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Molecular assay for the diagnosis of *Setaria cervi*

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Abstract
Present study deals with the diagnostic evaluation of *Setaria cervi* by PCR assay with the primers developed from "IpSdS" repeat sequence in the genome of *Setaria digitata*. Standrdized PCR assay has yielded 125bp, 332bp products from the genome of adult *Setaria cervi* and In-vitro harvested microfilariae. Among the products 125bp product is more prominent than 332b product. The PCR was found sensitive in detecting the DNA as low as 953 ag from template DNA of *S.cervi. Setaria* species may occur individually or coexist in their natural habitat and are distinct to each other. Differentiation of Setaria species is not crucial for the diagnostic purpose. Hence the PCR assay may be used in the Field diagnosis of bovine microfilariosis irrespective of species involved.

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

Introduction
Bovine microfilariosis is an emerging disease in India producing various clinical manifestations resulted in loss of body condition, reduced productivity with long convalescence period. Microfilariosis is caused by microfilaria (L_1stage) of *Setaria* inhabiting the bloodstream of bovines. Around 43 species of *Setaria* (Yatawara *et al*., 2007) have been identified so far in more than 100 countries of world including India. In India mainly 3 *setaria* spp were reported i.e., *S. digitata*, *S.* labiatopapillosa and *S. cervi*. (Mohan. 1975, Chauhan and Pande. 1980, Patnaik. 1989, Siddiqui *et al*., 1996) [9, 3, 11, 16].
S. cervi is more prevalent in Buffaloes (Siddiqui *et al*., 1996) [16] and predominantly found in pantnagar of uttarpradesh (Singh *et al*. 1973, Sharma and kumar. 1994, siddiqui *et al* 1996) [17, 15, 16] and coastal areas of Andrapradesh (Shastry *et al*. 1985., Venu *et al*. 2000, Pavan kumar *et al*. 2004) [14, 21, 12]. Incidence of *S.cervi* was also reported by Varma *et al* (1971) [20], Mohanty *et al* (2000) [10] and Sunder *et al* (2005) [18] from Bihar, Kerala and Karnataka respectively. *Setaria* species may occur individually or coexist in their natural hosts. Sunder and ravindran (2009) [19] reported mixed infections of *S. digitata*, *S. labiatopapillosa* and *S. cervi* in 16.04 % (30/187) of animals in Karnataka.
An important requirement to plan effective control strategies for these infections is the correct identification of the nematode at the species level both in the vertebrate host and in the vector. Molecular assays are necessary for the correct identification of filarial nematodes because larval stages of filarial species usually cannot be identified by classical morphology (Cancrini and Kramer 2001) [2]. Hence, molecular characterization allows the correct identification of the parasites in the bovine host and arthropod vectors. Highly repetetive DNA probes have been already developed for Brugia (Williams *et al*., 1993) [23] and Onchocerca species (Harnett *et al*., 1989) [5]. In contrast, relatively little progress has been achieved with respect to infections caused by filarial parasites of animals, particularly those among livestock. Hence, the present assay designed to develop a specific and sensitive PCR assay for the diagnosis of *S.* cervi microfilariosis.

Materials and methods
Collection of nematode worms
Thread like, milky white *Setaria* worms were collected from the bovines slaughtered in chengicherla, Hyderabad slaughter house in PBS 7.2, Worms were cleaned for several times in PBS pH 7.2 to remove host material and blood contamination (Dhas *et al* 1993) [4] etc,.

Microscopic examination
The anterior and posterior ends of the worm were cut and cleared for species identification as

per the procedure given by Kennedy (1979) [8]. Briefly, the cut head and tail ends of the worm were dehydrated in ascending grades of alcohol i.e. in 90% alcohol for 30 min, 100% alcohol for 15 min followed by clearing in lactophenol for 2 days to make the cuticle transparent. Cleared anterior and posterior ends of the worm were mounted in the same medium on a clean glass slide and observed under low power magnification.

Harvesting of microfilaria from single female *S.cervi*

Microfilariae were harvested from a single female *S.cervi* worm incubated in PBS7.2 at room temperature for 18 hrs. Once the microfilaria were seen, incubating fluid was centrifuged and pellet of microfilaria preserved for further studies

DNA extraction

Genomic DNA was isolated from the frozen middle portion of *S.cervi* and *In vitro* harvested microfilariae according to the procedure outlined by Sambrook *et al.* (1989) [13] with minor modifications. Briefly, the frozen middle portion of the worm was thawed, trituarated and centrifuged. cell lysis buffer was added to the sediment and to the pellet of microfilariae @ 600µl and incubated at 37^0C for 1hr which was followed by the addition of proteinase K (100 µg/ml) and incubation at 56^0C for 1 hr with periodic swirling. The lysed cell suspension was then layered over an equal volume of tris-saturated phenol, and homogenized by gentle repeated inversion and shaking. The tubes were then centrifuged at 2500 rpm. The upper aqueous phase was carefully picked up with a wide bored plastic Pasteur pipette and transferred to another tube containing tris-saturated phenol. The process of homogenization and extraction was repeated once again. The aqueous phase thus obtained was layered over an approximately equal volume of chloroform: isoamyl alcohol (24:1) and extracted twice. The final aqueous phase was carefully aspirated and the volume was measured. To the separated aqueous phase, 0.2 volumes of 10M ammonium acetate were added. This was overlaid with 2 volumes (of original aqueous phase) of ethanol at room temperature. The phases were mixed by gentle swirling of the tubes.

The precipitated and spooled DNA was collected, washed with 70% ethanol. DNA Pellet was dissolved in 50-100 µl of TE buffer (pH 8). The tubes were maintained in a water bath at 65oC for 10 min and stored at -20oC.

Polymerase chain reaction assay

The PCR was performed as per the protocol given by Wijesundera *et al* (1999) [22] with minor modifications.

The forward M2Fp, 5'CCGACATCAAGTTCATG 3' and reverse M2Rp, 5'GATTCAAGAACATGGTG 3' set of primers were custom synthesized by Genei™. Fifty microlitre reactions were set-up in each reaction. The reaction mixture consisted of 10 µl (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. cervi* 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 30sec extension at 72 °C, followed by a final extension of 15 min at 72 °C. The PCR products were stored at 4 °C until use. PCR products were electrophorosed in 1.5% agarose gel at 75 volts for 1hr and visualized in gel documentation system

Analytical sensitivity of PCR assay

Appropriate fourfold dilutions of template DNA 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.

Results and Discussion

Setaria cervi worms are thread like, milky white in colour. Anterior end of worms consists of a peribuccal crown with a pair of circular lateral lips and bifid dorsal and ventral projections at anterior end and tail end consists pair of lateral appendages near the tip which contained a knob with roughly bifurcated end with blunt spines. These morphological features are found in confirmity with the reports of Varma *et al*., 1971 [20].

The present PCR assay for molecular diagnosis of S.cervi was standardized with the primers specific to the repetitive element "IpSdS" in the genome of *S. digitata*. Genomic DNA was extracted from the eviscerated middle portion of adult female *S. cervi* from which microfilaria were harvested earlier and later confirmed as *S. cervi* by morphological studies. The phenol chloroform extraction method yielded 200ng of DNA per micro liter with A260/A280 ratio of 1.946 indicating the purity of DNA. DNA thus collected showed a single band without any smearing when run in agarose gel (0.7%) electrophoresis. One ng/µl of such DNA was used as template in PCR assay.

The PCR assay using primers specific to repetitive element "IpSdS" in the genome of *S. digitata*, performed under the conditions described previously for *S. digitata* has amplified 1 ng of genomic DNA of adult female *S. cervi* and also microfilarial DNA yielding two bands of 125 bp and 322 bp length without any band in the negative control when run in agarose (1.5%) gel electrophoresis. Among the both products 125bp product is more prominent than 332b product (Fig.1). Trials were conducted to evaluate the theoretically lowest quantum of parasite DNA that could produce a visible signal for diagnosis by using the DNA isolated from adult female *S. cervi*. The PCR assay could detect the DNA as low as 953ag from template DNA of *S cervi.* (FIG 2.)

The PCR assay using primers specific to repetitive element "IpSdS" in the genome of *S. digitata*, amplified two bands of 125 bp and 322 bp length in 1 ng of genomic DNA of adult female and microfilaria of *S. cervi* without any amplification in the negative control which could be due to sharing of similar sequences as opined by Jayasinghe, (2000) [6]. The above worker established a closer relationship between *S.digitata* and *S. cervi* than between *S. digitata* and *S. labiato-papillosa* by constructing a phylogenetic tree. Alasad *et al., 2012* [1] while working on phylogentetic study of *Setaria cervi* further confirmed *Setaria cervi* as member of the *Setaria* genus and formed a sister clade with *S. labiatopapillosa* and *S. cervi*. Wijesundera *et al*., 1999 [22] could not apply *S. digitata* specific PCR assay on *S. cervi* which could be due to non availability of the species in Sri Lanka. The present inference drawn could be supported by the statements of earlier workers of Sri Lanka (Wijesundera *et al*., 1999 and Jayasinghe and Wijesundera, 2003) [22, 7] who reported only *S. digitagta* and *S. labiato-papillosa* as Setaria species occurring

in Sri Lanka. In India *Setaria* species may occur individually or coexist with other species of setaria. Differentiation of *Setaraia* species is not crucial for the diagnostic purpose (Jayasinghe and Wijesundera., 2003) [7]. Species specific primers may help in the phylogenetic and taxonomic studies and also to study species specific virulence and pathogenicity. Hence, the present assay may be used in the field diagnosis of bovine microfilariosis irrespective of species involved.

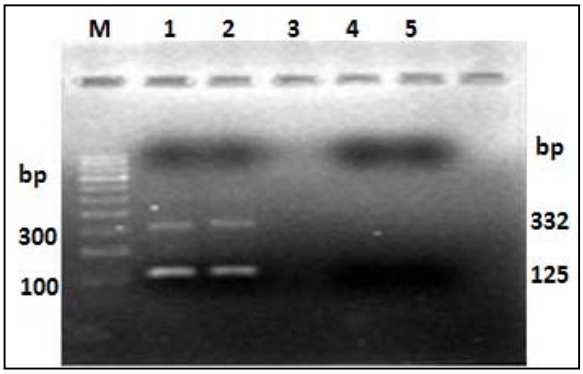

Fig. 1: PCR assay in detecting the genomic DNA of adult and microfilaria of *S. cervi*.

M: Gene ruler 100 bp DNA ladder plus
1: *S. cervi* (adult)
2: S. *cervi* (microfilaria)
3: Cattle (leukocyte)
4: Goat (leukocyte)
5: Negative control

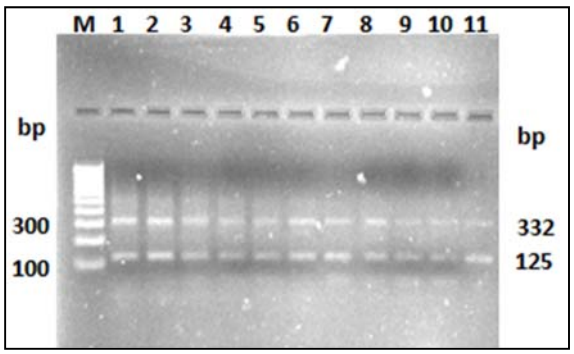

Fig 2: Analytical sensitivity of PCR assay with four fold dilutions of *S.cervi* (adult) genomic DNA.

M: Gene ruler 100 bp DNA ladder plus
1. 1ng
2. 250pg
3. 62.5pg
4. 15.625pg
5. 3.906pg
6. 976.56fg
7. 244.14fg
8.61.035fg
9. 15.258fg
10. 3.814fg
11. 953.6ag

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