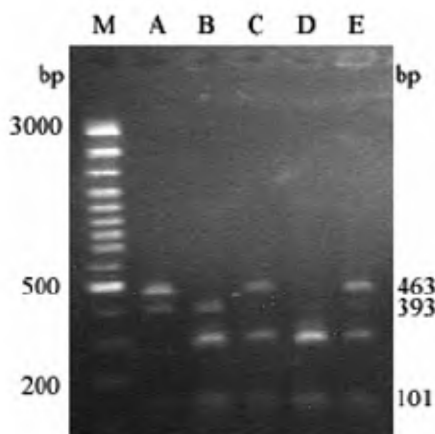


Figure 3. RFLP of rDNA-PCR products from mixed templates with *Cfr131*. Lane M, GeneRuler™ 100 bp DNA ladder plus; lane A, *B. bigemina* + *T. annulata*; lane B, *B. bigemina* + bovine leucocyte DNA; lane C, *T. annulata* + leucocyte DNA; lane D, Bovine leucocyte DNA, and lane E, *B. bigemina* + *T. annulata* + bovine leucocyte DNA.

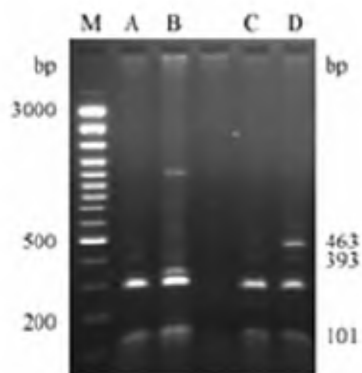


Figure 4. RFLP of rDNA-PCR products of blood samples digested with *Cfr131*. Lanes A–C, Blood samples from bovine calves detected as *B. bigemina*; lane D, Blood sample from bovine calf with mixed infection of *B. bigemina* and *T. annulata*.

Various diagnostic techniques are available for specific detection of the economically important tick-borne haemoprotozoans, viz. *B. bigemina* and *T. annulata*^{1,12,13}. In an endemic region for tick-borne diseases caused by *Theileria* spp. or *Babesia* spp., it would be desirable to have a common test such as a single-tube multiplex PCR assay for simultaneous detection of parasites in the blood of carrier cattle. This, however, requires knowledge of DNA targets conserved among the piroplasms, which contain enough genetic variation to facilitate the design of reliable species-identification protocols.

The analytical sensitivity of the PCR assay was 150 pg for *B. bigemina* and 10 pg for *T. annulata*. Variation in the sensitivity of this PCR assay could be due to the difference in the number of repetitive elements in the ribosomal DNA gene in the genome of the species. According to McLaughlin *et al.*⁹, the rDNA amplicons were apicomplexan taxon-specific. But the primers were found to co-amplify the leucocyte DNA of similar size. Genetic variability at the β -tubulin gene locus has been exploited for the discrimination of *Babesia* and *Theileria* parasites by PCR-RFLP technique, which works on the same principle¹⁴. Co-amplification of host DNA was seen due to strong conservation of the β -tubulin gene in this study. Similarly, in the present investigation, the utility of the conserved sequences and the informative restriction endonuclease markers for *T. annulata* and *B. bigemina* were demonstrated both from mixed DNA templates and whole-blood samples containing single and mixed species in the presence of host leucocyte DNA.

In India, *B. bigemina* and *T. annulata* are widely reported. The occurrence of *B. bovis* or *B. bovis*-like organisms is equivocal^{15–17}. In this scenario, the PCR-RFLP method described here shows a good potential for epidemiological screening and identification of the two common pathogenic tick-borne intracellular protozoa in the country. Further, the technique also has good sensitivity and specificity needed for use as a general surveillance tool for molecular epidemiology of these intracellular parasites.

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Genetic transformation of an elite Indian genotype of cotton (*Gossypium hirsutum* L.) for insect resistance

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***Agrobacterium*-mediated genetic transformation of an elite Indian genotype (Bikaneri Nerma) of cotton (*Gossypium hirsutum* L.) was achieved using shoot apical meristems isolated from seedlings as explants and a**

synthetic gene encoding Cry1Ac δ -endotoxin of *Bacillus thuringiensis*. Regeneration of shoots was carried out in selection medium containing kanamycin (100 mg/l) after co-cultivation of the explants with *Agrobacterium tumefaciens* (strain EHA 105). Rooting was accomplished on a medium containing naphthaleneacetic acid and kanamycin. Progeny obtained by selfing T₀ plants was grown in the greenhouse and screened for the presence of neomycin phosphotransferase (*nptII*), and *cry1Ac* genes by polymerase chain reaction (PCR) and Southern hybridization. Expression of Cry1Ac in the leaves of the transgenic plants was detected by Xpress strips and quantified by Quan-T ELISA kits (DesiGen). Insect bioassays were performed with the larvae of cotton bollworm (*Helicoverpa armigera*). Field tests of the most promising lines (T₂ and T₃ generations) were performed under contained conditions. Results of the field tests showed considerable potential of the transgenic cotton for resistance against cotton bollworm.

Keywords: *Agrobacterium*, genetic transformation, *Gossypium hirsutum*, insect resistance, shoot apical meristem.

COTTON is the most important source of natural fibre. India is the world's third largest cotton producer. One of the major limiting factors which affects cotton production in India, is the incidence of pests, especially bollworms, causing more than 50% yield loss¹. The limited genetic variability for bollworm resistance in cotton land/wild races makes the task of developing pest-resistant lines difficult. In the past decade insecticidal proteins of *Bacillus thuringiensis* (*Bt*), a Gram-positive soil bacterium, have been expressed in cotton and other crop species by genetic engineering with significant social, environmental and economic benefits to the farmers². In 2005, *Bt*-cotton expressing Cry1Ac protein of *Bt* was cultivated in an area of 20.0 million hectares in more than a dozen countries, including India³.

Introduction of foreign genes in elite genotypes is limited by the genotype-specific nature of gene transfer in cotton. Coker genotypes, which are amenable for regeneration *in vitro* by somatic embryogenesis, are widely used in genetic transformation experiments^{4–7}. However, alternate procedures to transform non-Coker genotypes have been reported^{8–11}. In the present study, we report successful introduction of *Bt-cry1Ac* gene in an elite Indian genotype of cotton following a modified shoot apical meristem procedure⁹ and significant protection from cotton bollworm in field conditions.

Cotton cv. Bikaneri Nerma (*Gossypium hirsutum*) was selected because of its high commercial value. Bikaneri Nerma, which is the female parent of the popular cotton hybrid, NHH-44, is also cultivated as a variety in Punjab, Rajasthan and Haryana.

Seeds were delinted with sulphuric acid and soaked in HgCl₂ (50 mg/l) for 30 min and kept for shaking (50 rpm)

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