RESEARCH ARTICLE Indian Journal of Animal Research, Volume 54 Issue 8: 1006-1011 (August 2020)

Evaluation of a SYBR Green Real-Time PCR Assay for Specific Detection of *Pasteurella multocida* in Culture and Tissue Samples from Sheep

Jyoti Kumar, G.G. Sonawane, Fateh Singh[1], S. Jegaveera Pandian, Rajiv Kumar[2] **10.18805/ijar.B-3774**

ABSTRACT

Pasteurella multocida is one of the bacterial species involved in cases of ovine respiratory complex that has been implicated to cause significant economic losses in sheep production system worldwide. The present study was undertaken with the aim of evaluating a SYBR Green dye based real time PCR assay targeting KMT1 gene for the detection of *P. multocida.* The analytical specificity and sensitivity of the PCR primers were evaluated. The test showed ten-fold more sensitivity than conventional PCR and detected down to 275.5 fg/ µl of genomic DNA concentration, equivalent to 100 copies of KMT1 gene of *P. multocida*. The real-time PCR was found to be specific for KMT1 gene of *P. multocida*, as no cross reactivity was detected with a variety of known bacterial isolates. A total of 52 ovine lung tissue samples were screened for *P. multocida*, which showed improved level of detection as compared to conventional PCR. It is concluded that, this assay may be used as a valuable diagnostic tool for the rapid and specific detection of *P. multocida*. By virtue of its high throughput format and its ability to accurately identify as well as quantify the bacterial DNA, the method may be useful in large scale epidemiological studies and clarification of pathogenesis.

Key words: *Pasteurella multocida,* PCR, Real time PCR, Sensitivity, Specificity, Sheep, SYBR green.

INTRODUCTION

Respiratory infections in sheep remains a major cause of concern in all types of production systems. India is home to diverse ovine germplasm with 65.07 million (5.54%) sheep out of 1172.833 million world sheep population (FAO, 2015; DADF-Annual report 2017). *P. multocida* in sheep can cause outbreaks of bronchopneumonia, septicaemia, otitis media and polyarthritis (Donachie 2007; Jensen *et al*., 1982; Rad *et al*., 2010; Petridou *et al*., 2011). It has been reported as a primary pathogen in pneumonia and septicaemic outbreaks from different countries (Cameron *et al*., 1978; Chandrasekhran *et al*., 1991; Hancock *et al*., 1991; Diker *et al*., 1994; Dimri *et al*., 1994; Davies *et al*. 2002).

The reports on *P. multocida* related to clinical illness of ovine pneumonic pasteurellosis are scanty despite its proven role in the development of respiratory diseases in lambs (Odugbo *et al*. 2006). Lung pathology due to these microorganisms has been recorded most often during the necropsy of dead sheep and is evident by gross changes like congestion and consolidation of the lung tissues. Failure to diagnose ovine pneumonia during ante mortem examination of the infected animals and gap in the accurate information related to the onset of lesions restrict our knowledge for its strategic control (Goodwin *et al*. 2004). Diagnosis of pasteurellosis is usually achieved based on the clinical findings, cultural isolation and phenotyping and capsular serotyping of the causative pathogens, which is laborious and time-consuming. However, the introduction of advanced genotypic methods that are more sensitive and specific helps to identify the bacteria and their genetic elements more accurately and rapidly (McPherson and Moller, 2002; Hassan *et al*., 2016). PCR based tests has several advantages compared to traditional diagnostic methods. PCR is highly sensitive, rapid and specific. Real-time PCR (qPCR) has further advantages with respect to rapidity, precision, sensitivity and accuracy. This work narrates a real-time assay using SYBR green chemistry for sensitive and rapid detection of *P. multocida* from bacterial cultures and ovine lung tissues.

Division of Animal Health ICAR-Central Sheep and Wool Research Institute, Avikanagar-304 501, Rajasthan, India.
[1]ICAR-National Institute of High Security Animal Diseases, Anand Nagar, Bhopal-426 022, Madhya Pradesh, India.
[2]Animal Genetics and Breeding Division, ICAR-Central Sheep and Wool Research Institute, Avikanagar-304 501, Rajasthan, India.

Corresponding Author: Jyoti Kumar, Division of Animal Health ICAR-Central Sheep and Wool Research Institute, Avikanagar-304 501, Rajasthan, India. Email: jyotivet@gmail.com

How to cite this article: Kumar, J., Sonawane, G.G., Singh, F., Pandian, S.J. and Kumar, R. (2020). Evaluation of a SYBR Green Real-Time PCR Assay for Specific Detection of *Pasteurella multocida* in Culture and Tissue Samples from Sheep. Indian Journal of Animal Research. 54(8): 1006-1011.

Submitted: 26-12-2018 **Accepted:** 01-04-2020 **Published:** 28-07-2020

MATERIALS AND METHODS

Sample collection and processing

For evaluation of the real-time PCR assay over clinical samples, fifty-two archived lung tissue samples collected from sheep that had died of respiratory disease during 2014-

2017 were included. The geographical location belongs to the semiarid eastern plain of Rajasthan at 26°12′ 52.2″ N latitude and 75°45′ 24.84″ E longitude at 320 meters above mean sea level.

Bacterial strains

The specificity of the PCR primer was evaluated using DNA 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. It included *P. multocida* (n=10) *Mannheimia haemolytica* (n=15), *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), *Proteus mirabilis* (n=1).

DNA extraction and PCR

Genomic DNA was extracted from *P. multocida* culture and lung tissue samples from sheep using Qiagen DNeasy Blood and Tissue kit, according to manufacturer's protocol. Genomic DNA was quantified using a Quawell UV-Vis Q5000 spectrophotometer (Quawell Technology Inc., San Jose, CA, USA). The extracted DNA samples were stored at - 20°C (to minimize degradation) till further use in the SYBR green real-time PCR and conventional PCRs assay.

Primers targeting KMT1 gene specific to *P. multocida* were synthesized from Sigma (Bengaluru, India). Primers used in this study were Forward 5′ - ATCCGCTATTTACCC AGTGG -3′ and Reverse 5′ - GCTGTAAACGAACTCGCCAC -3′ (Townsend *et al*., 1998). The optimized thermal cycling conditions were as follows; initial denaturation at 95°C for 5 min followed by 40 cycles of denaturation at 95°C for 45 s, annealing at 56°C for 45 s and extension at 72°C for 1 min, and a final elongation at 72°C for 10 min. The amplicon of approximately 460 bp was visualized after electrophoresis through 2% agarose gel run at 7.0V/cm and stained with ethidium bromide.

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

SYBR green Real-time PCR assay

Real-time PCR assay was performed over DNA template from culture and tissue samples with the same primer set as used for conventional PCR using Step One Plus 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 total volume of 10 µl containing 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.

A reference standard curve was constructed using *P. multocida* genomic DNA to determine the efficiency and detection limit of the assay. The amount of genomic DNA in a stock solution was calculated based on the size of the *P. multocida* genome being around 2543931 bp (Sahay *et al*., 2018) and the average molar mass per base pair being 650 (g/mol)/bp. The copy number was calculated using the calculator found at: https://cels.uri.edu/gsc/cndna. html. Reactions were carried out using seven serial 10-fold dilutions for the generation of standard curve.

Comparison of analytical sensitivity and specificity of real-time PCR with PCR

Real-time PCR and conventional PCRs were carried out under optimal reaction conditions using same primers set and their sensitivities were evaluated. Specificity of the developed real-time PCR and conventional PCR assays were assessed by performing the reaction with *P. multocida*, as well as other bacterial DNA.

RESULTS AND DISCUSSION

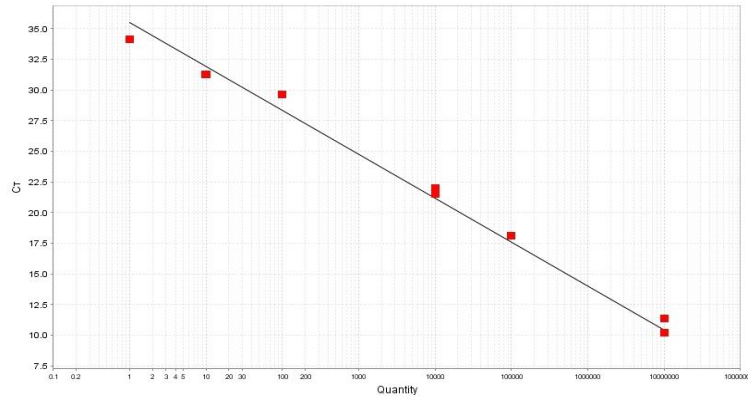
In general, the prevalence of *P. multocida* organism is usually underestimated due to cumbersome process of isolation and identification by conventional microbiological methods. A 10-fold dilution series of DNA from 1×10^7 to 1×10^0 genome copies per reaction were prepared, and four replicates of each concentration were tested to plot a standard curve. Although the qPCR is a widely used technique, recommendations on how to use a standard curve to estimate PCR efficiency are rare and a general consensus or guidelines are lacking. It is known that estimates of PCR efficiency may be influenced by several experimental factors. A properly designed assay shall, in the absence of interfering substances in the sample matrix, amplify target DNA with at least 90% efficiency (Broeders *et al*. 2014).

The performance of new assay needs to be tested by means of specificity, efficiency and sensitivity (sometimes also for limits of detection and quantification). The PCR efficiency is one of the most important indicators of the performance of a qPCR assay and is also required parameter for quantitative analysis. The PCR efficiency depends on many factors including: to primers and template sequences and structures, undesirable intra-molecular interactions affecting PCR efficiency, inhibitors and other interfering substances from the sample, reagents used and their concentrations etc. (Svec *et al*., 2015). The standard curve remains the most reliable, practicable and robust approach to estimate PCR assay efficiency that is broadly accepted by the community. The efficiency of the qPCR was calculated to be 90.19% and the limit of detection, in which all four replicates had a positive amplification curve, was

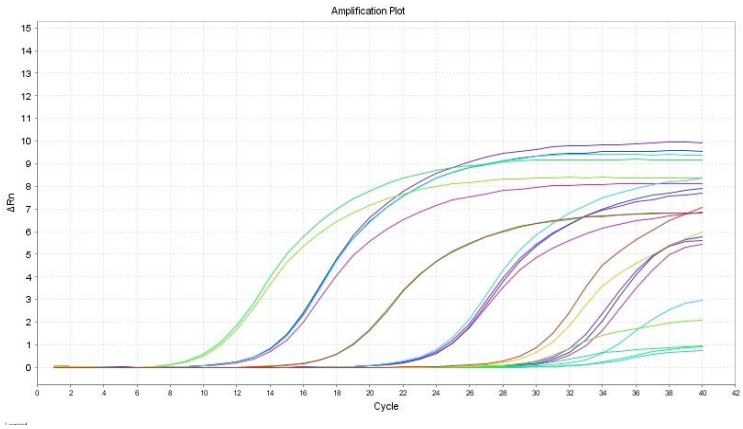
upto 100 copies of the genome/µl of reaction mix (Fig 1). A Cq >35 should be considered as suspect or indeterminate and retested as needed to determine clinical relevance.

Under the described trial conditions (1 µl of DNA extract per 10 µl assay), the detection limit of the qPCR test using DNA from pure bacterial culture was 2.755 pg per assay or 275.5 fg/ µl equivalent to 100 genome copies per µl (Fig 2). While the detection limit of the conventional PCR was 27.55 pg per assay or 2.755 pg/ µl equivalent to 1000 genome copies per µl. Therefore, our qPCR was ten times more sensitive than the conventional PCR. The limits of detection of other PCR tests used for *P. multocida* have not been determined (Miflin and Blackall, 2001; Liu *et al*., 2004; Sabiel *et al*., 2012; Singh *et al*., 2018). The threshold limit of detection of PCR for the KMT1, HSB, PSL and Miflin primer sets were 500 pg, 50pg, 50pg and 5ng of DNA of *P. multocida* per assay, respectively (Jonathan and Arora, 2012). In another PCR assay published by Jonathan and Arora (2012), IPFWD and IPREV primers sets demonstrated a higher sensitivity, but that is only specific for *P. Multocida* serotype B: 2 causing haemorrhagic septicaemia in cattle and buffaloes in India. In another PCR and multiplex assay previously developed by Townsend *et al*., (1998) and Register and de Jong (2006), the threshold limits for KMT1 primer sets were between 1-10 pg of DNA per assay. For the detection of P. Multocida in swine, the detection limit described by Lung *et al* (2015) was 10^3 copies/µl and that of loop-mediated isothermal amplification method was 22pg/ µl (Bhimani *et al* (2015).

On evaluation of specificity using genomic DNA extracted from different bacterial strains (*M. haemolytica, B.trehalosi, Escherichia coli, Staphylococcus aureus, C. pseudotuberculosis, M. avium subspecies paratuberculosis, P. aeruginosa, A. variabilis, P. mirabilis*) other than *P. multocida*, real-time amplification was seen only in positive control (*P. multocida)*. False positive signals observed 30-35 cycles on and were verified using melting curve. As gel electrophoresis revealed that no specific amplification products were formed, it was indicative of non-specific fluorescence of low molecular weight DNA. Additionally, sequence of the KMT1 amplicon showed high degree of similarity (98-100%) with the published sequence in the NCBI database. None of the other bacterial strains gave any amplification products indicating the high degree of specificity of this assay. Only positive control DNA produced amplification with a melting peak at approximately 81.1°C which is consistent with that expected for *P. multocida* under current reaction conditions (Fig 3a.). Similar results for



a



b

Fig 1: a. Standard curve of SYBR green real time PCR with 90.19% efficiency.
b. Amplification plot for kmt1 of *P. multocida* over different dilutions of template.

specificity testing were observed for the conventional PCR also (Fig 3b.). Under our study conditions, we found the KMT1 real time PCR 100% specific, limiting the risk of the false positive results. A similar specificity was reported with the qPCR and end point PCR assay published by Townsend *et al*., (1998) and Marois *et al* (2008).

A total of 52 ovine lung tissue samples were screened to detect and quantify *P. multocida*. Out of these 52 samples, five lung tissue samples (9.6%) were found positive for *P. multocida* by qPCR with genome copies ranging from 1000-7000/10 ng of tissue DNA as deduced from standard curve. However, four lung tissue samples (7.7%) were detected positive for *P. multocida* by conventional PCR. Although statistical sense cannot be drawn owing to limited sample size, improved detection percentage with SYBR green dye chemistry based qPCR is obvious and it warrants further evaluation over a large number of clinical samples.

Faster and sensitive detection of *P. multocida* is of significant clinical importance. The specific and highly sensitive SYBR green PCR assay performed in the present

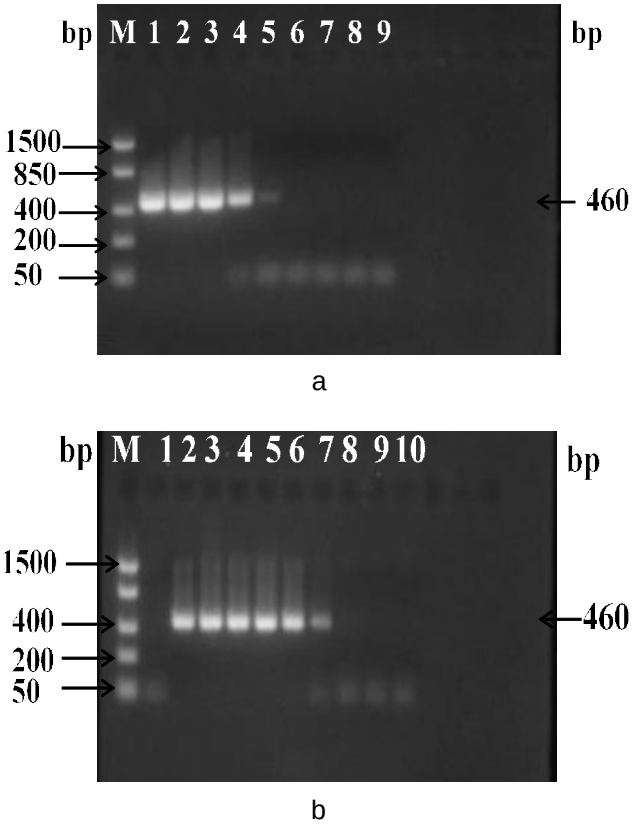


Fig 2: Analytical sensitivity of conventional PCR and SYBR green real-time PCR.

a. Agarose gel electrophoresis of conventional PCR product showing sensitivity upto 27.55 pg. Amount of P. multocida DNA in L2= 27.55 ng, L3= 2.755 ng, L4=275.5 pg, L5= 27.55 pg, L6= 2.755 pg, L7= 0.2755 pg, L8= 0.02755 pg, L1= positive control, L9= negative control, M= FastRuler Low Range DNA ladder (#SM1103).

b. Agarose gel electrophoresis of SYBR green real-time PCR product showing sensitivity upto 2.755 pg. Amount of P. multocida DNA in L3= 27.55 ng, L4= 2.755 ng, L5=275.5 pg, L6= 27.55 pg, L7= 2.755 pg, L8= 0.2755 pg, L9= 0.02755 pg, L10= 0.002755 pg L2= positive control, L1= negative control, M= FastRuler Low Range DNA ladder (#SM1103).

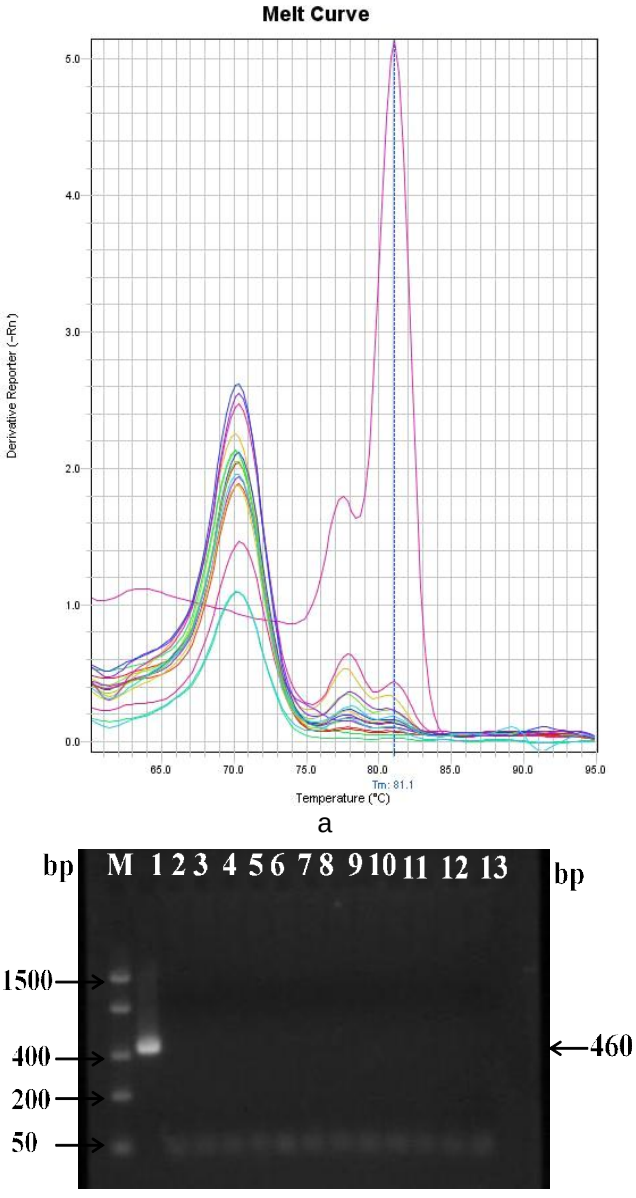


Fig 3: Evaluation of specificity of SYBR green real time PCR and conventional PCR.

a. Melt curve showing presence of single specific product of *P. multocida.*

b. A representative agarose gel electrophoresis showing specific PCR product of 460 bp in L1, L1=*P. multocida*, L2=*M. haemolytica*, L3= *B.trehalosi*, L4= *E. coli*, L5= *S. aureus*, L6= *C. pseudotuberculosis*, L7= *M. avium* subspecies *paratuberculosis,* L8= *P. aeruginosa,* L9= *A. variabilis,* L10= *P. mirabilis,* L11 and L12- negative control**.**

work may be suitable as a rapid and economic diagnostic tool to identify sheep carrying *P. multocida*. As the sample size was relatively small, this promising assay needs to be validated on a wider scale 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 minimize the cost of treatment.

ACKNOWLEDGEMENT

The authors are thankful to the Director, ICAR-CSWRI, Avikanagar for providing necessary facilities to carry out this study. The work has been conducted in the Network Project on Veterinary Type Culture Centre (ICAR-NRCE, Hisar).

COMPLIANCE WITH ETHICAL STANDARDS

CONFLICT OF INTEREST

The authors declare that they have no competing interests.

REFERENCES

Bhimani, M., Bhanderi, B. and Roy, A. (2015). Loop-mediated isothermal amplification assay (LAMP) based detection of *Pasteurella multocida* in cases of haemorrhagic septicaemia and fowl cholera. Vet. Ital. 51: 115-121.

Broeders, S., Huber, I., Grohmann, L., Berben, G., Taverniers I., Mazzara, M., Roosens, N. and Morisset, D. (2014). Guidelines for validation of qualitative real-time PCR methods. Trends Food Sci. Tech. 37 (2):115-126.

Cameron, C. M., Engelbrecht, M. M., and Vermueulen, A. S. M. (1978). The serological and immunological relationship of type strains A and D of *Pasteurella multocida* to field isolates from sheep. Onderstepoort J. Vet. 45: 215-220.

Chandrasekharan, S., Hizat, K., Saad., Z., Johara, M. Y. and Yeap, P. C. (1991). Evaluation of combined pasteurella vaccines in control of sheep pneumonia. Brit Vet J. 147: 437-443.

Davies, R. L., Watson, P. and Caffrey, B. (2003). Comparative analyses of *Pasteurella multocida* strains associated with ovine pneumonia and ovine vaginal tract. Vet Rec. 152: 7-10.

Department of Animal Husbandry, Dairying and Fisheries (DADF). Annual Report (2017-2018). Department of Animal Husbandry, Dairying and Fisheries, Ministry of Agriculture and Farmers Welfare, Government of India. 1st ed. New Delhi, India: DADF; 2018.

Diker, K. S., Akan, M. and Haziroglu, R. (1994). Antimicrobial susceptibility of *Pasteurella haemolytica* and *Pasteurella multocida* isolated from pneumonic ovine lungs. Vet Rec. 134: 597-598.

Dimri, U., Lonkar, P.S., Srivastaya, C. P. and Bhagwan, P. S. K. (1994). An outbreak of pneumonia due to *Pasteurella multocida* in sheep. Indian Vet J. 71: 1163-1167.

Donachie, W. (2007). Pasteurellosis. In: Diseases of Sheep. 4th edn. [Ed Aitken I. D]. Blackwell Science. pp 224-231.

Food and Agriculture Organization of the United Nations (FAO) (2015). Livestock. In: Gennari P, editor. FAO Statistical Pocket Book, World Food and Agriculture. Food and Agriculture Organization of the United Nations, Rome: FAO Statistics Division. pp. 30.

Gilmour, N. J. L. and Gilmour, J. S. (1989). Pasteurellosis of Sheep. In Pasteurella and Pasteurellosis. [Eds Adlam, C., Rutter, J. M., Academic Press. London, pp 223-262.

Goodwin, K. A., Jackson, R., Brown, C., Davies, P.R., Morris, R.S. and Perkins, N. R. (2004). Pneumonic lesions in lambs in New Zealand: patterns of prevalence and effects on production. New Zeal Vet J. 52: 175-179.

Hancock, R. D., Fallavena, L. C. and Rebeiro, L. A. O. (1991). Pneumonic pasteurellosis due to Pasteurella multocida in a flock of lambs in Brazil. Vet Rec. 128: 154-155.

Hassan, G. M., El-Feky, Z. A., Eissa, E. A. and Teleb, A. A. (2016). Rapid diagnosis of virulent *Pasteurella multocida* isolated from farm animals with clinical manifestation of pneumonia respiratory infection using 16S rDNA and KMT1 gene. Asian Pac J Trop Dis. 6: 21-26.

Jensen, R., Pierson, R. E., Weibel, J. L., Tucker, J. O. and Swift, B. L. (1982). Middle ear infection in feedlot lambs. J Am Vet Med A. 181: 805-807.

Jonathan, L. and Arora, A. K. (2012). Comparison of detection threshold of different *Pasteurella multocida* specific PCRs. Indian. J. Anim Res. 46(1):28-33.

Liu, D., Lawrence, M.L. and Austin, F.W., (2004). Specific PCR identification of *Pasteurella multocida* based on putative transcriptional regulator genes. J. Microbiol. Methods. 58: 263-267.

Lung, O., Ohene-Adjei, S., Buchanan, C., Joseph, T., King, R., Erickson, A., Detmer, S. and Ambagala, A. (2017). Multiplex PCR and microarray for detection of swine respiratory pathogen. Transbound. Emerg. Dis. 64(3): 834-848.

Marois, C., Cariolet, R., Morvan, H. and Kobisch, M. (2008). Transmission of pathogenic respiratory bacteria to specific pathogen free pigs at slaughter. Vet. Microbiol. 129: 325-332.

McPherson, M.J. and Moller, S. G. (2000). Polymerase Chain Reaction. BIOS Scientific Publishers Ltd.; Oxford, 1st ed., United Kingdom. pp 1-18.

Miflin, J.K. and Blackall, P.J. (2001). Development of a 23S rRNA-based PCR assay for the identification of *Pasteurella multocida*. Lett. Appl. Microbiol. 33: 216-221.

Odugbo, M. O., Odama, L. E., Umoh, J. U. and Lamorde, A. G. (2006). *Pasteurella multocida* pneumonic infection in sheep: prevalence, clinical and pathological studies. Small Ruminant Res. 66: 273-277.

Petridou E.J., Gianniki Z., Giadinis N.D., Filioussis G., Dovas C.I. and Psychas V. (2011). Outbreak of polyarthritis in lambs attributed to Pasteurella multocida. Vet Rec. 168 (2): 50.

Rad, M., Movassaghi, A. R., Sharifi, K., Naseri, Z. and Seifi, H. A. (2011). Two outbreaks of *Pasteurella multocida* septicemia in neonatal lambs. Comp Clin Path. 20(1): 57-59.

Register, K. B. and de Jong, K. D. (2006). Analytical verification of a multiplex PCR for identification of Bordetella bronchiseptica and Pasteurella multocida from swine. Vet. Microbiol. 117: 201-210.

Sabiel, Y.A., Musa, M.T. and Ann, V.Z. (2012). Identification of Mannhemia haemolytica and Pasteurella multocida by Polymerase Chain Reaction and Random Amplification of Polymorphic DNA. Sudan J. Vet. Res. 27: 55-59.

Sahay, S., Shome, R., Sankarasubramanian, J., Vishnu, U. S., Prajapati, A., Natesan, K., *et al* (2018). Insights into the

genome sequence of ovine *Pasteurella multocida* type a strain associated with pneumonic pasteurellosis. Small Ruminant Res. 169: 167-175.

Singh, F., Sonawane, G. G. and Meena, R. K. (2018). Molecular detection of virulent *Mannheimia haemolytica* and *Pasteurella multocida* in lung tissues of pneumonic sheep from semiarid tropics, Rajasthan, India. Turk. J. Vet. Anim. Sci. 42: 556-561.

Svec, D., Tichopad, A., Novosadova, V., Pfaffl, M. W. and Kubista, M. (2015). How good is a PCR efficiency estimate: Recommendations for precise and robust qPCR efficiency assessments. Biomolecular Detection and Quantification. 3: 9-16.

Townsend, K. M., Frost, A. J., Lee, C. W., Papadimitrious, J. M. and Dawkins, H. J. S. (1998). Development of PCR assays for species and type specific identification of *Pasteurella multocida* isolates. J. Clin. Mirobiol. 36: 1096 1100.