

*Original Research***Molecular Diagnosis of Bovine Dermatophilosis Using Species Specific Polymerase Chain Reaction****P. V. Tresamol, M. R. Saseendranath and Toms C. Joseph**

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Abstract

Present study involves use of a species specific PCR for molecular confirmation of *D. congolensis* isolates from cattle in Kerala. Thirty isolates of *Dermatophilus congolensis* isolated from dermatitis cases of cattle from Kerala were used in the study. Genomic DNA was extracted using the High Pure PCR Template preparation Kit. A species specific PCR to detect *D. congolensis* based on a 1029 bp internal, conserved region of the alkaline ceramidase gene was carried out using specific primers, 5'- GCG TAA CCG CTC CCT TCC TGC TCT TCC GCT TCC TC-3' (DCACIF) and 5'-GCC AAA CCA GCA GCC TCT TGC CGC ATA CAC TAT TCT C-3' (DCACIR). The use of this species specific PCR for molecular confirmation of *D. congolensis* isolates was found to be highly specific in detecting *D. congolensis* by discriminating from other bacteria.

Key words: Dermatophilosis, Molecular Diagnosis, PCR

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Introduction

Dermatophilosis, also known as streptothricosis, is an exudative, pustular dermatitis that affects mainly cattle, and caused by *Dermatophilus congolensis*. The disease is characterized by exudative, proliferative or hyperkeratotic dermatitis with matting of hairs and formation of thick and horny scabs, crusts and fissures with matted hair at their bases (Gebreyohannes and Gebresselassie, 2013; Tresamol and Saseendranath, 2014; Selvakumar *et al.*, 2017). It is an economically important disease which causes considerable loss in terms of skin damage, reduced meat and milk production, culling or death of affected animals and costs of control and treatment (Zaria, 1993). It has been reported by the Food and Agricultural Organization (FAO) as one of the important bacterial diseases which affect cattle and other

animals in the tropical and subtropical regions (Hashemi Tabar *et al.*, 2004). Dermatophilosis was reported to be associated with wide spread dermatitis among crossbred dairy cattle of various states in India (Pal, 1995; Sudhakara Reddy *et al.*, 2014). *D. congolensis* was identified as the etiology of widespread lower leg dermatitis among dairy cattle and buffaloes in Kerala (Tresamol *et al.*, 2015; Tresamol *et al.*, 2016).

Clinical signs of the disease are characteristic of chronic dermatitis which become visible where the hair on affected part of the skin is matted with exudates which harden when dry, presenting paint brush appearance. The common areas affected were lower limbs, udder, inguinal region, perineum and ventral abdomen (Tresamol and Saseendranath, 2014). Diagnosis of the condition is by demonstration of typical tram track appearance of the organism in stained skin scabs and confirmation by isolation and identification of organisms. But diagnosis by these methods is not useful from chronic and healing lesions and there is considerable difficulty in isolation and conventional biochemical characterisation of the organism (Tresamol and Saseendranath, 2013). Molecular diagnosis by using polymerase chain reaction (PCR) was described by several workers as a highly specific and sensitive test in comparison with the widely used conventional microbiological methods and validated its potential to be used in epidemiological as well as clinical studies (Han *et al.*, 2007; Shaibu *et al.*, 2010). Tresamol and Saseendranath (2015) also reported molecular diagnosis of dermatophilosis by PCR using primers targeting 16S rRNA gene of *D. congolensis*.

Dermatophilus congolensis synthesizes a number of exoenzymes such as proteases, keratinases and ceramidase which are having important role in its virulence and pathogenesis. In normal epidermis ceramidase catalyzes the cleavage of ceramide to fatty acids and sphingosine. An imbalance in the levels of these products may modify the features of epidermis including its permeability, immune functions and other physical characteristics determined by the normal proliferation and differentiation of epidermal keratinocytes (Ohnishi *et al.*, 1999). Garzia-Sanchez *et al.* (2004) identified alkaline ceramidase gene of *D. congolensis* and suggested its role in pathogenesis of dermatophilosis. Valipe (2011) described a sensitive and species-specific PCR targeting 1029 bp, unique sequence on alkaline ceramidase gene to detect *D. congolensis* and found that this PCR protocol can detect as low as 50×10^{11} μg of *D. congolensis* DNA within 4 hours. Present study involves use of this species specific PCR for molecular confirmation of *D. congolensis* isolates from cattle in Kerala.

Materials and Methods

Dermatophilus congolensis Isolates

Fifty cross bred cattle of various farms and households of Thrissur district of Kerala with history of dermatitis formed the study material. Microscopical examination of Giemsa and Gram stained smears of

the scab material from the lesions revealed characteristic Gram positive septate branching filaments with multiple rows of spherical to ovoid cocci, with typical 'tram-track appearance' suggestive of *D. congolensis* from 30 samples. Culture of the scab materials in sheep blood agar in presence of 10 per cent carbon dioxide yielded typical beta haemolytic colonies of *D. congolensis* from all these samples. The isolates were further confirmed by macroscopic and microscopic morphology of colonies and biochemical tests. The isolates were inoculated in to brain heart infusion broth and incubated overnight. About 200 µl of washed cells suspended in sterile distilled water was used for the DNA extraction.

DNA Extraction

Genomic DNA was extracted using the High Pure PCR Template Preparation Kit (Roche, Germany). The sample was centrifuged at 3000 g for 5 min. The pellet was re-suspended in 200 µl of Phosphate Buffered Saline (PBS) to which 200 µl of binding buffer was added followed by 40 µl of proteinase K. This was immediately mixed and then incubated for 10 min at 70°C. About 100 µl of isopropanol was added and properly mixed. This was then poured into a filter tube with a collection column and centrifuged at 8000g for 1 min. The collection column was discarded and a new one attached. About 500 µl of inhibition removal buffer was added to the filter tube and centrifuged at 8000g for 1 min. The column was discarded and a new one attached. About 500 µl of wash buffer was added to the filter tube and centrifuged at 8000 g for 1 min. The washing step was repeated and the column containing the eluent was discarded. A new 1.5 ml eppendorf tube was attached and 50 µl of pre warmed elution buffer was added to the tube containing the DNA and centrifuged at 8000g for 1 min. The eluted DNA was collected in the new 1.5 ml eppendorf tube and stored at -20°C until use.

Primers

The sequences of the primers were based on 1029 bp internal conserved gene of alkaline ceramidase of *D. congolensis*. Forward primer was 5'- GCG TAA CCG CTC CCT TCC TGC TCT TCC GCT TCC TC-3' (DCACIF) and Reverse primer was 5'-GCC AAA CCA GCA GCC TCT TGC CGC ATA CAC TAT TCT C-3' (DCACIR) (Valipe, 2011). The primers were custom synthesised (Sigma inc.) and reconstituted in sterile water for the assay.

Amplification of Alkaline Ceramidase Gene using PCR

A species specific PCR to detect *D. congolensis* based on a 1029 bp internal, conserved region of the alkaline ceramidase gene was carried out (Valipe, 2011) with minor modifications. The reaction was performed in a total reaction mixture of 25µl containing 2.5 mM MgCl₂, 200 µM of each nucleotide, 0.5µM of each primer and one unit of *Taq* polymerase and one microlitre of template DNA from the

samples. DNA isolated from *Staphylococcus aureus* formed the negative control. The tubes were spun briefly and placed in the Veriti 96 well Thermocycler (Applied Biosystems, USA).

The PCR programme was as follows-

Initial denaturation	94 ⁰ C for 2minutes	30 cycles
Denaturation	92 ⁰ C for 30 seconds	
Annealing	50 ⁰ C for 45 seconds	
Extension	72 ⁰ C for 30 seconds	
Final extension	72 ⁰ C for 10 minutes	

The PCR products were detected by electrophoresis in a 1.5 per cent agarose gel in TBE buffer (1X). Five microlitre each of the PCR products was mixed with one microlitre of 6x gel loading dye and the samples were loaded into the respective wells carefully. A one Kb DNA ladder was used as molecular size marker. Electrophoresis was carried out at 70V for 45 minutes. DNA amplifications were visualised under Ultra violet transilluminator and the results were documented in a gel documentation system (Alpha innotech Corporation, USA).

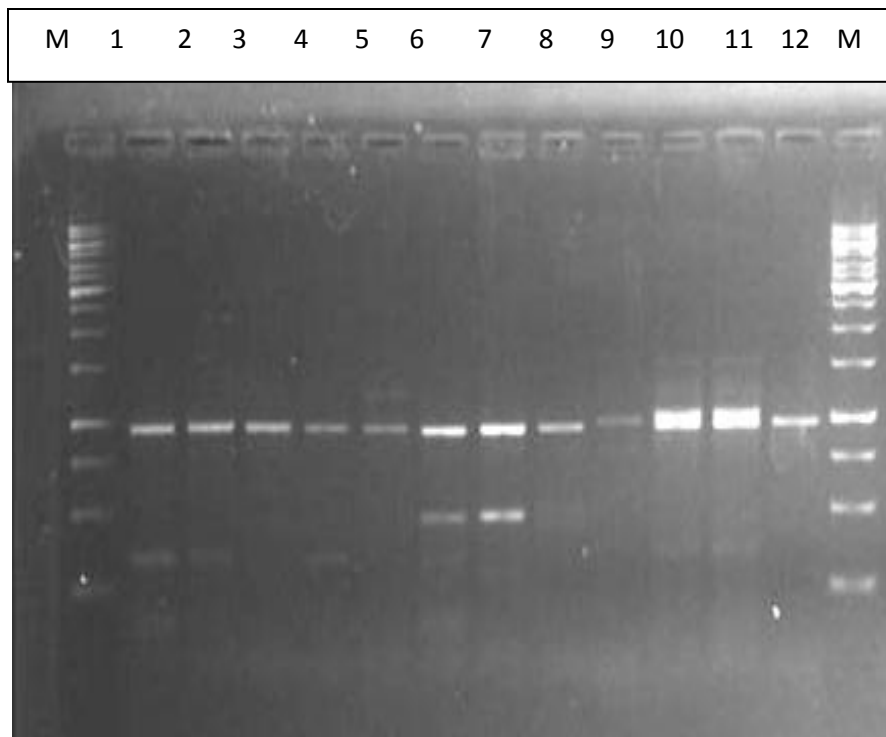


Plate1: Agarose gel electrophoresis of species specific PCR products of *D. congolensis*

Results and Discussion

The *D. congolensis* species specific PCR with primer pair DCACIF and DCACIR under the above mentioned cycling conditions yielded a specific PCR product of 1029 bp. No amplification was observed with the negative control. The assay amplified the 1029 bp target region from all 30 isolates. It is based

on the 1029 bp sequence of alkaline ceramidase gene of *D. congolensis* which was found to be a unique, reliable and specific target to detect *D. congolensis* from microbiological samples. This PCR was reported to be highly specific and sensitive and saved a significant amount of time and labour in comparison with microbiological methods (Valipe, 2011). Ceramides have important protective and cell regulatory roles in the epidermis Hence detection of this ceramidase may suggest their role in the pathogenesis of dermatophilosis.

In most of the laboratories the conventional methods such as direct skin scab smear examination and isolation of organisms are used for routine diagnosis of dermatophilosis. But many times the samples are contaminated with different species of bacteria which make culturing unsuccessful by inhibiting the growth of *D. congolensis* (Dalis *et al.*, 2010). Polymerase chain reaction was used by several researchers for rapid and accurate detection of *Dermatophilus congolensis* in clinical specimens (Buenviage *et al.*, 2000; Han *et al.*, 2007). Shaibu *et al.* (2010) also recommended PCR as a good diagnostic technique for *D. congolensis* isolates from cattle, sheep and goat. Sensitive and accurate molecular techniques enable rapid and prompt diagnosis so that appropriate and early treatment and control measures can be adopted to reduce the economic loss.

Conclusion

Present study describes usefulness of a species specific PCR based on a 1029 bp internal, conserved region of the alkaline ceramidase gene of *D. congolensis* for diagnosis of bovine dermatophilosis. Thirty isolates of *Dermatophilus congolensis* from lower leg dermatitis cases of cattle from Kerala were confirmed by using this PCR. The use of this species specific PCR for molecular confirmation of *D. congolensis* isolates was found to be highly specific in detecting *D. congolensis* by discriminating from other bacteria.

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