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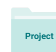

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REGULAR ARTICLES

Rapid detection of *Corynebacterium pseudotuberculosis* in clinical samples from sheep

Jyoti Kumar · Bhupendra Nath Tripathi · Rajiv Kumar · Ganesh Gangaram Sonawane · Shivendra Kumar Dixit

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Abstract *Corynebacterium pseudotuberculosis*, a Gram-positive bacterium is the causative agent of caseous lymphadenitis (CLA), a chronic disease of sheep, goats and other warm blooded animals. In the present study, a total of 1,080 sheep reared under semi-intensive system on organized farms situated in the semi arid tropical region of Rajasthan, India, was clinically examined. Pus samples from superficial lymph nodes of 25 (2.31 %) adult sheep showing clinical lesions similar to CLA were collected for laboratory analyses. On the basis of morphological, cultural and biochemical characteristics 12 (48 %) bacterial isolates from pus identified it as *C. pseudotuberculosis*. A polymerase chain reaction (PCR) assay targeting Putative oligopeptide/-dipeptide ABC transporter, nicotinamide adenine dinucleotide phosphate (NADP) oxidoreductase coenzyme F420-dependent and proline iminopeptidase (PIP) genes of *C. pseudotuberculosis* was developed that showed 14 pus samples as positive. All *C. pseudotuberculosis* isolates were also found positive for these genes in the PCR. The specificity of the PCR products was confirmed by sequencing of the amplified products that showed 98–100 % homology with published sequences available in the NCBI database. The present study shows the incidence of CLA as 2.31 %, 1.1 % and 1.29 % based on clinical, bacterial culture and direct pus PCR assay, respectively. The PCR assay was rapid, specific and as significant as bacterial culture in detecting bacteria directly in the clinical pus samples. The PCR assay developed in the study can be applied for the diagnosis and control of CLA. Furthermore, the assay can also be applied to detect *C. pseudotuberculosis* in various clinical samples.

Keywords *Corynebacterium pseudotuberculosis* · Caseous lymphadenitis · Polymerase chain reaction · Lymph nodes · Sheep · Pus

J. Kumar · B. N. Tripathi (✉) · G. G. Sonawane · S. K. Dixit
Division of Animal Health, Central Sheep and Wool Research Institute (CSWRI), Avikanagar,
Malpura, Rajasthan 304501, India
e-mail: bntripathi1@yahoo.co.in

R. Kumar
Animal Biotechnology Section, CSWRI, Avikanagar,
Malpura, Rajasthan 304501, India

Introduction

Corynebacterium pseudotuberculosis is a Gram-positive, mycolic acid containing, facultative intracellular actinomycete that causes caseous lymphadenitis (CLA), a chronic bacterial disease of sheep, goats and other warm blooded animals (Dorella et al. 2006). The disease is clinically manifested by the characteristic suppurative abscesses in the superficial and internal lymph nodes and other internal organs (Williamson 2001). CLA is of significant economic concern for sheep husbandry due to reduced wool, meat and milk yield, loss of fertility, culling of affected animals, condemnation and downgrading of affected carcass at slaughter and meat inspection (Williamson 2001; Guimaraes et al. 2011). Being a non-notifiable disease in many countries including India, its actual prevalence, extent of infection and associated losses have been largely underestimated. There are only few reports of its occurrences among sheep and goats in India (Mohan et al. 2008). The most feasible and reliable control strategy for this disease would include reduction of the intensity of infection in the flock by accurate identification and removal of the infected animals. The laboratory diagnosis of the CLA is generally achieved by the bacterial culture and its confirmation by biochemical, serological and nucleic acid based detection methods (Baird and Fontaine 2007; Guimaraes et al. 2011). Serological tests suffer from cross-reactivity due to infections with other bacteria such as

Corynebacterium spp., *Listeria monocytogenes* and *Mycobacterium avium* subspecies *paratuberculosis* (Dercksen et al. 2000; Manning et al. 2007).

Unfolding knowledge of a large number of bacterial toxins, virulence factors and organism's genome has led to an enhanced understanding of the mechanisms of bacterial pathogenicity. Since virulence factors are directly involved in the mechanisms of bacterial pathogenicity, their genes represent ideal targets for the accurate detection and identification of different medically relevant pathogenic bacterial species by molecular methods. Pathogenicity islands (PAIs) in bacterial genome encode virulence genes in large numbers are present in a broad range of bacterial pathogens and are central to their pathogenicity and lifestyle. The *C. pseudotuberculosis* genome includes seven putative PAIs, which contain several classical virulence factors, including genes for PIP and ABC transport system (Ruiz et al. 2011). Coenzyme F420-dependent enzymes have been implicated in pivotal redox reactions playing a key role in defence against oxidative and nitrosative stress in pathogenic mycobacterium and other actinomycete (Bashiri et al. 2010).

Polymerase chain reaction (PCR) is a robust technique and has been used frequently for direct detection of microbes from a variety of clinical and postmortem samples. However, the efficacy of the PCR for direct detection of *C. pseudotuberculosis* in clinical pus samples has been less commonly evaluated (Pacheco et al. 2007). Our PCR assay targeted putative oligopeptide/dipeptide ABC transporter, NADP (nicotinamide adenine dinucleotide phosphate) oxidoreductase coenzyme F420-dependent and proline iminopeptidase (PIP) genes of *C. pseudotuberculosis.* With the increase in PCR-based methods for the detection of pathogens the inclusions of internal amplification control (IAC) have been proposed for any PCR intended for diagnostic use (Hoorfar et al. 2004). In order to avoid the probable shortcoming of false negative, sheep-specific mitochondrial 12S rRNA gene was incorporated as a non-competitive IAC in the PCR assay. In the present study, we developed a PCR assay amplifying three genes for rapid and specific detection of *C. pseudotuberculosis* in clinical samples of suspected cases of CLA and its comparison with the bacterial culture.

Materials and methods

Animals and clinical examination

The animals of organized farms maintained under semi intensive system in semi-arid tropical region of Rajasthan at a longitude of 75°28′E, latitude of 26°26′N and an altitude of 320 m above mean sea level were included in the study. The highest mean monthly temperature during April to June is about 42 °C. Rainfall is erratic and ranged from 200 to 500 mm/year. Pure and crossbred genotypes of sheep have been maintained at the farms. A total of 1080 sheep belonging to different breeds (Malpura and its crosses) at farms was clinically examined for the presence of abscessation of superficial lymph nodes. The consistency of enlarged lymph nodes at various locations were either hard or soft and did not evinced pain on palpation. The location of the affected lymph nodes was also recorded.

Sample collection

A small area on the enlarged lymph nodes of affected sheep was shaved and cleaned with antiseptic to avoid environmental contamination during sampling. Creamy to caseated, odourless pus samples were aspirated aseptically from the abscessed lymph nodes suspected for CLA from 25 animals using sterile disposable syringe and needle (18 gauges). The samples kept on ice were brought to the laboratory for processing.

Bacterial isolation

Pus samples were directly inoculated on blood agar base (Himedia, Mumbai, India) supplemented with 5 % defibrinated sheep blood. Bacterial isolates with whitish, opaque, hemolytic and convex colonies obtained after 24–48 h of aerobic incubation at 37 °C were selected for further identification (Quinn et al. 2011). Pure cultures were obtained from these isolates after studying morphological characteristic of bacterial colonies and bacteria in Gram-stained smears. The 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 48 h. The resultant black colonies were subjected to routine biochemical tests, i.e., nitrate reduction, catalase and urease production and synergistic hemolysis with *Rhodococcus equi* (strain VTCC BAA67, courtesy Veterinary Type Culture Centre, Hisar, India) and antagonistic hemolysis with *Staphylococcus aureus* for phospholipase D (PLD) production (Guimaraes et al. 2011). Catalase, PLD and urease-positive and nitrate-negative cultures were considered positive for *C. pseudotuberculosis*.

DNA Isolation

DNA extraction from pus samples

Bacterial DNA was isolated from all pus samples that had already been subjected to cultural isolation. For isolation of bacterial DNA, approximately 100 mg pus was transferred into 1.5-ml Eppendorf tubes, and DNA was extracted using QIAGEN DNeasy Blood and Tissue Kit (Qiagen, Germantown, MD, USA) as per the manufacturer's

instructions. The DNA purity was checked on 0.8 % agarose gel electrophoresis and stored at −20°C until used.

DNA extraction from bacterial isolates

A few colonies from the characterized pure cultures of *C. pseudotuberculosis* from 24 to 48 h growth on blood agar plates were transferred into 1.5-ml Eppendorf tubes. The bacterial genomic DNA was extracted using QIAGEN DNeasy Blood and Tissue Kit as per the manufacturer's instructions (Qiagen). The DNA purity was checked on 0.8 % agarose gel electrophoresis and stored at −20°C until used.

Oligonucleotide primers and PCR

The oligonucleotide primers from three most conserved genes of *C. pseudotuberculosis* namely, Putative oligopeptide/dipeptide ABC transporter, NADP oxidoreductase coenzyme F420-dependent and proline iminopeptidase (PIP) genes were synthesized commercially from Sigma (UK) and used for PCR amplification (D'Afonseca et al. 2010). The oligonucleotide primer sequences for amplifying these genes along with their product sizes are listed in Table 1. A primer pair targeting sheep-specific mitochondrial 12S rRNA (Table 1), a housekeeping gene, was designed to act as an IAC for PCRs done with DNA extracted directly from clinical pus samples of suspected CLA. PCR carried out in a final volume of 25 μl of reaction mixture containing 1xPCR buffer, 2 mM $MgCl_2$, 200 μM dNTPs mix, 0.2 μM of each of forward and reverse primers, 1.25 units of Taq DNA polymerase (Sigma) and 5 μl of DNA template (DNA from pus or bacterial isolates) in a thermocycler (MJ Research PTC200). PCR conditions were optimized by putting gradient PCRs with annealing temperature ranging from 45°C to 65°C. Annealing temperature between 53.4°C and 59.6°C produced PCR products of expected length, of which 54°C was found to be optimum and therefore selected for further reaction. The PCR conditions used to amplify all four gene fragments included 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 and a final cycle at 72°C for 5 min. Negative control consisting of all component of reaction mixture except the DNA template was included in the PCR. The PCR products were analyzed by visualization of desired size of DNA bands in the ethidium bromide stained agarose gel (2.5 % w/v, 0.5× Tris borate EDTA buffer) under gel documentation system (Sambrook and Russell 2001).

Sequencing of PCR products

For the sequencing of the gene fragments standard PCRs were run using Pfu polymerase (Fermentas) and PCR products were resolved on 2.0 % agarose gel. The specific sized bands were excised from gel under UV light and were subsequently purified using minielute gel extraction kit (Qiagen). The purified products were sequenced by Imperial Life Science Laboratories (iLife Discoveries Pvt. Ltd.) and compared with sequence available in the NCBI database.

Results

On thorough clinical examination, 25 (2.31 %) out of 1,080 adult sheep were found to have affection of superficial lymph nodes at various stages of enlargement and abscessation (Table 2).

The cases were suspected for CLA based upon the characteristic overt presence of a palpable firm or slightly soft subcutaneous enlargement in the location of superficial lymph nodes. The affected lymph nodes were parotid, superficial cervical, mandibular, prescapular and prefemoral (Fig. 1 and Table 3). The size of the affected lymph nodes varied from 2 to 7 cm in diameter and were painless on palpation but with varying degree of hair/wool loss over or around it. All the cases yielded creamish-yellow pus samples of variable consistency. Of these 25 animals, 12 (48 %) were found to be positive for *C. pseudotuberculosis* based on cultural examination, giving an overall proportion of 1.1 % of animals examined clinically. All isolates of *C. pseudotuberculosis* were Gram-positive pleomorphic rod, catalase positive, urease positive, PLD positive and nitrate negative (Quinn et al. 2011). As all the *C. pseudotuberculosis* were negative for nitrate reduction test, they belonged to biovar *Ovis* (Guimaraes et al. 2011).

Table 1 The oligonucleotide primers used in this study

Target gene	Primers	Sequence (5′→3′)	Length of PCR products (bp)	Source/reference
Putative oligopeptide/dipeptide ABC transporter	Forward Reverse	ccttaccgagacaacgtcat gcctggtgcttatcattgat	285	D'Afonseca et al. (2010))
NADP oxido-reductase coenzyme F420-dependent	Forward Reverse	ctgcgacatagctaggcact ccgccagacttttctctaca	382	D'Afonseca et al. (2010)
Proline iminopeptidase	Forward Reverse	aactgcggctttctttattc gacaagtgggaacggtatct	551	D'Afonseca et al. (2010)
12S rRNA	Forward Reverse	taacccttgtmccttttgsatrrk agactaacttttaaagatacagtggg	270	This work

Table 2 Comparative result of clinical examination, bacterial culture and PCR (from pus and bacterial isolate)

	Clinical examination	Bacterial culture	PCR from pus	PCR from isolate
Number tested	1080	25	25	12
Number positive	25	12	14	12
Percentage	2.31	48	56	100
Overall percentage	2.31	1.1	1.29	1.1

Some of the other bacterial isolates from pus samples were identified as *Staphylococci* spp., *S. aureus* and *Erysipelothrix rushiopathiae* (Quinn et al. 2011). PCR was used for the detection of *C. pseudotuberculosis* directly from the clinical pus samples. Of 25 pus samples from CLA suspected sheep tested for the presence of Putative oligopeptide/dipeptide ABC transporter, NADP oxidoreductase coenzyme F420-dependent and PIP genes of *C. pseudotuberculosis* by PCR, 14 (56 %) were found to be positive (Fig. 2) giving an overall proportion of 1.29 % of animals examined clinically. Amplification of 12S rRNA target as IAC was obtained with each PCR reaction performed from DNA extracted directly from clinical pus samples (Fig. 2). All isolates were also positive for PCR (Fig. 3).

All three nucleotide sequences were deposited in the NCBI GenBank (accession no. JF901917, JF901919 and JF901921). Sequencing of the amplified products revealed identity of 99–100 %, 99–100 % and 100 % with published sequence of PIP, NADP oxidoreductase coenzyme F420-dependent and ABC transporter gene of *C. pseudotuberculosis* available in the NCBI database, respectively.

Discussion

The present study shows the incidence of CLA as 2.31 %, 1.1 % and 1.29 % based on the clinical examination, bacterial culture and pus PCR assay, respectively. In Turkey, Cetinkaya et al. (2002) reported an overall incidence of CLA to be 3.5 % and 3.4 % based on cultural isolation and PCR. The incidence of CLA in sheep was reported to be 1.6 % in Egypt (Mubarak et al. 1999; Al-Gaabary and El-Sheikh 2002), which was comparable to our results. However, in other studies from Australia (Paton et al. 2003) and Egypt (Al-Gaabary et al. 2009), higher prevalence of CLA has been reported on the basis of clinical and cultural examination.

Fig. 1 CLA infected sheep showing affected parotid lymph node

The characteristic of the organism and the insidious nature of infection in the animal make the diagnosis and control of CLA a difficult proposition. Clinical diagnosis of CLA was not always confirmed by the laboratory diagnostic methods such as ELISA and the bacterial culture (Ter Laak et al. 1992; Al-Gaabary et al. 2010). This was well evident from a number of previous and the present study. Bacterial culture requiring isolation and characterization by biochemical tests, synergistic hemolysis test with *R. equi* and antagonistic hemolysis test with *S. aureus* is a cumbersome and time consuming and less sensitive. Furthermore, due to challenges in making a consistently reliable interpretation of culture plates and biochemical characteristics, considerable performance variation is possible between diagnostic laboratories, even if they follow standardized procedures.

Therefore, in the present study, we used PCR assay based on three specific genes to confirm *C. pseudotuberculosis* isolates and to detect it directly in the pus samples. The PCR carried out on pus samples detected 14 cases (1.29 %) positive in comparison to the bacterial culture (12 cases, 1.1 %), thus showing a definite edge over the bacterial culture.

The PCR assay was in complete agreement with the bacterial culture in identifying all the 12 isolates as *C. pseudotuberculosis* as well as their direct detection in the culture positive pus samples. In previous studies, genes such as *rpoB*, *pld*, and 16S rRNA have been employed in the PCR for identification of *C. pseudotuberculosis* showing similar results (Cetinkaya et al. 2002; Pacheco et al. 2007; Pavan et al. 2012). The specific identification of *C. pseudotuberculosis* isolates based on PCR amplification of 16S rRNA gene fragment could not distinguish it from the bacterium *Corynebacterium ulcerans* and bio-var *equi* of *C. pseudotuberculosis* (Centinkaya et al. 2002) because of high degree of homology at genomic and biochemical levels (Khamis et al. 2004). In a similar study, Pacheco et al. (2007) reported a PCR sensitivity of 94.6 % in detecting bacteria from pus samples targeting 16S rRNA, rpoB and pld genes. The pus PCR analysis detected *C. pseudotuberculosis* in two clinical pus samples, but negative by culture. PCR methods are normally designed to identify genomic DNA, instead of viable cells, have been found to surpass the diagnostic sensitivity of bacterial culture by a varying degree from various clinical samples (Koskinen et al. 2010).

Table 3 Distribution of clinical lesion and diagnostic results

Sr. no	Abscessed lymph node	*C. pseudotuberculosis*	PCR	
		Isolation	Pus	Culture
1	Parotid	+	+	+
2	Parotid	−	−	−
3	Parotid and mandibular	+	+	+
4	Parotid and mandibular	−	+	N.A.
5	Parotid and mandibular	+	+	+
6.	Parotid	+	+	+
7	Parotid	+	+	+
8	Parotid	−	−	−
9	Parotid	−	−	−
10	Superficial cervical and parotid	+	+	+
11	Superficial cervical	+	+	+
12	Superficial cervical	−	−	−
13	Superficial cervical	−	−	−
14	Mandibular	+	+	+
15	Mandibular and prefemoral	+	+	+
16	Mandibular	−	−	−
17	Mandibular	+	+	+
18	Mandibular	−	−	−
19	Prescapular	+	+	+
20	Prescapular	−	+	N.A.
21	Prescapular	−	−	−
22	Prescapular	−	−	−
23	Prefemoral	+	+	+
24	Prefemoral	−	−	−
25	Supramammary	−	−	−

N.A. Not applicable

PCR is the most sensitive of currently available rapid methods to detect microbial pathogens in clinical specimens. It is known that the sensitivity and specificity of a

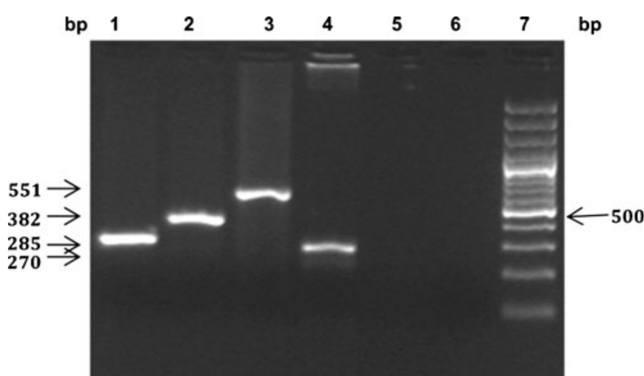

Fig. 2 PCR amplification profile of *C. pseudotuberculosis* from DNA isolated directly from pus. *Lane 1* 285-bp product of putative oligopeptide/dipeptide ABC transporter; *lane 2* 382-bp product of NADP oxidoreductase coenzyme F420-dependent; *lane 3* 551-bp product of PIP; *lane 4* sheep-specific 12S rRNA gene as internal amplification control; *lane 5 R. equi* negative for PIP gene; *lane 6* negative control; *lane 7* molecular size marker 100 bp plus (sm#0323, Fermentas)

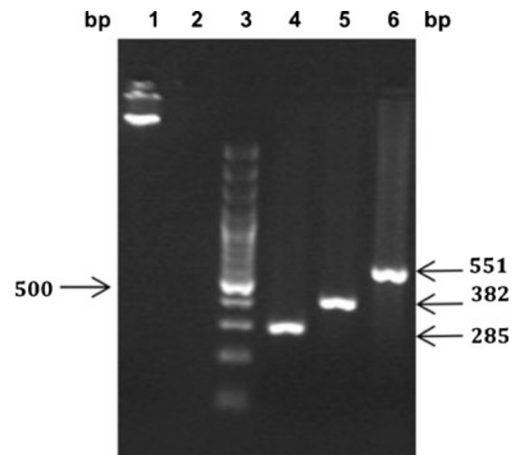

Fig. 3 PCR amplification profile of *C. pseudotuberculosis* isolates. *Lane 1 R. equi* negative for PIP gene; *lane 2* negative control; *lane 3* molecular size marker 100 bp plus (sm#0323, Fermentas); *lane 4* 285 bp product of putative oligopeptide/dipeptide ABC transporter; *lane 5* 385-bp product of NADP oxidoreductase coenzyme F420-dependent; *lane 5* 551-bp product of PIP gene

PCR assay is dependent on target genes, primer sequences, PCR techniques, DNA extraction procedures, and PCR product detection methods. PCR detection of different bacteria in clinical specimens such as pus has not yet been reviewed sufficiently. Since a variety of clinical specimens, such as pus, blood, urine, sputum, and CSF, vary in regard to the nature and character of the content, the design of the PCR assay for each specific specimen is likely to affect the sensitivity. Virulence genes, which are the central characteristic distinguishing the pathogenic from the non-pathogenic, are abundantly present in PAIs. PIP and Putative oligopeptide/dipeptide ABC transporter system are virulence factors encoded in recently reported PAIs in pathogenic C. *pseudotuberculosis* genome (Ruiz et al. 2011). Virulence association and distinct abundance of copies of PIP and Putative oligopeptide/dipeptide ABC transporter gene in *C. pseudotuberculosis* make them suitable molecular diagnostic target. Coenzyme F420-dependent enzymes including NADP oxidoreductase coenzyme F420-dependent has an important role in the metabolism of Archaea and Actinomycetes. Coenzyme F420-dependent enzymes have been implicated in several redox reactions playing a key role in defence against oxidative and nitrosative stress in pathogenic mycobacterium and other actinomycete (Bashiri et al. 2010). Inclusion of a non-competitive 12S rRNA target as IAC in the PCR assay allayed the critical shortcoming of false negative results due to the presence of inhibitory substances within the sample (Hoorfar et al. 2004).

In the present study, one-step PCR assay confirms that *C. pseudotuberculosis* and method involving the PCR employing three genes distinguishes *C. pseudotuberculosis* from other species of the genus *Corynebacterium* of veterinary importance and from genetically related pathogens such as *R. equi*. The assay provides a rapid and simple diagnostic tool for the

identification of *C. pseudotuberculosis* from sheep and goats and for the discrimination between this species and other related pathogenic bacteria. The assay also circumvents the problem of clinical diagnosis that was mostly dependent on less reliable serological tests and highly variable cultural and biochemical characteristic of *Corynebacterium* spp. used for differentiation (Hommez et al. 1999). The higher detection rate of *C. pseudotuberculosis* genome by PCR in clinical sample may be attributed to the detection of the dead or less number of organisms in the pus, which could not be detected by bacterial culture.

The specificity of the PCR assay can be confirmed by restriction enzyme (RE) analysis Southern blot hybridization and sequencing of the amplified products. In the present study, sequencing of the amplified products of all three genes showed 98–100 % homology with the published sequences in the NCBI database (CP003407.1, CP003385.1, CP003152.1, CP003540.1, CP003385.1, CP003152.1, CP003062.1, CP003061.1, CP003407.1, CP003385.1, CP003152.1, CP002251.1, etc.), thus confirming the specificity of the PCR assay of our study. Also the target sequence amplified with Pfu polymerase assured exclusion of error in the sequence obtained from amplicon (Vijayan and Tsou 2008). The specificity of the primers used in the present study ruled out the possibility of cross-reaction with closely related species (*C. ulcerans* and *C. diphtheriae*) (D'Afonseca et al. 2010).

It is evident from the results that all 25 clinically suspected cases did not turn out to be CLA cases, which suggests either less sensitivity of the bacterial culture and PCR or involvement of other bacteria. Meanwhile, it has been reported that recovery of live organisms in the bacterial culture depends on the stage and nature of infection, the immune status of the animal, antimicrobial therapy and distribution of organism within lesions as well as the presence of other bacterial agents (Al-Gaabary et al. 2009; Fontaine and Baird 2008). In our recent investigation, a few suspected cases of CLA in goat yielded *S. aureus* subsp. *anaerobius*, and in sheep yielded *Erysipelothrix rushiopathiae* (unpublished information). Suppuration of lymph nodes or subcutaneous tissues by other bacteria like *Staphylococcus* spp., *S. aureus* subsp. *anaerobius*, *Erysipelothrix* spp., and *Streptococci*, may complicate the diagnosis of the abscess conditions (Al-Gaabary et al. 2010; Seyffert et al. 2010). Thus, it is most likely that clinical diagnosis by morphological characteristics may lead to an overestimation of incidence of CLA. It is suggested that suspected CLA cases must be confirmed by bacterial culture or molecular test for correct diagnosis. This observation is supported by a number of previous studies, wherein estimates of CLA incidence on clinical basis have always been greater than bacteriological and molecular bases (Cetinkaya et al. 2002; Al-Harbi 2011). The PCR assay developed in the study can be applied for the diagnosis and control of CLA in other countries where it is a problem.

The findings of the present study suggested that all clinically cases indistinguishable from CLA could be confirmed by the bacterial culture and PCR. The PCR assay was rapid, specific and as significant as bacterial culture in detecting bacteria directly in the clinical pus samples. The PCR assay offers advantages over bacteriological culture in terms of reduced time and its ability to also detect non-viable bacteria in pus sample. Inclusion of three specific genes as target confers specificity and sensitivity to PCR for detection of *C. pseudotuberculosis* directly from the clinical samples as well for the confirmation of *C. pseudotuberculosis* isolates. The assay can be applied further to detect *C. pseudotuberculosis* from other form of clinical sample from other animal disease conditions and even from human's cases.

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