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Molecular detection of *Pasteurella multocida* isolated from pneumonic lungs of pigs

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Abstract
The study aimed to investigate the presence of *Pasteurella multocida* in pigs with pneumonia and identify its association with other bacterial isolates. A total of 180 pigs were subjected to post mortem examination, and lung samples with pneumonic lesions were collected. Bacteria were isolated from 127 samples, and suspected colonies of *Pasteurella multocida* were further analyzed using Polymerase Chain Reaction (PCR) for the detection of the *kmt1* gene. The different bacteria isolated were *Pasteurella multocida, S. suis, E. coli, Staphylococcus* spp., *Klebsiella* spp., and *Salmonella* spp. Among the suspected colonies of *Pasteurella multocida*, 27 isolates were confirmed to be positive for the *kmt1* gene. This study highlights the importance of understanding the role of *Pasteurella multocida* in pig pneumonia to develop effective control measures.

Keywords: *Pasteurella multocida*, pneumonic lungs, pigs

1. Introduction
The study aimed to investigate bacterial pneumonia in pigs, specifically focusing on the isolation, identification, and molecular detection of *P. multocida* from pneumonic lungs. *P. multocida* is a small, non-motile, gram-negative cocco-bacillus commonly found in the upper respiratory tract of pigs (Petrocchi-Rilo *et al*., 2019) [11]. It is a commensal and opportunistic pathogen of the oral, nasopharyngeal and upper respiratory tract (Garcia *et al*., 2011) [8]. *P. multocida* comprises of complicated antigenic structure and has 5 capsular serotypes (A, B, D, E and F) and 16 somatic serotypes (Pijoan C, 1992) [12]. In swine, capsular type A is the causative agent of pneumonia, pleuritis and abscessation (Zhao *et al*., 1995) [18]. Pneumonic pasteurellosis can be identified by chronic pneumonia, purulent bronchopneumonia and pleurisy (Quinn *et al*., 1994) [14].
P. multocida is considered as an opportunistic pathogen causing secondary bacterial pneumonia in pigs and aggravating pulmonary infections. Secondary bacterial infection determines the severity of the disease. Secondary infection by necrotizing, pus-forming bacteria are the usual cause of death and the secondary agents that are important include *Pasteurella multocida*, *Streptococcus suis* and *Actinobacillus suis* (Dosen *et al*., 2007) [6].
Bacterial pneumonia in pigs is a significant concern for both commercial and small-scale farmers, as it leads to various hindrances in pig production, including increased morbidity and mortality (Abubakar *et al*., 2017) [1]. In economically disadvantaged communities, such outbreaks can result in severe economic losses, as pig farming is often a primary source of livelihood. *P. multocida* is an important etiological agent of swine chronic pneumonia (Pijoan and F Trigo, 1982) [13]. Therefore, the study aimed to gain insight into bacterial pneumonia in pigs and implement effective control measures. This involved isolating and identifying major bacterial pathogens, with a specific focus on *P. multocida*. Molecular detection techniques were utilized to ensure accurate identification of *P. multocida* in pneumonic lungs. The findings from this study could contribute to the development and implementation of effective control measures to minimize the impact of bacterial pneumonia in pig populations.

2. Materials and Methods
The materials required for the present study comprising of tissue samples of lungs and upper respiratory tract were collected from various slaughter houses, field mortalities, private piggery farms located in and around Guwahati and from postmortem examinations carried out at Department of Pathology, College of Veterinary Science, A.A.U., Khanapara, Guwahati,

Assam for a period of one year i.e. from March, 2020 to February, 2021. A total of 180 pigs irrespective of age, sex were subjected to detailed post mortem examination. Based on gross examination, 127 pneumonic lungs samples were collected aseptically and subjected for bacteriological investigation.

2.1 Bacteriological Studies

The primary isolation of *Pasteurella* spp. involved using Brain Heart Infusion agar plates and Blood Agar Base medium and these plates were incubated at 37 °C for 24 hours (Jabeen *et al*., 2013) [9]. Gram's staining was performed on suspected colonies, revealing small, non-motile, gram-negative coccobacilli (Fig.2). Methylene blue staining was then done, showing bipolar characteristics (Fig.3).

To isolate *Streptococci*, the samples were streaked onto Brain Heart Infusion agar plates and incubated at 37 °C for 24 hours. The morphological, cultural, and biochemical characteristics were studied (Cowan and Steel, 1993; Tarradas *et al*., 1994) [4, 15].

For the isolation of *Staphylococcus* spp., the samples were streaked onto Mannitol Salt Agar plates. After incubation at 37ºC for 24 hours, yellow colonies were observed. Gram's staining revealed purplish spherical clusters resembling grapes.

In the case of *Escherichia coli*, *Klebsiella* spp., and *Salmonella* spp., the samples were directly inoculated onto MacConkey Lactose Agar plates for primary isolation. After incubation at 37 °C for 24 hours, the IMViC test was performed for further differentiation.

2.2 Molecular Detection by Polymerase Chain Reaction (PCR)

Confirmation of *Pasteurella multocida* was made on detection of *kmt1* gene by simplex PCR. The PCR was carried out using a thermocycler from Applied Biosystems. Once pure colonies were identified and confirmed, they were preserved in 80% glycerol broth at a temperature of -20 °C for future studies.

2.2.1 Extraction of bacterial DNA

Template DNA was prepared from each isolates of *Pasteurella multocida* for their molecular characterization in terms of detection of *kmt1* gene by simplex PCR.

BHI agar plate grown *Pasteurella multocida* cultures were enriched in 2 ml Brain Heart Infusion broth by incubation at 37 °C for 24 hours. The culture was then centrifuged at 3000 rpm for 5 minutes. The supernatant was discarded and the pellet was washed with 150 µl of tris-EDTA (TE) buffer with pH 8 by centrifuging and discarding the supernatant twice. To the pellet, 100 µl of sterile nuclease free water was added, mixed properly and transferred to 1.5 ml eppendorf tubes. The samples were placed on a vortex shaker for gently shaking. The suspension was boiled for 15 to 20 minutes, snap chilled on ice for 10 minutes and then centrifuged at 12,000 rpm for 10 minutes. The collected supernatant was used as templates and stored at -20 °C for further use. The extracted DNA concentration was measured in Nanodrop Spectrophotometer (Thermofisher scientific, USA).

Details of oligonucleotide primer sequences for *kmt1* gene are depicted in Table 1. The specific forward and reverse primers used in this study were commercially procured from Molbiogen.

Table 1: Primers Used to Characterize the *Pasteurella multocida* Isolates

Primer	Sequence (5′-3′)	Target	Product size	Reference
KMT1T7	ATC CGC TAT TTA CCC AGT GG	*kmt1*	460bp	Townsend *et al*. (1998) [16]
KMT1SP6	GCT GTA AAC GAA CTC GCC AC			

2.2.2 Reconstitution and dilution of primer

The primers that were obtained in lyophilized form were rehydrated by adding nuclease free water to achieve a concentration of 100 pmol/µl. The reconstituted primers were then mixed thoroughly by gently vortexing. From the primers' stock solution, a working solution with a concentration of 10 pmol/µl was prepared. This working solution was divided into 10 µl aliquots and each aliquot was stored at -20 °C for future use.

2.2.3 Amplification of *kmt1* gene by Polymerase Chain Reaction (PCR)

The template DNA extracted from suspected colonies were subjected to simplex PCR and the targeted *kmt1* gene was amplified using the specific primers (Townsend *et al*., 1998) [16].

In this experiment, PCR was performed to amplify the *kmt1* gene. The reaction mixture consisted of 12.5 µl of 2X Dream Taq master mix, which contains Taq DNA polymerase, MgCl2, and dNTPs in reaction buffer. The template DNA was added at a concentration of 100-150 ng, along with 1 µl each of the forward and reverse primers of the *kmt1* gene. Nuclease-free water was added to bring the final reaction volume to 25 µl.

The PCR reaction was carried out in a Thermal cycler from Applied Biosystems, following a previously reported thermocycling condition (as specified in Table 3). To confirm the specificity of the reaction, DNA lysate as positive control was included from the Department of Veterinary Microbiology, C.V.Sc., A.A.U., Khanapara. A non-template control (NTC) was also included as a negative control to detect any contamination or non-specific amplification.

2.2.4 Composition of the reaction mixture

The reaction mixture given below was added in a PCR tube and the tube was placed in a thermal cycler for amplification of the *kmt1* gene (Table 2).

Table 2: Composition of the Pcr Reaction Mixture for *kmt1* Gene Amplification

Template DNA	5 µl
Forward primer (10 pmol)	1 µl
Reverse primer (10 pmol)	1 µl
Master mix	12.5 µl
Nuclease free water	5.5 µl
Total	25 µl

Table 3: Thermo Cycling Conditions for Molecular Detection of *kmt1* Gene

Target gene	Conditions	Temperature	Duration	No. of cycles
kmt1	Initial denaturation	95 °C	3 min	1
	Denaturation	95 °C	1 min	35
	Primer Annealing	55 °C	1 min	
	Extension	72 °C	30 sec	
	Final Extension	72 °C	7 min	1

The agarose gel used for electrophoresis had a concentration of 1.5% and contained ethidium bromide. The electrophoresis was conducted in 1X Tris-Acetic acid- EDTA (TAE) buffer, which consisted of 40 mM Tris-HCl, 1 mM EDTA, and 0.1% glacial acetic acid with a pH of 8. The electrophoresis was run at a voltage of 80-100 V for a duration of 1 hour. After electrophoresis, the gel was visualized using UV light in a Gel Doc System manufactured by BioRad (USA). The images were captured using Imagelab software.

3. Results and Discussion

In this investigation, 31 isolates of *Pasteurella multocida* were obtained from pneumonic lungs of pigs. These isolates were subjected to PCR screening for the presence of the kmt1 gene. Out of the 31 isolates, 27 were found to be positive for the kmt1 gene, while 4 isolates did not show the presence of this gene. The isolates were characterized as Gram-negative rods with bipolar staining characteristics and were found to be non-haemolytic on sheep blood agar. They were also found to be aerobic to facultatively anaerobic and positive for indole, oxidase, catalase, and carbohydrate fermentation with slight gas production. (Bergey *et al*., 1994; Chawak *et al*., 2000; Anupama *et al*., 2003) [3, 5, 2]. During our study, the pneumonia cases were classified into different types based on the distribution, texture, color, appearance, and exudation of the affected lungs. *P. multocida* could be isolated from cases of bronchopneumonia (5), interstitial pneumonia (11), suppurative pneumonia (2), fibrinous pneumonia (7) and haemorrhagic pneumonia (1). Out of all the pathogens detected, *P. multocida* was found to be the most dominant, followed by *Streptococcus suis*. Other bacteria such as *E. coli*, *Staphylococcus* spp., *Salmonella* spp., and *Klebsiella* spp. were also isolated, and mixed bacterial infection was found in a significant number of cases. Several workers (Dosen *et al*., 2007; Lavanya *et al*., 2011; Fablet *et al*., 2012) [6, 10, 7] have also detected one or more of these pathogens in pigs died of pneumonia in different proportions. Mixed bacterial infection indicated etiological complexity involved in the pathogenesis of pneumonia as one or more bacteria were isolated from the pneumonic lungs of pigs.

The 31 pure isolates of *P. multocida* were further screened for the *kmt1* gene. Out of 31 samples, *kmt1* gene with amplicon size 460 bp was detected from 27 samples (Fig. 3). In routine diagnostic procedure, obtaining a pure culture of *P. multocida* from clinical samples can be difficult due to contaminants and/or death of organisms. Therefore, *P. multocida* species-specific PCR was used as an alternative method to identify *P. multocida* in these samples (Varte *et al*., 2014) [17]. It was demonstrated that the primers KMT1T7 and KMT1SP6 are highly specific for *P. multocida* isolates (Townsend *et al*. 1998) [16].

Overall, the study suggests that PCR using the KMT1T7 and KMT1SP6 primers is an effective method for detecting *P. multocida* in clinical samples, especially when obtaining pure cultures of the bacteria is challenging.

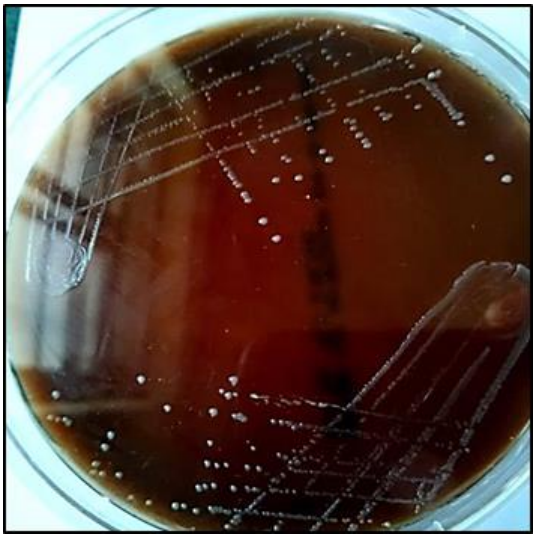


Fig 1: *Pasteurella multocida* showing dew drop colonies on blood agar

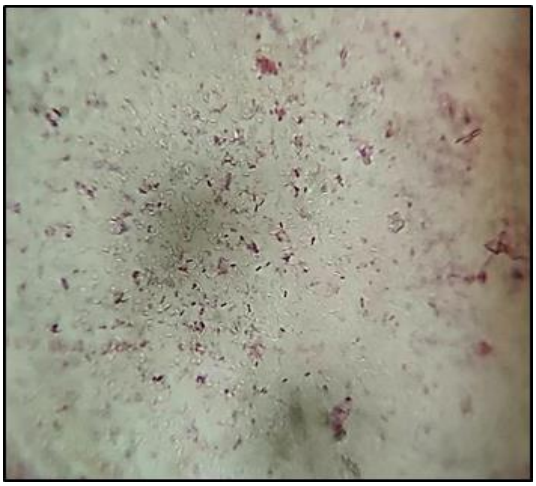


Fig 2: *Pasteurella multocida* orgamism in gram's staining (100x)

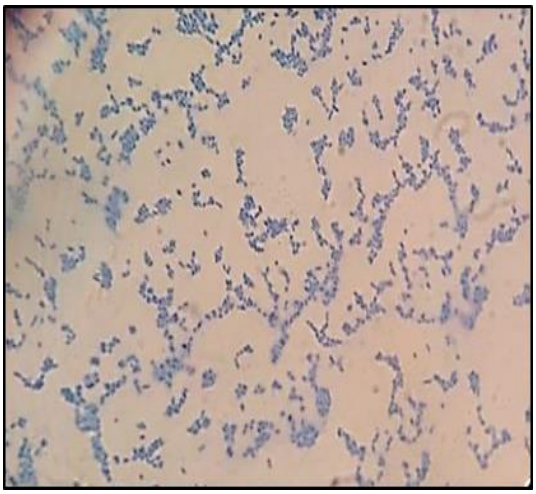
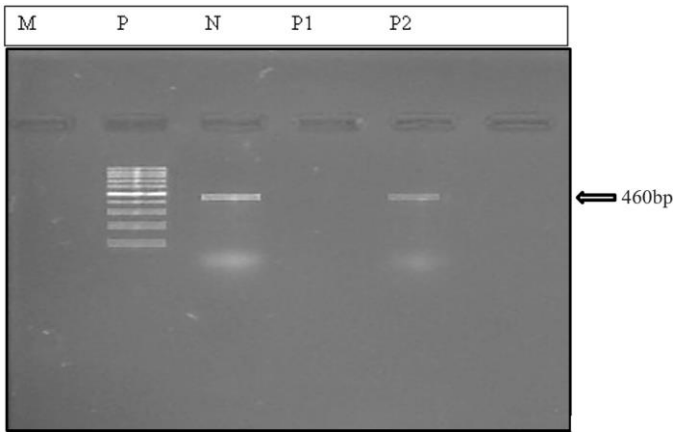


Fig 3: *Pasteurella multocida* organism in methylene blue staining showing bipolar reaction (100x)



M: 100bp ladder, Lane N: Negative control, Lane P: Positive control, Lane P1, P2: Sample tested

Fig 4: Detection of *kmt1* gene (460bp) of *Pasteurella multocida* by PCR

4. Conclusion

The findings of the present study suggest that *Pasteurella multocida* is the predominant bacterial pathogen associated with swine pneumonia. In the collected samples, *P. multocida* isolates accounted for 18.89% of all detected pathogens. The *kmt1* gene specific primers successfully amplified the DNA from 27 out of 31 *P. multocida* isolates, indicating that *P. multocida* is one of the causative agent of pig pneumonia.

The use of *P. multocida* species-specific PCR was found to be a rapid and reliable method for identifying *P. multocida* isolates. The primers KMT1T7 and KMT1SP6 were highly specific for *P. multocida*. However, further research is needed to understand the specific serotype(s) of *P. multocida* involved in the pathogenesis of swine pneumonia. Additional studies focusing on swine pasteurellosis would provide more comprehensive insight into the disease and its causative agents.

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6. References

1. Abubakar RH, Madoroba E, Adenubi O, Leather DM, Fasina FO. Bacterial pathogens of pigs with particular reference to *Escherichia coli*: A systematic review and meta-analysis. Journal of Veterinary Medicine and Animal Health. 2017;9(7):159-185.
2. Anupama M, Venkatesha MD, Yasmeen N, Gowda RNS. Evaluation of polymerase chain reaction (PCR) for identification of virulent *P. multocida* and its comparison with animal inoculation test. Indian J Anim. Sci. 2003;73:166-167.
3. Bergey DH, Harrison FC, Breed RS, Hammer BW, Huntoon FM. Bergey's Manual of determinative bacteriology. 9th Ed, Williams and Wilkins, Baltimore, 1994.
4. Cowan ST, Steel KJ. Cowan and Steel's manual for identification of medical bacteria. 3rd Ed, Cambridge University Press; c1993. p. 199-241.
5. Chawak MM, Verma KC, Kataria JM, Kamar AA. Characterization of indigenous isolates of avian *P. multocida*. Indian Comp. Microbiol. Immunol. Infec. Dis. 2000;21:111-114.
6. Dosen R, Prodanov J, Milanov D, Stojanov I, Pusic I. The bacterial infections of respiratory tract of swine. Biotechnology in Animal Husbandry. 2007;23(5-6):237-243.
7. Fablet C, Marois C, Dorenlor V, Eono F, Eveno E, Jolly JP, *et al*. Bacterial pathogens associated with lung lesions in slaughter pigs from 125 herds. Research in Veterinary Science. 2012;93(2):627-630.
8. Garcia N, Fernandez-Garayzabal JF, Goyache J, Dominguez L, Vela AI. Associations between biovar and virulence factor genes in *Pasteurella multocida* isolates from pigs in Spain. Vet Rec. 2011;169:362.
9. Jabeen A, Khattak M, Munir S, Jamal Q, Hussain M. Antibiotic susceptibility and molecular analysis of bacterial pathogen *Pasteurella multocida* isolated from cattle. Journal of Applied Pharmaceutical Science. 2013;3(04):106-110.
10. Lavanya K, Ramadevi V, Srilatha Ch. Pneumonia in pigs: A pathomorphological study. Indian Journal of Veterinary Pathology. 2011;35(2):206-208.
11. Petrocchi-Rilo M, Gutierrez-Martin CB, Mendez-Hernandez JI, Rodriguez-Ferri EF, Martinez-Martinez S. Antimicrobial resistance of *Pasteurella multocida* isolates recovered from swine pneumonia in Spain throughout 2017 and 2018. Veterinary and Animal Science, 2019, **7**.
12. Pijoan C. Pneumonic pasteurellosis. Diseases of swine 7th Ed, Lowa State University Press; c1992. p. 552-559.
13. Pijoan C, Trigo F. Pasteurelosis. In R. Necoechea and C. Pijoan (ed.), Diagnostico de las enfermendades del cerdo. R. Necoechea and C. Pijoan, Mimosa no. 53-7, Mexico; c1982. p. 511-513.
14. Quinn PJ, Carter ME, Markey BK, Carter GR. Veterinary Clinical Microbiology. Wolfe Publication, London, U.K; c1994. p. 254-258.
15. Tarradas C, Arenas A, Maldonado A, Luque I, Miranda A, Perea A. Identification of Streptococcus suis isolated from swine: Proposal for biochemical parameters. J. Clin. Microbiol. 1994;32:578-580.
16. Townsend KM, Frost AJ, Lee CW, Papadimitriou JM, Dawkins HJ. Development of PCR assays for species and type-specific identification of *Pasteurella multocida* isolates. J Clin Microbiol. 1998;36(4):1096-100.
17. Varte Z, Dutta TK, Roychoudhury P, Begum J, Chandra R. Isolation, identification, characterization and antibiogram of *Pasteurella multocida* isolated from pigs in Mizoram with special reference to progressive atrophic rhinitis. Veterinary World. 2014;7(2):95-99.
18. Zhao, Guosong, Thomas, Halbur, Duane C, Pankratz. Colonization of oropharynx and nasal cavity of CDCD pigs by a nontoxigenic strain of *Pasteurella multocicda* type D. J. Swine Health Prod.1 995;3(3):113-115.