Polymerase chain reaction assay for the diagnosis of bovine microfilariosis due to *Setaria digitata* in Hyderabad region of Telangana state

Lingala Raju Kumar, Manchukonda Udaya Kumar and GS Sreenivasa Murthy

**Abstract**

The present study deals with the diagnostic evaluation of PCR assay targeted to a repetitive element (IpSdS) in the genome of *Setaria digitata* in bovines suspected for microfilariosis. The PCR assay has yielded 125bp and 332 bp products. Similar banding pattern observed with both adult *Setaria digitata* worm, In-vitro isolated microfilariae. The standardized PCR assay found sensitive in detecting as little as 31.25pg of DNA microfilariae which is equivalent to 1/8 th of single microfilaria. The standardized PCR assay gave positive signals with 49 out of 326 bovine blood samples tested, indicating an overall prevalence of 15.03% of bovine microfilariosis. Comparatively a higher percentage of infection was recorded in buffaloes (17.54% (30/171) than in cattle (12.25% (19/155). A proportionate increase of 2.22%, 8.64%, 17.92% and 23.4%, of bovine microfilariosis was recorded in the age groups starting from <3 years (1/45), 3-6 years (7/81), 6-9 years (19/106), and above 9 years (22/94), respectively. Similarly, highest prevalence of bovine microfilariosis was recorded in lactating 32/146 (21.91%%) animals followed by non lactating 14/110 (12.72%) and heifers 3/42 (7.14%). The PCR assay was found to be superior than Modified Knots technique and Giemsa staining which detected 49, 20 and 13 positive cases of bovine microfilariosis out of 326 samples screened, showing 15.03%, 6.14% and 3.98% of positivity, respectively.

**Keywords:** *Setaria digitata*, Microfilariae, IpSdS sequence, PCR.

**1. Introduction**

Bovine microfilariosis is a vector borne, chronic wasting disease of cattle and buffaloes caused by microfilariae of *Setaria*. In India 3 *Setaria* spp i.e., *S.digitata, S.cervi, S.labiatopapillosa* have been reported but infections due to S.digitata is more common in bovines (Binosunder et al. 2005) [3]. The disease is more common in tropical countries and is transmitted by mosquitoes belonging to genera *Aedes, Culex, Anopheles, Armigeres* (Tung et al. 2004) [20]. It is an emerging disease producing clinical manifestations of microfilariosis as anemia with an increase in erythrocyte sedimentation rate (ESR), total leukocyte (TLC), lymphocyte and eosinophil counts (Sharma And Joshi 2002) [13] along with loss of appetite, debility, edematous swelling in dependant parts of the body, congested conjuctiva with mucopurulent discharges from eyes, intermittent fever, lusterless and rough hair coat, decrease in milk yield and chronic wasting (Sharma And Joshi 2002, Sharma and Kumar 1994) [13 14]. Sometimes infective stages are transmitted to abnormal hosts lead to serious and often fatal cerebrospinal nematodiosis (CNS) (Bazargani et al. 2008; Innes et al. 1952) [2, 5], thus causing substantial economic losses to animal husbandry. The diagnosis and disease surveillance are necessary for the rapid detection of the infected animals and to plan effective disease control measures because Losses due to latent and clinical parasitism are considered to be higher than that caused by the microbial agents (Sharma et al. 1980; Sharma et al. 1985) [12, 13].

PCR based assays offers the possibility of increased sensitivity and specificity in diagnosis over traditional Microscopic and serological tests (Wijesundera et al. 1999) [22]. Repeat DNA sequences are ideally suited for the Detection of the parasite DNA because the high copy number of these elements greatly increases the sensitivity of the detection assay. Earlier, Lizotte et al. (1994) [7] used primers targeting Hha I repetitive sequence in the genome of *B.malayi* where as Siridewa et al. (1996) [16], Williams et al. (1996) [23] and Toe et al. (1998) [19] targeted 490bp repeat sequence, SapI-PCR and O-150 in the diagnosis of *W. bancrofti*, human wuchereriosis and onchocerciosis, respectively.
Wijesundera et al. (1996) [21] developed a probe using one (IpSdS) of the two (IpSdM and IpSdS) repetitive sequences which have been cloned and sequenced from the genome of *S. digitata*. Hence, the present study conducted in order to develop simple, rapid and sensitive PCR assay for the diagnosis of microfilariosis of *S. digitata* in cattle and buffaloes.

2. Materials and methods

*Setaaria* worms were collected from the peritoneal cavities of the slaughtered bovines and cleaned several times in PBS pH 7.2 to remove host material and blood contamination (Dhas et al. 1993) [4] etc.

2.1. Harvesting of microfilariae from the Adult *S. digitata* worm:

Microfilariae were in-vitro harvested from a single female worm incubated individually in PBS (pH 7.2) in clean, sterile separate petridishes at room temperature (22 °C) for 18hrs. following the harvesting of microfilaria, the cut head and tail end of the worms cleared for the morphological confirmation of the *S. digitata* worms.

2.2. Isolation and analysis of genomic DNA from the female worm and microfilariae harvested from the *S. digitata*;

Genomic DNA was isolated from the frozen middle portion of *S. digitata* and 1000 microfilariae harvested from the same worm according to Sambrook et al. (1989) [10] with minor modifications.

Briefly, pellet of triturated worm sediment and aliquots of microfilaria was lysed by cell lysis buffers and proteins deactivated by adding Proteinase K. Later DNA was extracted by phenol: chloroform: isoamylalcohol extraction and precipitated by 10M ammonium acetate and 100% ethanol. The precipitated and spooled DNA was collected, washed twice with 70% ethanol, air dried and final DNA pellet was dissolved in 50-100 µl of TE buffer (pH 8). The tubes were maintained in a water bath at 65°C for 10 min and stored at -20°C.

1ng/1µl working solutions of DNA were prepared, then held at 65°C for 10 min in a water bath and stored at -20°C. The template DNA of *Setaria digitata* thus isolated was used in all PCR reactions as reference positive control.

2.3. PCR assay

The PCR was performed according to the procedure described by Wijesundera et al. (1999) [22] with minor modifications. The forward M2Fp, 5’CCGACATCAAGTTCATG 3’ and reverse M2Rp, 5’GATTTAGGAGATGGTG 3’ set of primers were custom synthesized by GeneiTm. The reaction mixture consisted of 1µl (1 ng) of template DNA, 5 µl of 10 x PCR buffer, 10µl of 400µm dNTP mix, 5 µl (10 pmol) each of forward and reverse primers, 2.5 unit of Taq DNA polymerase and the reaction volume was made up to 50 µl with autoclaved deionized triple distilled water (ATDW). Positive and negative controls with or without *S. digitata* DNA, respectively, were run with each reaction. The cycling conditions adjusted were as follows: 30 cycles of 30 sec denaturation each at 94 °C, 20 sec annealing at 53 °C and 30 Sec extensions at 72 °C, followed by a final extension of 15 min at 72 °C. The PCR products were stored at 4°C until use and they were analyzed in ethidium bromide stained (0.5 µg/ml) 1.5% agarose gel at 75 volts for 1hr. The gels were visualized by gel documentation system (Syngene) and documented.

2.4. Analytical sensitivity of PCR assay

Appropriate fourfold dilutions of template DNA of *S. digitata* and Microfilaria made in ATDW, starting from a concentration of 1 ng up to 14 atto grams were run by PCR assay, as per the conditions described above. The analytical sensitivity of PCR assay was detected by running the PCR products in agarose gel electrophoresis, as described earlier.

2.5. Field validation of PCR assay in the diagnosis of bovine microfilariosis

The suspected bovine blood samples were collected from the different areas suspected for microfilariosis with the previous history and geographical areas warm and humid in nature. The DNA from bovine blood samples was extracted according to the procedure described by Wijesundera et al. (1999) [21]. Fifty microlitres of blood was taken into an eppendorf tube, RBC were lysed with 1ml of distilled water followed by centrifugation at 12000g for 5 min. After discarding the supernatant the process of hemolysis was repeated once again to completely lyse the RBC. After completely removing water, 50µl of TE buffer containing 0.45%NP-40 detergent was added to the pellet. The sample was digested by adding Proteinase k @1mg/ml to the final volume followed by incubation at 56 °C for 1.5hr. Proteinase k was inactivated by heating the sample in boiling water for 10min. The sample was then allowed to cool to room temperature and 20µl of the above digest was used as template in PCR assay. A total of 326 blood samples were screened in the present study.

2.6. Diagnosis of bovine microfilariosis by blood examination

Dehaemoglobinised thick blood smears were examined by routine Giemsa staining technique and the whole blood collected in heparinized blood collecting tubes and preserved at 4 °C in refrigerator was examined by Modified Knots technique as described by Sloss et al. (1994) [17]. The results of PCR assay were compared with those of blood smear examination and knots technique in the detection of bovine microfilariosis due to *S. digitata* infection.

3. Results and discussion

*Setaria digitata* is a slender worm with a spirally coiled, tapering end being female worms (104±8.0mm) were comparatively longer than the male worms (35±2mm). Anterior end of worms had a chitinous peribuccal crown bearing dorsal and ventral projections and a pair of lateral lips adjacent to buccal aperture. The posterior end is tapering, with a smooth knob with a pair of lateral appendages anterior to the knob. Morphological features were found similar to the reports of Rhee et al. (1994) and Subhachalat et al. (1998)

The DNA isolated from the adult worm and microfilariae yielded 336ng and 250 ng per micro liter respectively with A260/A280 ratio of 1.8 & 1.9 respectively indicating the purity of DNA. Agarose gel electrophoresis of both adult worm and microfilariae extracted DNA showed single band without smearing (Fig 1).

PCR amplification of the *Setaria digitata* repetitive element “IpSdS” yielded 2 products of 125bp and 332bp length (Fig 2). The amplification of two bands in the present assay could be
attributable to tandemly arranged nature of repeating element (IpSdS) in the genome of *S. digitata* from which the primers were designed as opined by Wijesundera *et al.* (1999) [22] who also reported an extra band of 519bp length. However, an improvement of reducing the extra band of 519bp could be achieved by increasing the annealing temperature to 53 °C in our study. The problem of primer related artefacts, primer dimmers (fast moving broad band) due to less concentration or absence of target DNA reported by Wijesundera *et al.* (1999) [22] could be eliminated in our study by minor modifications to the cycling conditions described by Wijesundera *et al.* (1999) [22].

The assay has yielded the same banding pattern with adult worm and corresponding microfilariae. Amplification did not see in negative controls and cattle, goat blood DNA (FIG 4) and the assay could detect as low as 31.25pg of microfilaria DNA which is equivalent to 1/8th of one microfilaria (Fig 3). The PCR assay was found positive in 49 out of 326 blood samples tested indicating an overall prevalence of 15.03% of bovine microfilariosis (Fig 4).

Comparatively higher percentage of infection was recorded in buffaloes (17.54% i.e. 30/171) than in cattle (12.25% i.e. 19/155) in our study (Fig 5). The higher rate of incidence in buffaloes could be due to agro climatic and seasonal factors favourable to the vectors and more proximity of buffaloes to stagnant water due to their wallowing habit. A proportionate increase of 2.22% (1/45), 8.64% (7/81), 17.92% (19/106) and 23.4% (22/94) of incidence was recorded among age groups starting from below 3 years, 3-6 years, 6-9 years and above 9 years of age, respectively (FIG 6). Similar reports of higher incidence of microfilariosis in aged animals was reported by Rao *et al.* (2005) in Andhra Pradesh, India.

Based on the physiological status of the animal, significantly (*P*≤ 0.05) highest prevalence of bovine microfilariosis was recorded among lactating 21.91% (32/146) (18.01%) animals followed by non lactating 12.72% (3/42) animals and heifers 7.14% (3/42) out of 326 female animals screened by PCR assay(Fig 7).

The results of PCR assay were compared with those of traditional techniques viz., Giemsa staining and Modified Knots techniques in detecting bovine microfilariosis. Out of 326 samples screened, 49, 20 and 13 were found positive showing 15.03%, 6.14% and 3.98% positivity by PCR assay, modified knots technique and Giemsa’s staining technique in detecting bovine microfilariosis, respectively. Comparatively less percent of incidence recorded in Giemsa’s staining and Modified knots technique could be due to non-availability of microfilaria during day time as opined by Mukherjee (1965) and Kumar *et al.* (2004) [6] who noticed maximum number of microfilaria in peripheral blood circulations from 6pm to 12pm. However, Sastry *et al.* (1985) [11] reported a non-periodic nature of microfilaria of *S. digitata*. The higher percentage of positive cases recorded in PCR assay compared to traditional techniques could be due to the amplification of free DNA liberated from microfilaria irrespective of the periodicity behavior of microfilaria as opined by Mukherjee. (1965), Abbasi *et al.* (1996) [3] and Wijesundera *et al.* (1999) [22]. Hence, the assay may be recommended in the diagnosis of bovine microfilariosis of *Setaria digitata*.  

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**Fig 1:** Agarose gel (0.7%) electrophoresis - Genomic DNA of hosts and *S. digitata* M: Molecular Marker (Lambda Hind III digest) 1: Cattle (leukocyte) 2: Goat (leukocyte) 3: Goat (brain tissue) 4: *S. digitata* (adult) 5: *S. digitata* (microfilaria)

**Fig 2:** Standardization of PCR assay for the diagnosis of *S. digitata* M: Gene ruler 100 bp DNA ladder plus 1: *S. digitata* (adult) 2: *S. digitata* (microfilaria) 3: Cattle (leukocyte) 4: Goat (leukocyte) 5: Negative control

**Fig 3:** Analytical sensitivity of PCR assay with double dilutions of genomic DNA of microfilaria harvested from *S. digitata* M: Gene ruler 100 bp DNA ladder plus 1:1ng 2: 500pg 3: 250pg 4: 125pg 5: 62.5pg 6: 31.25pg
4. Conclusion

Results from the aforementioned study indicate the standardized PCR assay is specific and sensitive in diagnosing the bovine microfilariosis of *Setaria digitata*.

Acknowledgements

First author is thankful to Sri Venkateswara Veterinary University, Tirupati, Andhra Pradesh, India for the financial support in the form of P.G. stipend and for providing facilities to carry out the research work.

References