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DETECTION OF *MANNHEIMIA HAEMOLYTICA* IN CULTURE AND LUNG TISSUE OF LAMBS BY REAL-TIME POLYMERASE CHAIN REACTION ASSAY

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ABSTRACT

Mannheimia haemolytica is one of the bacterial species involved in cases of ovine respiratory complex, causing significant economic loss to sheep production worldwide. In the year 2016-17, a study was undertaken with the aim to evaluate a SYBR Green dye based real-time polymerase chain reaction (PCR) assay targeting O-sialo glycoprotein endopeptidase (*gcp*) gene for the detection of *M. haemolytica* in the DNA isolated from culture and pneumonic lung tissue of neonatal lambs. The test showed thousand times more sensitivity than conventional PCR and detected down to 100 fg of genomic DNA of pure *M. haemolytica*. The real-time PCR was found specific for *gcp* gene of *M. haemolytica*, as no cross reactivity was detected with a variety of known bacterial isolates characterized previously by species specific or 16S rRNA PCR. The real-time PCR was employed to screen *M. haemolytica* in 41 ovine lung tissues collected from neonatal lambs, which showed increased level of detection as compared to the conventional PCR. The specificity of the PCR products was confirmed by sequencing of the amplified products of 227 bp size that showed 99-100% homology with the published sequences available in the NCBI database. It was concluded that the assay may be used as a valuable diagnostic tool for the rapid and specific detection of *M. haemolytica* in clinical samples.

Key words: Lamb, Lung, *Mannheimia haemolytica*, Pneumonia, Real-time polymerase chain reaction, Sensitivity

Mannheimia haemolytica is one of the bacterial species involved in ovine respiratory diseases and implicated in significant economic losses in sheep production worldwide (Gilmour and Gilmour, 1989). *M. haemolytica* causes serious outbreaks of acute pneumonia in neonatal, weaned and growing lambs. Outbreaks occur at any time of the year, and in any age group of animals, but mostly in lambs less than a year old (Mohamed and Abdelsalam, 2008; Deressa et al., 2010). In India, respiratory infections in sheep remain a major cause of concern. However, reports on *M. haemolytica* related of clinical ovine pneumonia are scanty despite its proven role in respiratory diseases of lambs (Mishra et al., 2000; Kumar et al., 2015; Gonzalez et al., 2013). Usually lungs with gross changes like congestion and consolidation have been recorded on necropsy of sheep died due to this microorganism. Failure to diagnose ovine pneumonia at an early stage in infected animals and lack of accurate information on causes of the disease hinders timely intervention (Goodwin et al., 2004).

Diagnosis of *M. haemolytica* infection is usually achieved on the basis of clinical findings, cultural isolation and extensive phenotyping and capsular serotyping of the causative pathogens, which is laborious, time-consuming and encounters

problems of validity and reproducibility of results (Angen et al., 1999). However, the introduction of molecular tools that are more sensitive and specific, help to identify the bacteria and their genetic elements more accurately and rapidly (McPherson and Moller, 2002; Hawari et al., 2008). Polymerase chain reaction (PCR) based tests have several advantages compared to traditional diagnostic methods. PCR is highly sensitive, rapid and specific (Ieven, 2007). Real-time PCR has further advantages with respect to rapidity, precision, sensitivity and accuracy (Kralik and Ricchi, 2017). A study was undertaken with the aim to evaluate a SYBR Green dye based real-time PCR assay targeting O-sialo glycoprotein endopeptidase (*gcp*) gene for the detection of *M. haemolytica* in DNA isolated from culture and pneumonic lung tissue of neonatal lambs.

MATERIALS AND METHODS

A total of 41 archived lung tissue samples collected from neonatal lambs that had died of respiratory affections during 2016-2017 were included for evaluation of the real-time PCR assay. A homogenous tissue suspension 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 that were previously characterized by species-specific or 16S rRNA PCR and amplicon sequencing were included to evaluate the specificity of the PCR primer. It included DNA samples from *M. haemolytica* (n=10), *Pasteurella multocida* (n=5), *Bibersteinia trehalosi* (n=5), *Escherichia coli* (n=5), *Staphylococcus aureus* (n=5), *Corynebacterium pseudotuberculosis* (n=5), *Mycobacterium avium* subspecies *paratuberculosis* (n=5), *Pseudomonas aeruginosa* (n=4), *Acinetobacter variabilis* (n=1) and *Proteus mirabilis* (n=1).

Genomic DNA was extracted from *M. haemolytica* pure culture and lung tissue samples using Qiagen DNeasy Blood and Tissue kit, according to manufacturer's protocol. The DNA content was quantified by use of a Quawell UV-Vis Q5000 spectrophotometer (Quawell Technology Inc., San Jose, CA, USA). The extracted DNA samples were stored at -20°C till further use in the real-time PCR and conventional PCR assays. Primers targeting *gcp* gene specific to *M. haemolytica* were synthesized from Sigma (Bengaluru, India). Primers used in the study were Forward 5′- TGGGCAATACGAACTACTCGGG -3′ and Reverse 5′-CTTTAATCGTATTCGCAG-3′ (Shanthalingam et al., 2014). The PCR reaction mixture (10 µl) consisted of 200 µM of dNTP mix, 0.2 µM of each primer, 1.5 mM of $MgCl_2$ and 0.5 unit of Taq DNA polymerase (Sigma-Aldrich, USA) in 1X PCR buffer. The optimized thermal cycling conditions were initial denaturation at 95°C for 5 min followed by 40 cycles of denaturation at 95°C for 30 s, 54°C for 30 s, and 72°C for 30 s, and a final extension at 72°C for 10 min. The amplicons of 227 bp was visualized after electrophoresis through 2% agarose gel run at 7.0 V/cm and stained with ethidium bromide. For sequencing of the amplicons, standard PCRs were run and PCR products (50 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, Ahmedabad, India), and compared with the sequences available in the National Center Biotechnology Information (NCBI) database.

Real-time PCR assay was performed on DNA samples from cultures and tissue samples with the same primer set as used for conventional PCR using StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, USA). The reaction parameters for primer and annealing temperature were optimized and optimized reactions were run. Each PCR reaction mix comprised of the total volume of 10 µl containing 0.10 µl each of forward and reverse primers (100 nm final concentration), 1µl template DNA, and 5 µl 2 X QuantiFast SYBR Green PCR Kit (Qiagen, CA,

USA) and 3.8 µl nuclease-free PCR grade water. The SYBR green real time PCR conditions were initial denaturation at 95°C for 5 min, followed by 40 cycles of 95°C for 10 s and combined annealing/extension at 60°C for 30 s. Real-time PCR and conventional PCRs were carried out under set reaction conditions using same primers and their analytical sensitivities were estimated. Specificity of the developed real-time PCR and conventional PCR assays were assessed by performing the reaction with *M. haemolytica*, as well as other available bacterial DNA.

RESULTS AND DISCUSSION

The prevalence of *M. haemolytica* organism is usually underestimated due to tedious process of isolation and identification by conventional microbiological methods. The identification of *M. haemolytica* with conventional methods becomes challenging in difficult situations (antibiotic treatment, frozen clinical samples, autolytic material, multi-bacterial samples etc.) (Angen et al., 2002; Ilhan and Keles, 2007). Moreover, serotyping does not represent a reliable method for identification of *M. haemolytica,* mainly because of non-serotypable nature of isolates and non-availability of typing sera in most diagnostic laboratories in India. In the current study, we evaluated a highly sensitive and specific real time assay for the detection of *M. haemolytica*. The performance of an assay needs to be tested by means of specificity and sensitivity. To obtain a good sensitivity, specificity and rapidity of an assay, selection of appropriate target gene and primers must be considered. We chose the *gcp* gene as a target for the detection of *M. haemolytica*. The *gcp* gene was reported to be a conserved and associated with virulence and pathogenesis in *M. haemolytica* infection (Abdullah et al., 1992). A ten-fold dilution, serial dilution of DNA from 10 ng to 1 fg per reaction was prepared, and used to estimate detection limit or sensitivity of the PCR tests (Fig. 1).

Under the described trial conditions (1µl of DNA extract per 10 µl assay), the detection limit of the SYBR green real time PCR test using DNA from pure

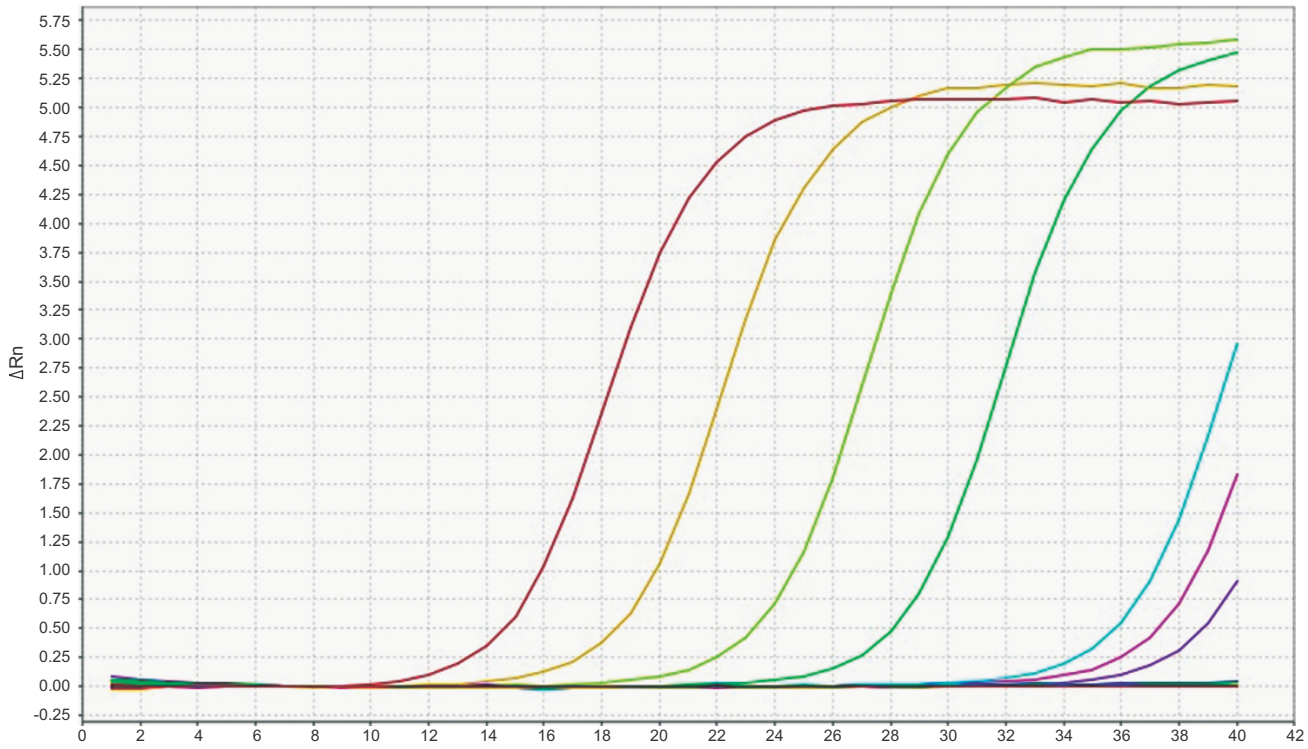
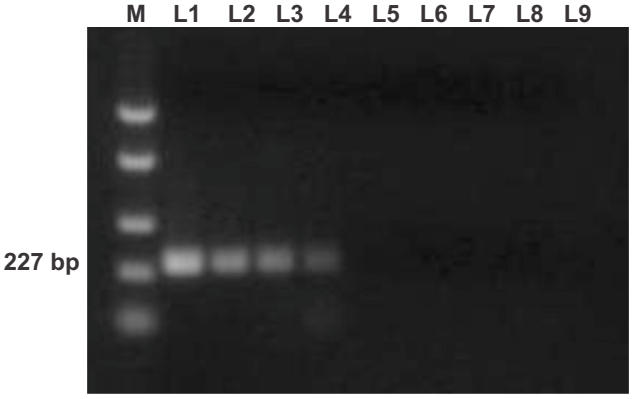
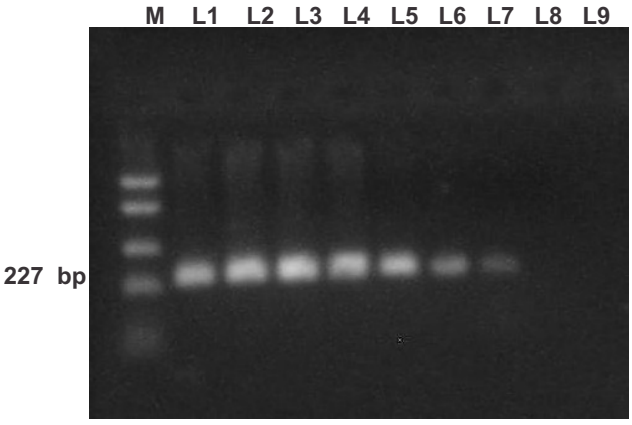


Fig. 1. Amplification plot for gcp gene of *M. haemolytica* over different dilutions of template DNA

bacterial culture was 100 fg (Fig. 2a). While the detection limit of the conventional PCR was 100 pg per assay (Fig. 2b). Therefore, real-time PCR was thousand times more sensitive than the conventional PCR. The limits of detection of other PCR tests used for *M. haemolytica* have not been determined (Dag et al., 2018; Singh et al., 2018; Sabiel et al., 2012). The *sodA* gene based real-time PCR showed similar results for sensitivity (Guenther et al., 2008).



a. Agarose gel electrophoresis of conventional PCR product showing sensitivity upto 100 pg (L1- positive control, Amount of *M. haemolytica* DNA in L2- 10 ng, L3- 1 ng, L4- 100 pg, L5- 10 pg, L6- 1 pg, L7- 100 fg, L8- 10 fg, L9- negative control, M- FastRuler low range DNA ladder)



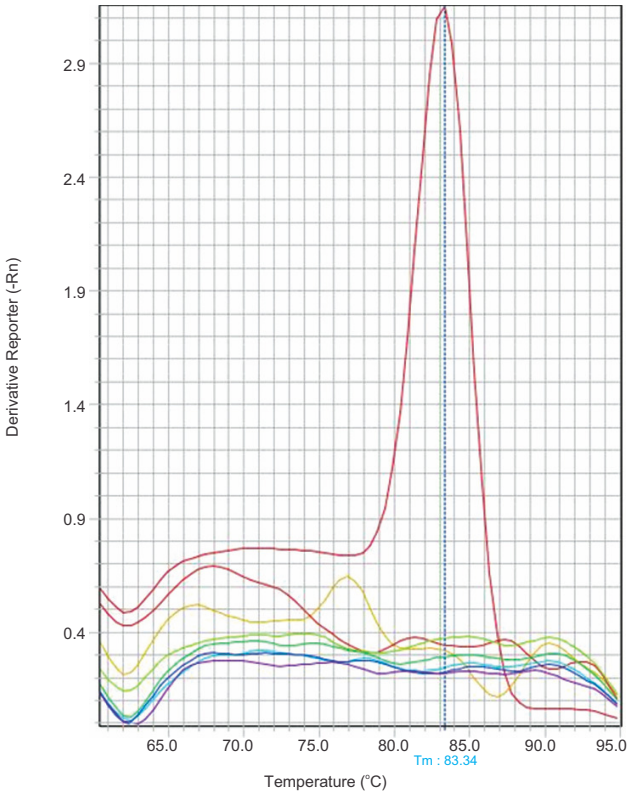
b. Agarose gel electrophoresis of SYBR green real-time PCR product showing sensitivity upto 100 fg (L1- positive control, Amount of *M. haemolytica* DNA in L2- 10 ng, L3- 1 ng, L4- 100 pg, L5- 10 pg, L6- 1 pg, L7- 100 fg, L8- 10 fg, L9- negative control, M- FastRuler low range DNA ladder)

Fig. 2. Analytical sensitivity of conventional PCR and SYBR green real-time PCR

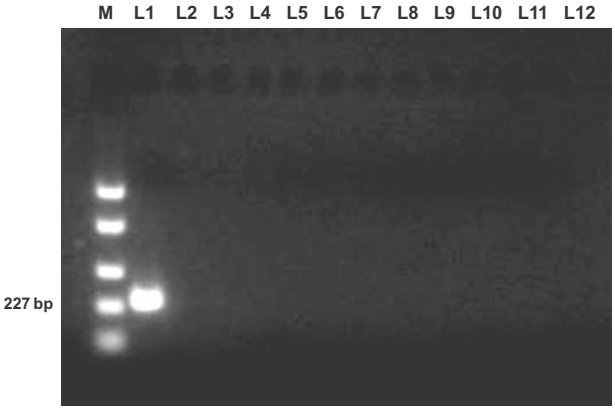
On evaluation of specificity using genomic DNA extracted from different bacterial strains *(P. multocida*, *B. trehalosi, E. coli, S. aureus, C. pseudotuberculosis, M. avium subspecies paratuberculosis, P. aeruginosa, A. variabilis, P. mirabilis)* other than *M. haemolytica,* real-time amplification was seen only in positive control (*M. haemolytica)*. False positive signals observed on 30 to 35 cycles and were verified using melt curve analysis. Alternatively, also the gel electrophoresis of the real-time PCR products was used to rule out non-specific fluorescence of low molecular weight DNA. Additionally, nucleotide sequence of the *gcp* amplicon showed high degree of similarity (99-100%) with the published sequence**s** in the NCBI database. None of the other bacterial strains showed amplification products indicating the high degree of specificity of this assay (Shanthalingam et al., 2014). Only positive control DNA produced amplification with a melting peak at approximately 83.34°C which is consistent with that expected for *gcp* gene amplicon of *M. haemolytica* under current reaction conditions (Fig. 3a). Similar results for specificity testing were observed for the conventional PCR also (Fig. 3b).

Out of 41 neonatal lamb lung tissue samples, DNA from seven lung tissue samples were found positive for *M. haemolytica* by real-time PCR. However, six lung tissue samples were detected positive for *M. haemolytica* by conventional PCR. With this limited sample size, the statistical significance cannot be established. However improved detection with SYBR green dye chemistry based real-time PCR is obvious and need further evaluation over a large number of tissue and clinical samples.

Faster and sensitive detection of *M. haemolytica* 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 *M. haemolytica*. As the sample size was relatively small, this promising assay needs to be tested on a wider scale and over a variety of samples to further assess its suitability in the field to enable its adoption for screening and early detection of



a. Melt curve showing presence of single specific product of *M. haemolytica*



b. A representative agarose gel electrophoresis showing specific PCR product of 227 bp in L1 (L1- *M. haemolytica*, L2- *P. multocida*, L3- *B. trehalosi*, L4- *E. coli*, L5- *S. aureus*, L6- *P. aeruginosa,* L7- *P. mirabilis*, L8- *C. pseudotuberculosis*, L9- *A. variabilis,* L10- *M. avium* subspecies *paratuberculosis,* L11 and L12- negative control)

Fig. 3. Evaluation of specificity of real-time PCR and conventional PCR

diseased / carrier animals in a healthy flock and further to minimize the cost of treatment.

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