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Development of a non-radioactive probe generated by RAPD-PCR for the detection of *Theileria annulata*

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ABSTRACT

A highly reproducible, dominant and monomorphic fragment of 963 base pair (bp) was amplified from the genome of *Theileria annulata* by random amplified polymorphic DNA - polymerase chain reaction (RAPD-PCR) using a arbitrarily selected primer AP_{17} (5'-GGTGACGCAG-3'). This fragment was reamplified using the same random primer (AP_{17}), gel purified and labeled with digoxigenin (DIG). Dot-blot hybridization of total genomic DNA with the probe detected Parbhani and Izatnagar strains of *T. annulata* with a threshold detection level of 10 pg of parasite template DNA. No cross-hybridization was observed with *Babesia bigemina, Trypanosoma evansi* and the bovine host DNA. The sensitivity of the DIG labeled probe developed in this study is at least 6 to 65 times higher than the other DIG labeled probes developed in India and therefore considered highly suitable for diagnosis of carrier animals especially when they are imported or exported from the country.

Key words: DNA probe, RAPD-PCR, *Theileria annulata*

Theileria annulata, the causative agent of bovine tropical theileriosis in India, is cyclically transmitted by *Hyalomma anatolicum anatolicum* (Bhattacharyulu *et al*. 1975). The true economic impact of theileriosis is difficult to assess in India with the changing epidemiology of the disease (Minjauw and McLeod 2003). The disease, continued to be a major threat till late nineties, however, during the last 10 years, there is an appreciable decline in the number of disease outbreaks, understandably due to enzootic stability factors operating with the host, parasite or the vectors (Das and Ray 2003). With the result there is a reservoir and/or carrier status prevalent in the country causing sporadic outbreaks due to biotic and abiotic stress factors. Therefore, there is a greater need for improved capability to disease diagnosis (D'Oliveira *et al.* 1995).

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The low sensitivity of microscopic detection of the parasite technique does not permit its use in epidemiological studies (Almeria *et al*. 2001). The indirect fluorescent antibody test (IFAT), using either cultured macroschizonts or piroplasm as the antigen has the limitation of cross-reactivity with antibodies directed against other *Theileria* sp. (Burridge *et al*. 1974, Kiltz *et al*. 1986). Also since, the specific antibodies tend to disappear in chronic carriers, these tests may provide negative results even when the piroplasms present in them can still infect ticks and spread the infection (D'Oliveira *et al.* 1995).

The molecular technique RAPD-PCR (Williams *et al*. 1990, Welsh and McClelland 1990) has shown its utility to not only to discern genetic variation among closely related organisms but was also found to be useful in designing diagnostic markers. The technique is used for identification of species/strain-specific fragments, which in turn may be used as probes (Hardrys *et al*. 1992, Basagoudanavar *et al*. 2001, Ravindran *et al*. 2006). The present communication reports the development of highly specific and sensitive nonradioactive probe generated from a monomorphic fragment of RAPD – PCR for the molecular detection and identification of *T. annulata.*

MATERIALS AND METHODS

Parasite strains and genomic DNA extraction: Parbhani (PBN) and Izatnagar (IZT) strains of *T. annulata* originally

isolated from the states of Maharashtra and Uttar Pradesh, respectively, were maintained as cryopreserved stabilates of ground-up tick tissue sporozoites (GUTTS) of infected tick, *H. a. anatolicum* at the Division of Parasitology, Indian Veterinary Research Institute, Izatnagar, and used in this study. Four young bovine calves (3–4 months) showing no haemoprotozoan infection in their blood smears and sero negative for theilerial antibodies in ELISA were inoculated subcutaneously near the left prescapular lymphnode with 5 tick-equivalent GUTTS of each of PBN and IZT strains. Smears made from peripheral blood and lymph node biopsy were stained by Giemsa and examined daily. The animals were also examined daily for pyrexia.

Blood was collected in the presence of anticoagulant, from the infected animals showing a parasitaemia of 40–80%. Purification of intraerythrocytic piroplasms of *T. annulata* was done according to Ray *et al*. (1998). DNA was extracted from leukocyte-free purified piroplasms using the method described by Sambrook *et al*. (1989).

RAPD - PCR and identification of monomorphic fragment: The RAPD-PCR reaction was set up in 25 µl reaction volume containing 20 ng of template DNA from a single parasite strain, 15 pmol of primer, 200 µmol of each dNTP and 1.5 U Taq DNA polymerase in 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 1.5 mM $MgCl_2$ and 0.01% w/v gelatin. The final volume was made to 25 µl with autoclaved Milli Q distilled water. The random 10-mer AP_{17} (5'-GGTGACGCAG-3') with 70% GC content was used for the assay. The reactions were performed on a Thermal cycler PTC-200 with a preheated lid.

The cycling conditions used were initial denaturation at 94°C for 5 min, followed by 45 cycles each of denaturation at 94°C for 1 min, annealing at 36°C for 45 sec, and elongation at 72°C for 1 min. This was followed by a final extension step at 72°C for 5 min. After the reaction the products were analyzed by electrophoresis in 1.4% ethidium bromide stained agarose gel. Highly intense and monomorphic product was identified and this was isolated from agarose gel using QIA quick gel extraction kit as per the manufacturer recommendations. The DNA fragment was reamplified using the same amplification conditions described earlier for RAPD-PCR.

Digoxigenin DNA labeling: DNA labeling of the reamplified fragment was carried out by incorporation of Digoxigenin-11-dUTP during PCR using DIG-DNA labeling and detection kit according to manufacturer's recommendations. PCR reaction was set up in 25 µl reaction volume containing 20 ng of DNA, 15 pmol of primer AP_{17}, PCR-DIG probe synthesis mix (200 µmol of each of dNTPs replacing the dTTP at 30% level with digoxigenin and 1.5 U Taq DNA polymerase in 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 1.5 mM $MgCl_2$ and 0.01% w/v gelatin. The reactions were performed on a thermal cycler PTC-200 with a preheated lid. Cycling conditions were the same as described above for RAPD-PCR. The success of the labeling was confirmed by comparing the electrophoretic mobility of 3 µl of labelled product in relation to unlabelled product on 1.4% agarose gel.

DNA blotting and hybridization conditions: For DNA blotting, the template DNA at various concentrations (1 pg to 25 ng) in 100 µl of 0.1 mM Tris-EDTA (pH 8.0) was boiled for 5 min. To each dilution, 5 µl of 10N NaOH was added and incubated at room temperature for 10 min. Then an equal volume (105 µl) of 2 M ammonium acetate was added and applied on to the nylon membrane using minifold dot-blotting apparatus. Similarly to check the specificity, equal concentration (50 ng each) of genomic DNA of *T. annulata* (PBN strain), *Babesia bigemina, Trypanosoma evansi* and bovine host DNA were blotted. The blots were dried and soaked in 2X SSC, and air dried before subjecting to UV cross-linking (120 $MJcm^{-2}$ energy) for 5 min, using a UV cross-linker.

After prehybridisation (68°C, 3 h), hybridization was carried out at 68°C overnight, in a hybridization oven using 25 µl of freshly denatured DIG-labeled probe. The membrane was then washed with 2X SSC, 0.5% (w/v) SDS at room temperature for 10 min, followed by two more washing steps at 68°C for 15 min each in 0.1% SSC, 0.1% (w/v) SDS under constant gentle agitation. The hybridized probe was immuno detected using anti-digoxigenin-AP conjugate (1:1000) in 1% blocking solution and visualized using the colorimetric substrate (NBT/BCIP).

RESULTS AND DISCUSSION

The purity of the DNA samples used in the RAPD assays was tested by bovine insulin–like growth factor binding protein 3 ($IGFBP_3$) gene specific PCR (Maciulla *et al*. 1997). No amplification was seen with *T. annulata* genomic DNA (PBN or IZT strain) templates, suggesting that the DNA fingerprints amplified by random primers were of parasite origin and devoid of possible host DNA contamination (data not shown).

RAPD profile amplified by primer AP_{17} (GGTGACGCAG) on two *T. annulata* strains were analyzed (Fig.1), the highly intense, monomorphic band (~ 963 bp) was selected and used for preparation of probe.

Dot-blot hybridization, using the DIG labelled reamplified product revealed specific signal only with *T. annulata.* No detectable signal was evident against *B. bigemina, T. evansi* and bovine host DNA (Fig.2a).

Hybridization signals were detected up to a minimum level of 10 pg *T. annulata* DNA. Negative control (Salmon sperm DNA, however, did not produce hybridization signal (Fig. 2b). The DNA templates of *T. annulata* IZT strain also revealed similar sensitivity detection levels (data not shown).

The RAPD-PCR technique has shown great promise in identifying polymorphic DNA markers and is mainly used to assess the genetic variation among the closely related

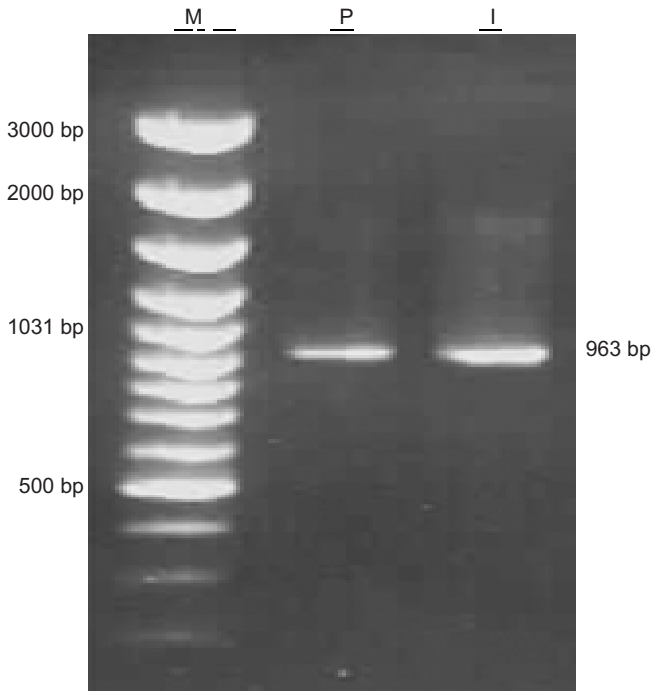


Fig.1. Agarose (1.4%) gel showing the DNA fingerprint profiles of *T. annulata* strains with primer Ap_{17}. Lanes P and I indicate Parbhani and Izatnagar strains respectively. Lane M, DNA molecular size marker100 bp ladder plus

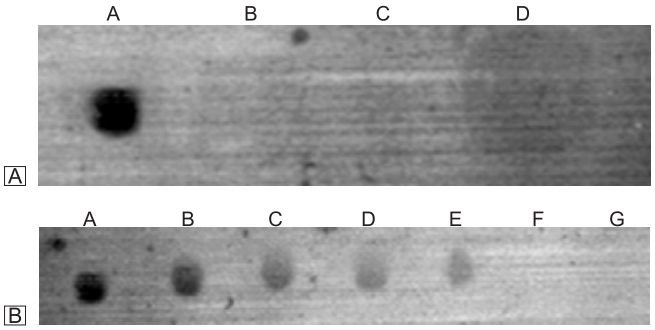
species/strains. The technique was extensively used for genotyping parasites (Basagoudanavar *et al*. 1999, Omanwar *et al*. 2001). RAPD-PCR was also previously used as a quick method for developing species/strain specific diagnostic probes (Basagoudanavar *et al*. 2001, Ravindran *et al*. 2006, Reddy *et al*. 1998).

In the present study, a highly intense, monomorphic ~963 bp RAPD fragment amplified by the 10-mer 5'-GGTGACGCAG-3' specific to *T. annulata* was selected and tested as probe for its sensitivity and specificity. The probe detected genomic DNA of *T. annulata* to a minimum of 10 pg, while it did not identify the DNA of host or other common haemoparasites in the study area.

The development of DNA probes based on identification of repetitive elements, unique genes or space DNA sequences and their cloning and/or *de novo* synthesis of the oligonucleotide sequence is time consuming. The advent of PCR for generation of RAPD using randomly chosen single oligonucleotide primers has simplified the identification of genetic markers for a number of parasites without the requirement of prior knowledge of their genomic nucleotide information (Macpherson and Gajadher 1994, Reddy *et al*. 1998).

Nucleic acid probes were earlier developed for *Theileria* species (Allsopp *et al*. 1993, Bishop *et al*. 1995). But, these assays were used merely for differentiation of species rather than for detection of the parasite in carrier animals.

Non-radioactive probes were described previously using



Figs 2A–2B. **2A.** Specificity assay of RAPD-PCR generated ~963 bp DIG-labeled probe of *T. annulata* by dot-blot hybridization. Lane A-D indicate genomic DNA of *T. annulata* (A), *B. bigemina* (B), *T. evansi* (C) and bovine host DNA (D). **2B.** Sensitivity assay: A-G indicate the concentration of DNA. A, 25 ng; B, 10 ng; C, 1 ng; D, 100 pg; E, 10 pg; F, 1 pg; G, negative control**.**

small subunit ribosomal RNA gene (Roy *et al*. 1999) or *EcoR*1 digested genomic DNA of *Theileria annulata* (Upadhyay *et al*. 2003). The threshold sensitivity for these probes was 64 pg (Upadhyay *et al*. 2003) or 650pg (Roy *et al*. 1999). The sensitivity of the DIG labelled probe described here is at least 6 to 65 times high and thus considered highly suitable for field diagnosis of carrier animals especially when they are imported or exported from the country.

Non-isotopic probes have great potential for use in developing countries like India as they are stable, not restricted by a short half-life and health hazards associated with the radioactive probes. Moreover the non-isotope probes can be used repeatedly with proper storage.

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REFERENCES

Allsopp B A, Bayliss H A, Allsopp M T E P, Cavalier-Smith T, Bishop R P, Carrington D M, Sohanpal B and Spooner P. 1993. Discrimination between six species of *Theileria* using oligonucleotide probes which detect small-subunit ribosomal RNA sequences. *Parasitology* **107**: 157–65.

Almeria S, Castella J, Ferrer D, Ortuno A, Estrada-Pena A and Gutierrez J F. 2001. Bovine piroplasms in Minorca (Balearic Islands Spain): a comparison of PCR based and light microscopy detection. *Veterinary Parasitology* **99**: 249–59.

Basagoudanavar S H, Rao J R, Omanwar S, Tiwari A K, Singh R K, Kataria R S and Butchaiah G. 2001. Identification of *Trypanosoma evansi* by DNA hybridization using a non-radioactive probe generated by arbitrary PCR. *Acta Veterinaria Hungerica* **49**: 191–95.

Basagoudanavar S H, Rao J R, Singh R K and Butchaiah G. 1999. Random amplification of polymorphic DNA fingerprinting of *Trypanosoma evansi*. *Veterinary Research Communication* **23**: 249–55.

Bhattacharyulu Y, Chaudhri R P and Gill B S. 1975. Transstadial transmission of *Theileria annulata* through common Ixodid ticks infesting Indian cattle. *Parasitology* **71**: 1–7.

Bishop R, Allsopp B, Spooner P, Sohanpal B, Morzaria S and Gobright E. 1995. *Theileria*: improved species discrimination using oligonucleotides derived from large-subunit ribosomal RNA sequences. *Experimental Parasitology* **80**: 107–15.

Burridge M J, Brown C G D and Kimber C D. 1974. *Theileria annulata*: cross reactions between a cell culture schizont antigen and antigens of East African *Theileria* species in the indirect fluorescent antibody test. *Experimental Parasitology* **35**: 374–80.

Das G and Ray D. 2003. PCR based detection of *Theileria annulata* infection in ticks collected from cattle of West Bengal, India. *Journal of Veterinary Parasitology* **17**: 11–14.

D'Oliveira C, Van Der Weide M, Habela M A, Jacquiet P and Jongejan F. 1995. Detection of *Theileria annulata* in blood samples of carrier cattle by PCR. *Journal of Clinical Microbiology* **33**: 2665–69.

Hardrys H, Balik M and Schierwater B. 1992. Application of random amplified polymorphic DNA (RAPD) in molecular ecology. *Molecular Ecology* **1**: 55–63.

Kiltz H H, Uilenberg G, Franssen F F J and Perie N M. 1986. *Theileria orientalis* occurs in Central Africa. *Research in Veterinary Medicine* **40**: 197–200.

Maciulla J H, Zhang H M and DeNise S K. 1997. A novel polymorphism in the bovine insulin-like growth factor binding protein-3 (IGFBP3) gene. *Animal Genetics* **28**: 375.

Macpherson J M and Gajadher A A. 1994. Specific amplification of *Sarcocystis cruzi* DNA using a randomly primed polymerase chain reaction assay. *Veterinary Parasitology* **55**: 267–77.

Minjauw B and McLeod A. 2003. Tick-borne diseases and poverty. The impact of ticks and tick-borne diseases on the livelihood of small-scale and marginal livestock owners in India and eastern and southern Africa. *Research report, DFID Animal Health Programme*, Centre for Tropical Veterinary Medicine, University of Edinburgh, U K.

Omanwar S, Rao J R, Singh R K and Butchaiah G. 2001. DNA polymorphism in *Trypanosoma evansi* by randomly amplified polymorphic DNA-PCR. *Veterinary Record* **148**: 244–46.

Ravindran R, Rao J R, Mishra A K. 2006. Detection of *Babesia bigemina* DNA in ticks by DNA hybridization using a nonradioactive probe generated by arbitrary PCR. *Veterinary Parasitology* **141**: 181–85.

Ray D, Bansal G C and Dutta B. 1998. Purification of intraerythrocytic piroplasms of *Theileria annulata* from infected bovine blood. *Indian Journal of Animal Sciences* **68**(11): 1167–68.

Reddy Y A, Rao J R, Butchaiah G and Sharma B. 1998. Random amplified polymorphic DNA for the specific detection of bubaline *Echinococcus granulosus* by hybridization assay. *Veterinary Parasitology* **79**: 315–23.

Roy K C, Ray D and Bansal G C. 1999. Detection of *Theileria annulata* using DNA hybridization. *Indian Journal of Animal Sciences* **69**(10): 1002–04.

Sambrook J, Fritsch E F and Maniatis T. 1989. *Molecular Cloning: A Laboratory Manual.* Cold Spring Harbor Laboratory, Cold Spring, Harbor, New York.

Upadhyay D, Ray D and Bansal G C. 2003. Use of labeled genomic DNA for detection of *Theileria annulata* in bovine carriers. *Indian Journal of Animal Sciences* **73**: 219–21.

Welsh J and McClelland M. 1990. Fingerprinting genomes using PCR with arbitrary primers. *Nucleic Acids Research* **18**: 7213–18.

Williams J G K, Kubelik A R, Livak K J, Rafalski J A and Tingey S V. 1990. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucleic Acids Research* **18**: 6531–35.