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BRIEF COMMUNICATION

Epidemiological, bacteriological and molecular studies on caseous lymphadenitis in Sirohi goats of Rajasthan, India

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Abstract *Corynebacterium pseudotuberculosis* is the causative agent of caseous lymphadenitis (CL), a chronic debilitating disease of goats. In the present study, a total of 575 goats of Sirohi breed on an organized farm situated in the semi-arid tropical region of Rajasthan, India were clinically examined. Pus samples from superficial lymph nodes of 27 (4.7%) adult goats presenting clinical lesions suggestive of CL were collected for bacteriological and molecular analyses. Of these goats, 51.9% yielded *C. pseudotuberculosis* on the basis of morphological, cultural and biochemical characteristics. A polymerase chain reaction (PCR) assay targeting proline iminopeptidase gene specific to *C. pseudotuberculosis* was developed that confirmed all 14 bacterial isolates. The specificity of the PCR product was confirmed by sequencing of the 551-bp amplicon in both senses, showing 98–100% homology with published sequences. Thus, overall prevalence rate based on clinical, bacterial culture and PCR assay were found to be 4.7%, 2.4% and 2.4%, respectively. The PCR assay developed in this study was found to be specific and rapid, and could be used for confirmation of CL in goats as an alternative method to generally cumbersome, time-consuming and less reliable conventional methods.

Keywords Caseous lymphadenitis · *Corynebacterium pseudotuberculosis* · Proline iminopeptidase · Polymerase chain reaction · Lymph nodes · Goat

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Introduction

Caseous lymphadenitis (CL) is a chronic bacterial infectious disease of goats and other small ruminants caused by *Corynebacterium pseudotuberculosis*. These are Gram-positive, non-motile pleomorphic rods that often present a characteristic Chinese letter or palisade arrangement in the smear. *C. pseudotuberculosis* is a mycolic acid-containing facultative intracellular parasite that multiplies inside the macrophage (Baird and Fontaine 2007). The commonly occurring external form of CL is characterized by abscess formation in the superficial lymph nodes, while in the visceral CL abscessation occurs in various lymph nodes and other internal organs (Dorella et al. 2006; Fontaine and Baird 2008). A significant proportion of the world goat population is found in the countries defined by FAO as net food-importing countries (44%), low-income food-deficit countries (86%) and the least developed countries (31%, FAOSTAT 2011). The Indian subcontinent has approximately 28% of the total world goat population. The disease is widely distributed in goats and sheep of these countries, and farmers accrue significant financial losses due to reduced meat and milk yield, loss of fertility, culling of affected animals and their condemnation and downgrading at the time of slaughter and inspection (Williamson 2001; Guimaraes et al. 2011).

There are only sporadic published reports of occurrence of CL in the goat and sheep population of India (Mohan et al. 2008). Systematic study has not been conducted in most regions of India regarding its prevalence, extent of infection and associated socioeconomic impact on goat husbandry. In goats, the typical pattern of clinical manifestation in the form of abscessation of superficial lymph nodes in several animals of the flock suggests presence of *C. pseudotuberculosis* infection. The laboratory confirmation of the infection is usually achieved by bacterial isolation and biochemical tests, which

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produce variable results (Baird and Fontaine 2007). In the present study, we estimated the prevalence of *C. pseudotuberculosis* infection on clinical grounds, bacterial isolation and characterization and a specific PCR assay employing proline iminopeptidase (PIP) gene in a Sirohi goat flock maintained in semi-arid tropical region of Rajasthan state of India.

Materials and methods

Clinical examination and sample collection

The study was carried out on an organized goat farm maintained under semi-intensive system in the semi-arid tropical region of Rajasthan, India. The study was conducted over a period of 12 months (April 2010 to March 2011). A total of 575 goats belonging to Sirohi breed were clinically examined for the presence of enlarged and abscessed superficial lymph nodes. Pus samples from 27 adult goats with lesions suggestive of caseous lymphadenitis were aspirated aseptically using sterile disposable syringe and needle (18 gauges) after shaving and cleaning the swollen lymph node with 70% ethanol. The samples were identified and were immediately transported to the laboratory for processing.

Bacterial culture

Pus samples were directly inoculated on blood agar base (Himedia, Mumbai, India) supplemented with 5% defibrinated sheep blood. Bacterial isolates with whitish, dry, opaque, hemolytic, convex, friable consistency and freely moving colonies obtained after 72 h of aerobic incubation at 37°C were selected for further identification (Quinn et al. 2011). Pure cultures were prepared from these isolates after studying macroscopic characteristic of bacterial colonies and morphology in Gram-stained smears. Isolates were further inoculated on cystine tellurite blood agar (cystine tellurite agar base added with 5% defibrinated sheep blood and 5% *v*/*v* of 1% potassium tellurite) and incubated at 37°C for 72 h for its characteristic growth. The resultant black colonies were subjected to routine biochemical tests, i.e. nitrate reduction, catalase, urease and synergistic hemolysis with *Rhodococcus equi* and antagonistic hemolysis with *Staphylococcus aureus* for phospholipase D (PLD) production (Guimaraes et al. 2011). The culture strain VTCC BAA67 of *R. equi* was kindly provided by Dr. Praveen Malik, Incharge, Veterinary Type Culture, National Research Centre on Equines, Hisar, India. Catalase, urease and PLD-positive and nitrate-negative cultures were considered positive for *C. pseudotuberculosis*.

Bacterial DNA extraction

A few colonies from the pure cultures of *C. pseudotuberculosis* from 72-h growth on blood agar plates were transferred into a 1.5-ml microfuge tube. Bacterial DNA was extracted using HiPura kit (Himedia, Mumbai, India) as per the manufacturer's instructions. DNA purity was checked on 0.8% agarose gel electrophoresis. The DNA was stored at −20°C until used.

Polymerase chain reaction assay

The oligonucleotide primers amplifying 551-bp fragment of the most conserved PIP gene of *C. pseudotuberculosis* was synthesized from Sigma (UK). The forward and reverse primers were 5′-AACTGCGGCTTTCTTTATTC-3′ and 5′-GACAAGTGGGAACGGTATCT-3′, respectively (D'Afonseca et al. 2010). The PCR was carried out in 25 μl of reaction mixture containing 1× PCR buffer, 2 mM $MgCl_2$, 200 μM dNTP mix, 0.2 μM of each of forward and reverse primers, 1.25 units of Taq DNA polymerase (Sigma, UK) and 5 μl of DNA template in a thermal cycler (MJ Research PTC200). PCR conditions standardized and used for amplification were: a cycle of 95°C for 5 min, followed by 35 cycles each of 94°C for 30 s, 54°C for 45 s and 72°C for 45 s. A final cycle 5 min at 72°C was allowed for extension. The PCR products were resolved on 2.0% agarose gel electrophoresis with 0.5× Tris–borate–EDTA buffer and visualized with ethidium bromide staining. The PCR products of 551 bp were considered for identification as *C. pseudotuberculosis*.

Sequencing of PCR product

PCR products for sequencing were amplified with the use of Pfu DNA polymerase by using the standard protocol mentioned earlier. The amplicons were resolved on 2.0% agarose gel, and bands were excised from the gel under UV light. The DNA fragments were subsequently purified by minielute gel extraction kit (Qiagen, Germantown, MD, USA). The purified product was sequenced by Imperial Life Science (iLife Discoveries Pvt. Ltd.; GenBank accession no. JF901922) and compared with the PIP gene sequence available in the NCBI database.

Results and discussion

Of 575 goats examined clinically, 27 (4.7%) adult goats showed variable enlargement and abscessation of superficial lymph nodes that were not painful on palpation. In the recently affected goat, enlarged lymph nodes were generally of harder consistency. The commonly affected superficial lymph nodes were parotid, prescapular, submandibular,

cervical, prefemoral and brisket region lymph nodes. Abscessed lymph nodes yielded soft, pasty and odourless pus. Of these 27 animals, 14 (51.9 %) were found to be positive for *C. pseudotuberculosis* in the bacterial culture (Quinn et al. 2011). The nucleotide sequence was deposited in the NCBI GenBank (accession no. JF901922). All 14 bacterial isolates were Gram-positive pleomorphic rods, catalase positive, urease positive, nitrate negative and produced PLD, which are characteristic features of *C. pseudotuberculosis*. As all the isolates were negative for nitrate reduction, they belonged to biovar *Ovis* (Guimaraes et al. 2011).

The overall prevalence of 4.7%, 2.4% and 2.4% were recorded among animals at farm based on clinical examination, bacterial culture and PCR, respectively. Similar prevalence rates of CL in goats (4.81 %) based on clinical examination were reported from Assiut farms and abattoirs from Egypt (Mubarak et al. 1999). In other studies, prevalence rates varying from 0.2% to 13.5% have been reported in sheep and goats on the basis of clinical examination (Al-Gaabary et al. 2009, 2010; Al-Harbi 2011). Based on bacteriological examination in slaughtered sheep and goats, prevalence rates of 1.1% to 2.2% (Centikaya et al. 2002; Ghanbarpour and Khaleghiyan 2005) and 5.5% to 32.6% (Al-Gaabary et al. 2010; Zavoshti et al. 2010; Hassan et al. 2011) have been reported in previous studies carried out in Egypt, Iran and Saudi Arabia. It has been observed that recovery of live organisms on laboratory media depends on the stage and nature of infection, immune status of the animal as well as on the presence of other bacterial agents in the clinical samples (Al-Gaabary et al. 2009). In the present study, we could isolate *C. pseudotuberculosis* from 51.9% of the cases, which was comparable to those reported previously (Chirinozarraga et al. 2006; Al-Gaabary et al. 2010). However, in a recent study, a higher rate of isolation of *C. pseudotuberculosis* (90.07%) was reported in goats and sheep from Egypt (Al-Gabbary et al. 2009). Thus, the disease has economic significance in all agro-ecological zones especially the tropical, subtropical and arid areas across Asia, Africa, Central and South America, wherein over 80% of the world's goat population is located (FAOSTAT 2011).

The lower prevalence rate of the infection observed in the current study may partly be attributed to the better managemental practices as well as the harsh climatic conditions of the region, such as higher temperature during summer (up to 48° C), which is likely to limit the survival of the bacteria in the soil and external environment. In addition, another reason for reduced recovery of bacteria in culture could be the spatial distribution of organisms in the suppurative parenchyma of the lymph nodes. In a recent study, Fontaine and Baird (2008) demonstrated scattered clumps of bacteria in the zone of immature fibrosis, lying in between the outermost zone of mature fibrosis and innermost zone of coagulative necrosis by immunohistochemical technique analysis. It is possible that in our study, some of the pus samples collected might have contained more of dead and degenerated rather than live bacteria. Therefore, it is suggested that aspiration of pus samples should be attempted and perfected from the middle zone of immature fibrosis of the abscessed lymph node, where concentration of live bacteria could be greater.

Since the clinical examination could only detect external lesions, some cases of visceral CL cases might have gone undetected; therefore, the prevalence rate obtained in the current study could be an underestimate. Using the *C. pseudotuberculosis*-specific PCR for identification of 14 isolates, amplification of 551 bp fragment targeting PIP gene was obtained for all the isolates (Fig. 1). The sequencing of the amplified product revealed 98–99% identity with published sequence of PIP gene of *C. pseudotuberculosis* (GenBank accession no. CP003062.1, CP002924.1, CP001809.2, CP002251.1, CP001829.1, CP002097.1, CP003061.1 and CP003082.1) available in the NCBI database.

In a previous study, PCR amplification of 16S rRNA gene fragment was used for specific identification of *C. pseudotuberculosis* isolates (Centinkaya et al. 2002), which could not distinguish the bacterium *Corynebacterium ulcerans* and biovar *equi* of *C. pseudotuberculosis*. Other genes such as rpoB, pld and 12S rRNA have been employed in PCR for identification of *C. pseudotuberculosis* (Pacheco et al. 2007; Pavan et al. 2011). *C. pseudotuberculosis* and *C. ulcerans* have been reported to possess a high degree of homology at genomic and biochemical levels (Khamis et al. 2004). Specificity of primers used in the present study ruled out the possibility of amplification of gene fragments of closely related species of bacteria *C. ulcerans* and *Corynebacterium diphtheria* (D'Afonseca et al. 2010). Thus a PCR assay employing specific

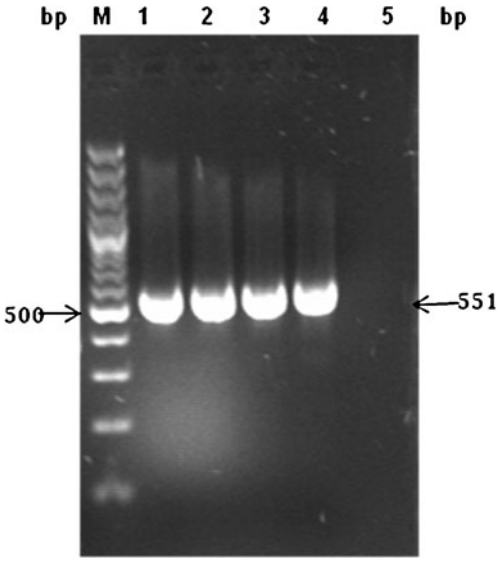

Fig. 1 PCR amplification of 551-bp fragment encoding PIP gene of *C. pseudotuberculosis*. *Lane M* molecular size marker (100 bp ladder), *lanes 1–4* 551 bp product of PIP gene of *C. pseudotuberculosis* isolates, *lane 5* negative control. Samples in lanes 1–4 are positive

primers of PIP gene of *C. pseudotuberculosis* offers several advantages. Firstly, it can be used for rapid and direct identification of cultured isolates, and secondly, it obviates the need of resorting to very cumbersome and often non-specific biochemical and less reliable serological tests for *Corynebacterium* spp. differentiation and diagnosis.

It is concluded that this is the first time that a study of *C. pseudotuberculosis* infection in Sirohi goats of India is performed. The prevalence rate of CL in Sirohi goats were 4.7%, 2.4% and 2.4% based on clinical examination, bacterial culture and PCR, respectively. All bacterial isolates were confirmed by PIP gene-specific PCR. This PCR assay offers advantage over bacteriological and biochemical characterization in terms of accuracy, sensitivity and rapidity for detection of *C. pseudotuberculosis* isolates. The findings of this study can be applied in other goat-producing regions for diagnosis and control of CL.

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