

Original Research Article

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Detection of *Corynebacterium pseudotuberculosis* by SYBR Green Real Time PCR

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ABSTRACT

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Corynebacterium pseudotuberculosis, a Gram positive bacterium is the causative agent of caseous lymphadenitis (CLA), a chronic debilitating disease of sheep, goats and other warm blooded animals. Definite diagnosis of CLA primarily relies on microbiological examination, followed by biochemical identification of isolates. In an effort to facilitate *C. pseudotuberculosis* detection, the present study was undertaken with the aim of evaluating a SYBR Green real time PCR assay targeting ABC transporter gene for the detection of *C. pseudotuberculosis* in DNA isolated from culture and pus samples from sheep and goats. The analytical sensitivity and specificity of the PCR primers were evaluated. The test demonstrated tenfold more sensitivity than conventional PCR and detected down to 100 femtogram of genomic DNA of pure *C. pseudotuberculosis*. The real-time PCR was found to be specific for ABC transporter gene of *C. pseudotuberculosis*, as no cross reactivity was detected with a variety of known bacterial isolates. The real time PCR was employed for direct detection of *C. pseudotuberculosis* in pus samples collected from clinically suspected cases of caseous lymphadenitis. It is concluded that, this real time assay may be used as a valuable diagnostic tool for the rapid and specific detection of *C. pseudotuberculosis* in clinical samples, large scale epidemiological studies and clarification of pathogenesis.

Introduction

Corynebacterium pseudotuberculosis is a Gram-positive bacterium that causes caseous lymphadenitis (CLA), a chronic bacterial disease of sheep, goats and other warm blooded animals (Dorella *et al.*, 2006). The disease during its course causes characteristic

suppurative abscessation in the superficial and internal lymph nodes and other internal organs (Williamson 2001). CLA caused by *C. pseudotuberculosis* is a highly contagious disease, and is of significant economic concern for sheep and goat husbandry due to reduced wool and meat quality (Dorella *et al.*, 2006; Guimaraes *et al.*, 2011).

In India, the detailed epidemiological studies of this disease are lacking, only few published reports of its occurrences among sheep and goats in India is available. The reliable control strategy for this disease would be rapid and early identification and removal of the infected animals from the herd or flock. The laboratory detection of *C. pseudotuberculosis* is done by microbiology, biochemical, serological and nucleic acid based detection methods (Baird and Fontaine 2007; Guimaraes *et al.*, 2011; Quinn *et al.*, 2011)). Serological tests are not so conclusive and suffer from cross reactivity with other bacteria (Manning *et al.*, 2007).

Polymerase chain reaction (PCR) based methods have been used frequently for direct detection of microbes in culture, clinical and postmortem samples. PCR based assay has many advantages compared to traditional diagnostic methods. A well designed PCR is highly sensitive, rapid and specific. Furthermore, real time PCR has additional advantages with respect to rapidity and sensitivity. The present study was to evaluate a real-time assay based on SYBR green for detection of *C. pseudotuberculosis* in bacterial cultures which can also be applied for direct detection in clinical or postmortem samples of CLA suspected animals.

Materials and Methods

Samples and bacterial DNA

A total of twenty archived pus samples collected from clinically suspected cases of caseous lymphadenitis in sheep and goats were included for evaluation of the real time PCR assay. A homogenous suspension of pus samples was made in 1 ml of autoclaved distilled water and transferred in 1.5 ml sterile plastic tubes for isolation of DNA. The DNA extracted from a variety of known bacterial isolates from sheep and available in our laboratory was included to evaluate the

specificity of the PCR primer. It included DNA samples from *Mannheimia haemolytica*, *Pasteurella multocida*, *Bibersteinia trehalosi*, *Escherichia coli*, *Staphylococcus aureus*, *C. pseudotuberculosis*, and *Rhodococcus equi* (strain VTCC BAA67, courtesy ICAR-National Centre for Veterinary Type Culture Centres, Hisar, India).

DNA extraction

The Genomic DNA was extracted from pure culture of *C. pseudotuberculosis* and pus samples using Qiagen DNeasy Blood and Tissue kit, according to manufacturer's instruction. The DNA content was quantified by use of a nanodrop (Quawell Technology Inc., USA). The quantified DNA samples were stored at - 20 °C till further use in the molecular assay.

Primers and PCR

Oligonucleotide primers targeting Putative oligopeptide/ dipeptide ABC transporter gene specific to *C. pseudotuberculosis* were synthesized from Sigma. Sequences of the primers used in this study were forward 5'- CCTTACCGAGACAACGTCAT -3' and reverse 5'- GCCTGGTGCTTATCATTGAT -3' (D'Afonseca *et al.*, 2010). The 10 µl conventional PCR reaction mixture consisted of 200µM of dNTP mix, 0.2 µM of each primer, 1.5mM of MgCl₂ and 0.5 U of Taq DNA Polymerase (Sigma-Aldrich, USA) in 1X PCR buffer. The optimized thermal cycling conditions were as follows; initial denaturation step at 95 °C for 5 min followed by 40 cycles of denaturation at 95 °C for 30 s, 54 °C for 45 s, and 72 °C for 45 s, and a final extension at 72 °C for 10 min. Negative control was also included during the PCR reaction set up. The amplicon of 285 bp was visualized after electrophoresis through 2% agarose gel containing ethidium bromide (Sambrook and Russell, 2001).

For sequencing of the representative amplicon standard PCRs were run and PCR products (60 µl) were resolved on 2% agarose gel. The specific sized bands were excised from agarose gel under UV light and were subsequently purified using MinElute Gel Extraction Kit (Qiagen). The purified products were sequenced (Xcelris Genomics, India) and compared with the published sequences available in the NCBI online database.

Real time PCR

The SYBR green real-time PCR assay was performed over DNA samples from cultures and pus samples with the same primer set as used for conventional PCR using StepOnePlus Real-Time PCR System (Applied Biosystems, USA). Each 10 µl PCR reaction mix had 0.10 µl each of forward and reverse primers (100 nm final concentration), 1 µl template DNA, and 5 µl 2 X Maxima SYBR Green/ROX qPCR Master Mix (Thermo scientific, USA) and 3.8 µl nuclease-free PCR grade water. The SYBR green real time PCR conditions were as follows: initial denaturation at 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and annealing/extension at 60 °C for 60 s.

Sensitivity and specificity of the PCR and real time PCR

The analytical sensitivities of the primers used in real-time PCR and conventional PCR were carried out by 10 fold dilution of the DNA isolated from well characterized and pure *C. pseudotuberculosis* isolate. Specificity of the real-time PCR and conventional PCR assays were assessed by performing the reaction with *C. pseudotuberculosis*, as well as DNA from a variety of bacteria isolated from sheep and goats.

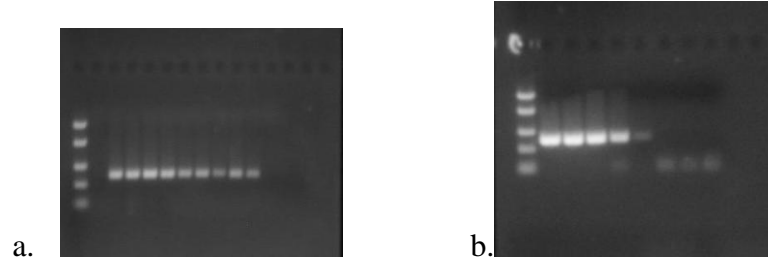
Results and Discussion

Infection caused by *C. pseudotuberculosis*, remains a cause of concern for sheep and goat farming system globally (Baird, 1997; Williamson, 2001; Paton *et al.*, 2003; Dorella *et al.*, 2006). Because of highly contagious nature of the agent, test and cull strategy has been the suggested method to control the spread of the disease (Baird, 1997; Williamson, 2001). As conventional methods are not always forthright, the improved method of rapid and specific detection may facilitate the control of CLA.

In the current study, we evaluated a sensitive and specific SYBR green real time assay for the detection of *C. pseudotuberculosis*. The performance of an assay was tested for its specificity and sensitivity. To obtain a good sensitivity and specificity of the assay selection of appropriate target gene and primers must be considered. In the current study, we chose the ABC transporter gene as a target for the detection of *C. pseudotuberculosis*. The ABC transporter gene is reported to be a conserved and associated with virulence and pathogenesis in *C. pseudotuberculosis* infection (Ruiz *et al.*, 2011). A 10-fold dilution serial dilution of DNA from 10 ng to 1 fg per reaction were prepared and used to estimate limit of detection also called as sensitivity of the PCR tests (Fig. 1).

Under the described reaction conditions (1 µl of DNA extract per 10 µl assay), the detection limit of the SYBR green real time PCR test using DNA from pure bacterial culture was 100 fg (Fig. 1a). While the detection limit of the conventional PCR was 1 pg per assay (Fig. 1b). Therefore, the real time PCR was ten times more sensitive than the conventional PCR.

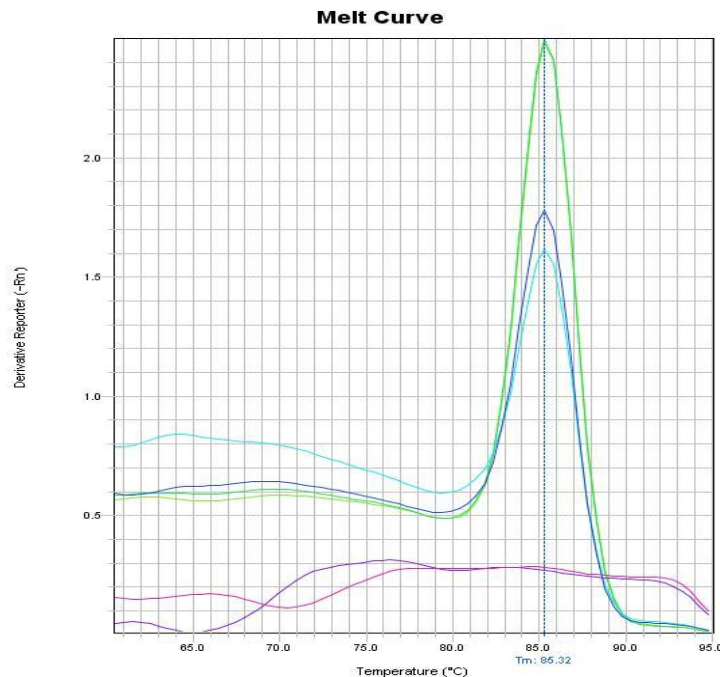
Fig.1 Analytical sensitivity of SYBR green real-time PCR and conventional PCR



(a). Agarose gel electrophoresis of SYBR green real-time PCR amplicon (285 bp) showing sensitivity upto 100 fg. Amount of *C. pseudotuberculosis* DNA in L1=FastRuler Low Range DNA ladder (#SM1103),= L2= negative control, L3=100 ng Sheep isolate, L4=50 ng goat isolate, L5=30 ng of stock DNA used for dilution, L6= 10 ng, L7= 1 ng, L8=100 pg, L9= 10 pg, L10= 1 pg, L11= 100 fg, L12=10 fg and L13= 1 fg.

(b). Agarose gel electrophoresis of conventional PCR product (285 bp) showing sensitivity upto 1 pg. Amount of *C. pseudotuberculosis* DNA in L1= FastRuler Low Range DNA ladder (#SM1103), L2= 10 ng, L3= 1 ng, L4=100 pg, L5= 10 pg, L6= 1 pg, L7= 100 fg, L8= 10 fg and L9= negative control

Fig.2 Representative Melt curve showing presence of single specific real time PCR product specific to *C. pseudotuberculosis*



The limits of detection of many other PCR tests used for *C. pseudotuberculosis* have not been determined. In another study by Pacheco *et al.*, 2006, the sensitivity of the multiplex PCR for genomic DNA from pure *C.*

pseudotuberculosis culture was 1 pg, similar as in our conventional PCR assay.

On evaluation of specificity using DNA from different bacterial species (*P. multocida*, *E.*

coli, *S. aureus*, *M. haemolytica*, *B.trehalosi*, *R. equi*) other than *C. pseudotuberculosis*, real-time amplification was seen only in DNA isolated from *C. pseudotuberculosis*. Similar result for specificity has been reported in a study targeting *rpoB* and *pld* gene of *C. pseudotuberculosis* (Pacheco *et al.*, 2006; Khamis *et al.*, 2004). False positive signals observed later in the amplification cycles were verified using melt curve analysis (Fig. 2). Alternatively, the agarose gel electrophoresis of the real time PCR amplification products was used to preclude non-specific amplification signals emanating from low molecular weight DNA like primer dimers. The specificity of the assay can be confirmed by nucleotide sequencing of the amplified products. Nucleotide sequence of the ABC transporter amplicon showed high degree of similarity (98-100 %) with the published sequence in the NCBI database. None of the other bacterial strains showed amplification products indicating the high degree of specificity of this assay (D'Afonseca *et al.*, 2010). Only positive control DNA produced right amplification with a melting peak at approximately 85.32°C which is consistent with that expected for targeted ABC transporter gene amplicon of *C. pseudotuberculosis* under current reaction conditions (Fig. 2). Same result for specificity testing was observed for the conventional PCR also.

A total of twenty pus samples were tested for the presence of *C. pseudotuberculosis*. Out of these, DNA from twelve pus samples was found positive for *C. pseudotuberculosis* by real time PCR. The conventional PCR also detected the same pus samples positive for *C. pseudotuberculosis* as detected positive by real time PCR. However, with this limited sample size and that too all from clinically obvious cases, the superiority of the real time PCR over conventional PCR could not be conclusively established or estimated in

percent terms. The improved detection with SYBR green dye chemistry based real time PCR is obvious as its sensitivity being on higher side and need further evaluation over a large number and variety of tissue and/or clinical samples.

Speedy and sensitive detection of *C. pseudotuberculosis* is of significant clinical importance. The specific and highly sensitive SYBR green PCR assay performed in the present work may be suitable as a rapid and economic diagnostic tool to identify the sheep carrying *C. pseudotuberculosis*. As the clinical sample size was relatively small, this promising assay needs to be tested on a large number of samples and over a variety of samples to further assess its suitability in the field to enable its adoption for the screening and early detection of diseased/carrier animals in a healthy herd and further to control the CLA.

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