Indian J. Vet. Med. Vol. 46, No. 1, 2025 pp. 16-19

Quadriplex PCR assay for diagnosis of haemoprotozoan diseases in bovines

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Abstract

The present study was conducted to standardize a quadriplex PCR assay for rapid, sensitive, specific and simultaenous diagnosis of major haemoprotozoan diseases of bovines. Primers targeting *cytochrome b* gene, repetitive nucleotide sequences, gene encoding carbomyl phosphate synthetase II and small subunit rRNA were used for detection of *Trypanosoma evansi*, *Theileria annulata*, *Babesia bovis* and *Babesia bigemina* generating amplicon of 257bp, 312bp, 446bp and 689bp, respectively. Multiplex PCR assay was optimized in a final volume of 25 µl containing 12.5 µl dream taq green master mix (2X), 0.5 µl of each cytob1 primer, 0.4 µl of each TR3/TR4 primer, 0.4 µl of Bb1/Bb2 primer and 0.6 µl of Bg3/Bg4 primer, 4µl of template DNA and rest NFW. Twenty DNA samples extracted from field blood samples were screened by quadriplex PCR assay. Primers were found specific as did not produce amplicon with non-target DNA. Limit of detection was determined using tenfold serial dilution of parasitic DNA. Quadriplex PCR assay could able to amplify DNA of *Theileria annulata* and *Babesia bigemina* at copy number 1000 whereas of *Babesia bovis* and *Trypanosoma evansi* at copy number 100, respectively. Out of 20 samples, *T. annulata* was seen in 15% sample, *B.bigemina* and *B.bovis* in 5 % sample and *B. bigemina* + *T. annulata* in 10% samples, rest sample were negative. Assay was found to be highly specific and can be used for simultaneous diagnosis of major haemoprotozoan diseases.

Keywords: Babesia, haemoprotozoan disease, quadriplex PCR assay, Theileria, trypanosoma

Introduction

In India, economic losses due to ticks and tick borne diseases have been projected to be Rs 3873.06 crores annually (Narladkar *et al.,* 2018). Both Giemsa staining and serological assay methods have limitation that they cannot diagnose disease in sample having low parasitiemia and carrier state. Serological assay failure to differentiate between past and present infections and can result in false positive result further leading to economic loss to farmer (Sumbria *et al.,* 2015).. PCR assays can detect the parasite within 6 hours after blood collection with higher sensitivity and specificity (Charaya et al., 2016). The present study describes the standardization of a novel multiplex PCR assay for simultaneous detection of *T. annulata*, *B.bigemina*, *B. bovis* and *T. evansi* in bovine blood samples and evaluates its use in detecting parasites from field samples.

Materials and Methods

Five microlitre blood samples positive for respective haemoprotozoans by conventional microscopic technique were collected from jugular vein in EDTA vials and used as positive controls. DNA from these samples were extracted using pure link DNA extraction kit (InVitrogen, USA) as follows. To 200µl blood sample, 200µl alkaline lysis buffer is added alongwith 20µl proteinase K ,mix and incubated at 55ºC for 30 minutes. To the mixture 200µl alcohol was added and after thorough mixing the content is transferred to spin column. Spin column was centrifuged at 12000rpm for minutes and column is transferred to new collection tube and 500 µl of wash buffer 1 was added and again centrifuged at same rate and time. Two washing with 500 µl of wash buffer 2 were done and empty column was again centrifuged at same rate and time. Finally DNA was eluted in 30 µl elution buffer. DNA obtained was stored at -20 ºC till further used.

Details of oligonucleotide primers used in standardization of multiplex PCR assay has been mentioned in table 1. Primer melting temperatures (Tm) and the potential for self-annealing, i.e. hair-pin loop and primer-dimer formation, were bioinformatically tested using oligonucleotide analyser software and finally primers were procured from Sigma Company. Multiplex PCR assay was standardized using variable conditions of annealing temperature (gradient PCR

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https://doi.org/10.56093/ijvm.v46i1.174338

assay), primer concentrations, number of PCR cycles and amount of DNA as template. Reaction conditions comprised an initial denaturation step of 95 ºC for 2 min followed by 35 cycles of denaturation (95 ºC for 30 s), primer annealing (gradient for 30 s) and extension (72 ºC for 1min). A final extension at 72 ºC for 10min was performed. For each reaction, 10 µl of PCR product was analysed by gel electrophoresis on a 2% agarose gel, as described above.

To determine the detection limit of the simplex PCR, copy number was calculated as follows. Average mass of nucleotide was taken as 650 daltons meaning one mole of nucleotide weighs 650g. This means 650g contains Avogadro number of nucleotide or 1g of nucleotide contains Avogadro number /650 nucleotide (Factor A). Amount of DNA was estimated using nanodrop and multiplied with factor A to get number of nucleotide in sample (Factor B). Finally Factor B is divided by length of product to get the number of copy number. For determination of sensitivity of Multiplex PCR assay equal amount (10^{10}) of copy number of *B. bovis*, *B. bigemina*,*T. annulata* and *T. evansi* was added in a mixture and ten fold serial dilution was prepared. Multiplex PCR assay was run using optimised conditions. Twenty samples were collected from a cross bred dairy herd for determining the utility of quadriplex in detecting the parasites. DNA was extracted and subjected to multiplex PCR assay. Simplex PCR assay for detection of individual parasites was employed on all the samples to compare the sensitivity of simplex PCR assay with that of quadriplex PCR assay. Microscopic examination of stained blood smear prepared from all the animals were examined and results were compared with both simplex and quadriplex assay.

Results and Discussion

Multiplex PCR assay standardized using published primer set in present study can simultaneously detect these diseases within 6 hour after receiving the samples. Assay was standardized using gradient PCR assay for simultaeneous detection of *Trypanosoma evansi*, *Theileria annulata*, *B. bigemina* and *Babesia bovis* could produce amplicons of 257 bp, 312 bp, 689bp and 446 bp, respectively at 55 ºC annealing temperature as shown in in Fig. 1 without any non specific amplification. The optimised multiplex PCR was performed in a final volume of 25 µl containing 12.5 µl dream taq green master mix (2X), 0.5 µl of each cytob1 primer, 0.4 µl of each TR3/TR4 primer, 0.4 µl of Bb1/Bb2 primer and 0.6 µl of Bg3/Bg4 primer, 4µl of template DNA and rest NFW. Gradient PCR assay result showed clear distinguished bands of all four parasite from 52 to 58°C and best at 55.8°C (Fig 1). Reaction conditions comprised an initial denaturation step of 95 ºC for 2 min followed by 35 cycles of denaturation (95 ºC for 30 s), primer annealing (55 ºC for 30 s) and extension (72 ºC for 1min). Bands were easily differentiated on gel electrophoresis. Primers were found specific as did not produce amplicon with non target DNA. Fragments of expected size were generated from template DNA representing *T. annulata, T.evansi, B. bovis* and *B.bigemina* isolates using each of the species-specific primer sets, while non-target species DNA displayed no evidence of fragment amplification as shown in fig. 2. This indicated that each primer set is capable of specifically amplifying the target locus in a single PCR reaction. Sensitivity of quadriplex PCR assay was checked by ten fold serial dilution of DNA mix containing equal copy number of all the parasites. Quadriplex PCR assay could able to amplify DNA of *Theileria annulata* and *Babesia bigemina* at copy number 1000 whereas of *Babesia bovis* and *Trypanosoma evansi* at copy number 100, respectively as shown in Fig 3. Quite similar to our study, Ashuma *et al.,*2014 developed and evaluated multiplex PCR assay for diagnosis of three parasites viz. *Theileria annulata, Trypanosoma evansi and Babesia bigemina* whereas in contrast we have standardized multiplex PCR assay with added parasitic identification *Babesia bovis*. Kundave *et al.,* 2018 developed and evaluated multiplex PCR assay for simultaneous detection of *Theileria annulata , Babesia bigemina and Anaplasma marginale* infections in bovines using diferent sets of primers and different species of parasites. On analyzing twenty DNA samples by quadriplex PCR assay, it was found it could detect mixed infection in 3 samples. Out of 20 DNA samples, *T. annulata* was seen in 15% sample, *B.bigemina* and *B.bovis* in 5 % sample and *B. bigemina* + *T. annulata* in 10% samples, rest sample were negative. Microscopic analysis revealed 4 samples positive for *Theileria annulata* and 3 samples positive for *Babesia* sp. None of the samples were found positive for *Trypanosoma evansi*. *Theileria annulata* was detected in 7 samples (35%) by simplex PCR assay whereas only 6 samples by multiplex PCR assay. The sample found positive for *B. bigemina* + *T. annulata* was also found positive microscopically for both parasites and by PCR assay. No disparity in results of simplex PCR assay and quadriplex PCR assay was observed. None of the samples were found

positive for *Trypanosoma evansi* by either simplex or multiplex PCR assay. The combination of parasites detected and primer combination used in the present study is novel for development of multiplex PCR assay. Multiplex PCR assay was found to be highly specific and can be used for easy, early, sensitive, specific and simultaneous diagnosis of haemoprotozoan diseases in epidemiological survey as a robust tool.

Table 1: Details of Primer used for detection of parasites DNA

Parasite	Targeted gene/ sequences	Sequence	Product size	Reference
T. annulata	Cytochrome b	F:5'ACTTTGGCCGTAATGTTAAAC 3' R:5'CTCTGGACCAACTGTTTGG 3'	312bp	1
T. evansi	Repetitive nucleotide sequences	F:5' GCGCGGATTCTTTGCAGACGA 3' R:5'TGCAGACACTGGAATGTTACT 3'	257bp	2
B. bovis	Gene encoding enzyme carbamoyl phosphate synthetase II	F: 5' TTTGGTATTTGTCTTGGTCAT 3' R: 5' ACCACTGTAGTCAAACTCACC 3'	446bp	2
B. bigemina	SSU r RNA	F: 5' TAGTTGTATTTCAGCCTCGCG 3' F: 5' AACATCCAAGCAGCTAHTTAG 3'	689bp	2

1 Bilgiç *et al*. (2013); 2; Ananyutthawongese *et al*. (1999)

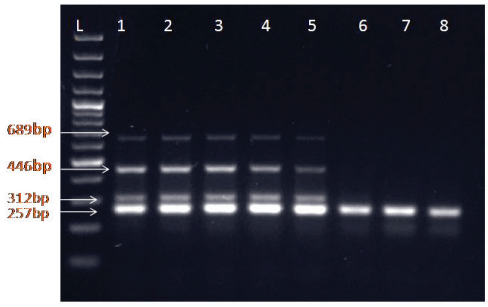

Fig. 1. Agarose gel electrophoresis results of gradient quardriplex PCR assay for simulateanous detection of *Theileria annulata*, *Babesia bovis, Babesia bigemina* and *Trypanosoma evansi*. L: 100bp ladder (New England biolab, USA); *Lane 1: 52°C;2: 52.7°C ; 3: 53.9°C;4: 55.8°C;5: 58.1°C;6: 60°C;7: 61.2°C; 8: 62°C*

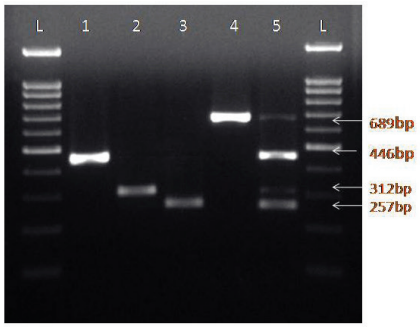

Fig. 2. Agarose gel electrophoresis results showing specificity of Quadriplex PCR assay with known positive DNA samples. L: 100bp ladder (New England biolab, USA) ; Lane 1: positive for *B. bovis*; Lane 2: Positive for *T. annulata*; Lane 3: Positive for *T. evansi*; Lane 4: Positive for *B. bigemina*; Lane 5: 106 dilution of stock solution prepared for checking multiplex sensitivity

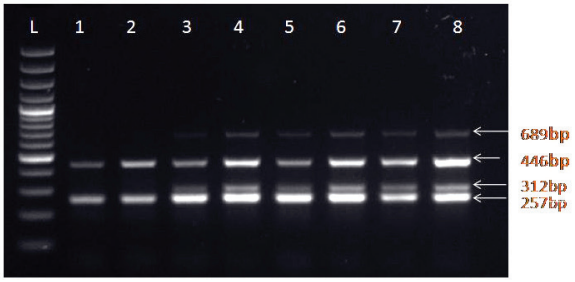

Fig. 3. Agarose gel electrophoresis results for determination of sensitivity of quardriplexPCR assay for simulateanous detection of *Theileria annulata*, *Babesia bovis, Babesia bigemina* and *Trypanosoma evansi* at 55 °C annealing temperature. L: 100bp ladder (New England biolab, USA) ; *Lane 1 to 8 :* Ten fold serial dilution from $10^1$to 10^8

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Received : 19.12.2025 **Accepted : 04.06.2026** **Published : 30.06.2026**