Footrot on a sheep breeding farm in the Himalayan state of Jammu and Kashmir

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Submitted for publication: 6 October 2008
Accepted for publication: 9 June 2010

Summary
In the present study ovine footrot was detected clinically on a sheep farm in the Himalayan state of Jammu and Kashmir. *Dichelobacter nodosus* was confirmed by culture and polymerase chain reaction (PCR) using species-specific 16S ribosomal RNA primers. When cultured, the organism appeared as flat colourless colonies having a fine granulated structure with irregular margins, and showing characteristic Gram-negative rods with swollen ends. Detection by PCR from cultured bacteria resulted in amplification of a 783 base pairs (bp) product. Serogrouping by multiplex PCR using group (A-I)-specific primers revealed the presence of serogroup B-specific bands of 283 bp.

Keywords

Introduction
Footrot is a specific contagious disease of the feet of sheep, goats and other ruminants caused by *Dichelobacter nodosus* (1, 9). It is characterised by an exudative inflammation followed by necrosis of the epidermal tissues of the hoof with a foul odour. Depending on their degree of virulence, isolates of *D. nodosus* are categorised as virulent, intermediate or benign (9). The affected animals show symptoms of lameness, reduced grazing, loss of body weight, reduced wool production and decreased fertility, leading to significant economic losses (5). *D. nodosus* is fastidious in nature and difficult to grow in culture, making its isolation and identification very difficult (8). Footrot is distributed worldwide but it has a major impact in those regions that have a temperate climate (9). Sheep husbandry is practised extensively in the northern Himalayas, which has a temperate climate. Until recently, despite repeated outbreaks and clinical presence, there was little information available on the detection, isolation and characterisation of *D. nodosus* from India, particularly in J & K state (2, 11). In the present study, ovine footrot was detected clinically and *D. nodosus* was isolated from a sheep farm in the J & K region of India. The organism was confirmed by culture and polymerase chain reaction (PCR) using species-specific 16S rRNA primers.

Materials and methods
Primary isolation
An outbreak of footrot was reported at the Sheep Extension Centre, Raithan, in the Budgam district of Kashmir. This centre, which houses approximately 100 crossbred Australian Merino sheep, is professionally managed by veterinarians and trained field staff. The affected sheep showed general loss of condition, lameness, loss of weight and reluctance to graze. Individual feet of the sheep were examined. Lesions of infected hooves varied from mild interdigital inflammation to severe interdigital lesions.
Samples from the lesions of 12 affected animals were collected on cotton swabs (Hi Culture Transport Swabs w/ Cary Blair Medium, Hi Media Labs Pvt. Ltd, Mumbai, India) and inoculated onto 4% hoof agar (HA) plates for isolation (7, 10). The plates were placed immediately in Anaero jars (Hi Media, Gujarat, India) containing Anaero Gaspack along with anaerobic indicator tablets (Hi Media, Gujarat, India) and transferred to the laboratory. The jars were placed in an incubator at 37°C within one to two days of sample collection. During transport they were kept at an ambient temperature of 15°C to 25°C. After three to four days of incubation, suspected colonies from each sample were subcultured on 4% HA plates to separate them from contaminating organisms. Isolated colonies were subjected to Gram staining.

**Detection of 16S ribosomal RNA gene by polymerase chain reaction**

Well-isolated bacterial colonies were picked up with an autoclaved pipette tip, suspended in 250 μl phosphate buffered saline (PBS), boiled for 5 min and immediately cooled on ice for 10 min. The suspension was then centrifuged at 16,110 g for 1 min, and 2 μl of the supernatant was used as a template. The PCR amplifications were performed in 25 μl volumes in 0.2 ml thin-walled PCR tubes. The PCR mixture contained a final concentration of 10 mM Tris-HCl, pH 9.0, 50 mM KCl, 3 mM MgCl₂, 0.01% BSA, 0.5 μM of each primer (5’GGGTTATGTAAGCCTGC3’ and 5’TGGTAGCGAGCTTTGCTACCT3’ [5]), 0.2 mM concentrations of each 2’-deoxynucleoside 5’-triphosphate, and 1.0 U of Taq DNA polymerase (Bangalore Genei Pvt. Ltd, Bangalore, India). The PCR products were electrophoresed in 0.8% agarose gels, stained with ethidium bromide, visualised under ultraviolet illumination and photographed with a gel documentation system (Biometra, Germany). A 2 μl volume of sterilised distilled water served as a negative control for the PCR.

**Serogroup-specific multiplex polymerase chain reaction**

Samples positive on PCR were subjected to serogroup-specific multiplex PCR using a common forward primer specific for the *D. nodosus* fimA gene and nine serogroup-specific reverse primers, as detailed by Dhungyel et al. (3). The PCR amplification was performed using three reverse primers and one forward primer in a single reaction tube.

The reaction mixture consisted of 20 mM Tris-HCl, pH 9.0, 50 mM KCl, 3 mM MgCl₂, 200 μM concentrations of each 2’-deoxynucleoside 5’-triphosphate, and 1.0 U of Taq DNA polymerase (Bangalore Genei Pvt. Ltd, Bangalore, India). The concentration of each primer (Table I) was 0.25 μM to 0.5 μM. Oligonucleotide primers were procured from Bangalore Genei Pvt. Ltd. The PCR products were analysed in 2.0% agarose gels, visualised and photographed as described above.

**Table I**

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Nucleotide sequence</th>
<th>Position in fimA</th>
<th>Product size (bp)</th>
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</thead>
<tbody>
<tr>
<td>FP</td>
<td>5’CCCTTAATCGAATCTGATGTTG3’</td>
<td>26-46</td>
<td>–</td>
</tr>
<tr>
<td>RA</td>
<td>5’AGTGTGCGCCTCCATTATTT3’</td>
<td>421-441</td>
<td>415</td>
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<td>RB</td>
<td>5’CGGATCGCCAGCTTCTGTCTT3’</td>
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<td>283</td>
</tr>
<tr>
<td>RC</td>
<td>5’AGAAGTGGCGCTTCATTATTT3’</td>
<td>331-351</td>
<td>325</td>
</tr>
<tr>
<td>RD</td>
<td>5’TGCAACAATTTCCCTGACCT3’</td>
<td>325-345</td>
<td>319</td>
</tr>
<tr>
<td>RE</td>
<td>5’CAGCTTTCGATCGGACCTG3’</td>
<td>367-389</td>
<td>363</td>
</tr>
<tr>
<td>RF</td>
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<td>241</td>
</tr>
<tr>
<td>RG</td>
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<td>283-305</td>
<td>279</td>
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<td>RH</td>
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<td>412-435</td>
<td>409</td>
</tr>
<tr>
<td>RI</td>
<td>5’CGATGGGTAGCTGACGACG3’</td>
<td>194-215</td>
<td>189</td>
</tr>
</tbody>
</table>

bp: base pair

**Results**

**Primary isolation and colony morphology**

All samples cultured on HA medium resulted in heavy bacterial growth of mixed morphologies. These were subcultured two to three times on 4% HA agar plates to remove the contaminating organisms. Bacterial growth from four samples from four different sheep showed characteristic colourless colonies that appeared as flat concentric zones with a finely granulated surface texture. These were hardly visible to the naked eye and varied in size from 0.5 mm to 2 mm in diameter with irregular margins. On Gram staining, characteristic Gram-negative rods with blunt swollen ends were observed (Fig. 1).

**16S ribosomal RNA polymerase chain reaction analysis of *Dichelobacter nodosus***

All four suspected bacterial cultures were subjected to the 16S ribosomal (r)RNA PCR assay in order to detect the presence of *D. nodosus*. All the cultures yielded an amplified product of the expected size of 783 base pairs (bp) (Fig. 2).
Serogroup-specific multiplex polymerase chain reaction

Four bacterial cultures showing typical features of *D. nodosus* and positive by 16S rRNA PCR were subjected to serogroup-specific PCR, and all the specimens yielded a single band of 283 bp characteristic of serogroup B of *D. nodosus*.

Discussion

In their initial efforts to isolate the bacteria, the authors picked samples from grade four or five lesions only. This was done to give a better chance of recovery of *D. nodosus*, because virulent footrot is usually diagnosed in flocks that show under-running of the hoof. However, initially they encountered a very heavy growth of contaminating organisms. Subsequent survey of the literature revealed that isolation of *D. nodosus* from early cases of footrot is more successful from low-grade lesions (4, 6). For further confirmation the authors performed colony touch PCR from suspected isolated colonies. Out of the suspected cultures, four (isolated from four different sheep) were found to be positive for *D. nodosus*-specific 16S rRNA by colony touch PCR.

Variations in the DNA sequence of the *fimA* gene of *D. nodosus* have been exploited to develop a simple and rapid serogroup-specific PCR (3, 12). The aim of the study was also to determine the serotype prevalent in this region. Samples positive on the colony touch 16S rRNA PCR were subjected to serogroup-specific PCR. This revealed the presence of a single band of 283 bp characteristic of serogroup B of *D. nodosus*, which is in agreement with an earlier report by Wani et al. (11).

Conclusion

The present work documents the isolation of *D. nodosus*, its detection by 16S rRNA PCR, and the detection of the B serogroup in a Himalayan region. Extensive sheep husbandry is practised by poor farmers in this region. In order to control ovine footrot there is an urgent need to formulate appropriate control strategies through rapid diagnosis, vaccination and management practices. However, in order to define the pathogenic nature of *D. nodosus*, its survival and transmission, and its specific role in sheep breeds of the state, systematic studies need to be undertaken.

Acknowledgements

The authors acknowledge funding for the project ‘Development of non-culture diagnostic techniques for *Dichelobacter nodosus*’ by the Department of Biotechnology, Government of India and facilities provided by SKUAST-J.
Détection de l’agent du piétin dans un élevage ovin de l’état himalayen du Jammu-et-Cachemire

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Résumé
Les auteurs présentent les résultats d’une étude conduite suite à l’observation de signes cliniques de piétin dans un élevage ovin situé dans l’état himalayen du Jammu-et-Cachemire. L’identification de l’agent causal, *Dichelobacter nodosus*, a été réalisée en culture et confirmée au moyen de la technique d’amplification en chaîne par polymérase (PCR) en utilisant des amorces spécifiques de l’ARNr 16S de la bactérie. En milieu de culture, le micro-organisme produit des colonies non pigmentées et plates, présentant une structure finement granulée aux bords irréguliers et une forme en bâtonnets aux extrémités renflées, caractéristique des bacilles Gram-négatifs. La PCR réalisée à partir de la bactérie mise en culture a conduit à l’amplification d’une séquence de 783 pb. La caractérisation du sérogroupe auquel appartiennent les souches isolées a été réalisée au moyen d’une PCR multiplexe utilisant des amorces spécifiques de groupes (A-I), qui a révélé la présence de bandes de 283 pb spécifiques du sérogroupe B.

Mots-clés

Dermatitis interdigital en una explotación de cría ovina del estado de Jammu y Cachemira, en el Himalaya

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Resumen
Los autores describen un estudio en el que se detectó dermatitis interdigital en una explotación ovina situada en el estado himalayo de Jammu y Cachemira. Tras el cultivo y la aplicación de una reacción en cadena de la polimerasa (PCR) con cebadores de ARN ribosómico 16S específicos de *Dichelobacter nodosus*, se pudo confirmar la presencia de este microorganismo. En cultivo, la bacteria formaba colonias planas e incoloras con una fina estructura granulada de contorno irregular, en las que se observaban los típicos bacilos gram negativos con los extremos abultados. La aplicación de la PCR a las bacterias cultivadas resultó en la amplificación de una secuencia de 783 pb. La tipificación de serogrupos por PCR múltiple, utilizando cebadores específicos de cada grupo (A-I), reveló la presencia de bandas de 283 pb características del serogrupo B.

Palabras clave
References


