The Pharma Innovation Journal 2021; SP-10(12): 561-565

www.ThePharmaJournal.com

The Pharma Innovation

ISSN (E): 2277- 7695
ISSN (P): 2349-8242
NAAS Rating: 5.23
TPI 2021; SP-10(12): 561-565

www.thepharmajournal.com
Received: 14-10-2021
Accepted: 16-11-2021

Nidhi Rawat
Department of Veterinary Microbiology, College of Veterinary Science & AH, Anjora, Durg, Chhattisgarh, India

SD Hirpurkar
Department of Veterinary Microbiology, College of Veterinary Science & AH, Anjora, Durg, Chhattisgarh, India

Nitin Gade
Department of Veterinary Physiology, College of Veterinary Science & AH, Anjora, Durg, Chhattisgarh, India

Jasmeet Singh
Centre for Wildlife Health and Forensic Science, DSVCKV, Durg, Chhattisgarh, India

C Sannat
Department of Veterinary Microbiology, College of Veterinary Science & AH, Anjora, Durg, Chhattisgarh, India

Sonali Prusty
Department of Animal Nutrition, College of Veterinary Science & AH, Anjora, Durg, Chhattisgarh, India

Corresponding Author
Nidhi Rawat
Department of Veterinary Microbiology, College of Veterinary Science & AH, Anjora, Durg, Chhattisgarh, India

Isolation and identification of *Pseudomonas aeruginosa* from clinical cases of bovine mastitis in and around Durg District, Chhattisgarh, India

Nidhi Rawat, SD Hirpurkar, Nitin Gade, Jasmeet Singh, C Sannat and Sonali Prusty

Abstract
Pseudomonas aeruginosa is gram-negative bacilli, known to be one of the causative micro-organisms of mastitis in dairy cattle. A study was conducted to assess the cases of clinical mastitis in dairy cattle associated with the *Pseudomonas aeruginosa* infection in and around Durg district of Chhattisgarh. A total of 72 milk samples from clinical mastitis cases of cattle were collected after screening by Californian mastitis test. Isolation of *Pseudomonas aeruginosa* was performed on Pseudomonas agar selective media. Seventeen bacterial isolates showing the blue-green or brown pigmentation were presumptively identified as *Pseudomonas aeruginosa* which were further subjected to Grams staining and biochemical tests. Molecular confirmation for *Pseudomonas aeruginosa* was carried out using species specific 16S rRNA gene which revealed 14 confirmed positive isolates. Further, lecithinase activity, gelatinase production, haemolysis and DNase test were also conducted to study the virulence characteristics of confirmed *Pseudomonas aeruginosa* isolates. This study concludes that *Pseudomonas aeruginosa* is one of the causative agents commonly reported in clinical mastitis.

Keywords: Clinical mastitis, molecular characterization, *Pseudomonas aeruginosa*, virulence

Introduction
Mastitis is disease of major concern among dairy farmers owing to huge economic loss as well as adverse effects on animal health and quality of milk. Annual economic loss due to bovine mastitis was reported in year 2009 to be around Rs. 7165.51 crores per annum (Reshi *et al.*, 2015) [17]. The major challenge is the analogous increase in incidences of mastitis with the development of high milk producing breeds of cows. *Pseudomonas aeruginosa* is one of the causative micro-organisms among multiple infectious bacterial etiological agents of bovine mastitis. Banerjee *et al.* (2017) [3] observed that approximately 5.4% cases of bovine subclinical mastitis infection in South Bengal were associated with *Pseudomonas aeruginosa*. It is generally considered to be a ubiquitous environmental bacterium, that is capable to establish opportunistic infections in animals, humans, and plants (Mathee *et al.*, 2008) [11]. Infection with *Pseudomonas* spp. has been found to be associated with contaminated water supplies in milking parlours, contaminated udder infusion vials, cannulas, or syringes and filthy environment (Moroni *et al.*, 2018) [13]. *Pseudomonas aeruginosa* expresses plethora of virulence determinants which are either cellular and/or extracellular components *viz.* pili, flagella, lipopolysaccharide, elastase, alkaline proteases, pyocyanin, pyoverdin, haemolysins, phospholipase C and rhamnolipids. Additionally, most of *Pseudomonas aeruginosa* cells form biofilms, leading to antibiotic resistance. Many workers have conducted study on isolation and identification of *Pseudomonas aeruginosa* with the aim to determine the prevalence rate of the infections associated with the bacteria (Barrasa *et al*, 2000; Szmolka *et al* 2012; Shahid Malik, 2004) [10, 23, 20]. In present study, isolation and identification of *Pseudomonas aeruginosa* from bovine mastitis milk was carried out to assess the cases of clinical mastitis in dairy cattle credited to the organism.

Materials and methods
Collection of samples
A total of 72 milk samples from dairy cattle showing signs of clinical mastitis were collected after screening by California Mastitis Test (Fig. 1) from various dairy farms in and around Durg district of Chhattisgarh. During sample collection, due precautions were taken to prevent

external contamination as far as possible. Samples were kept in insulated box and carried over ice pack and received at laboratory within 2hr of collection.

A Swollen udder, B Collection of Mastitic milk, C Positive California Mastitis Test

Fig 1: Sample collection from bovine clinical mastitis cases

Isolation of *Pseudomonas aeruginosa*

Every individual milk sample was opened carefully in a laminar air flow bench and loopful of sample was inoculated on CFC (Cephalothin-Sodium Fusidate-Cetrimide) broth base supplemented with CetriNix Supplement (cetrimide FD281) and incubated at 25ºC till visible growth appeared in the tubes. Then loopful of culture showing growth was streaked on *Pseudomonas* agar base supplemented with CetriNix supplement at 37°C for 24-48 hr for selective isolation of *Pseudomonas aeruginosa*. The sample showing no growth till 48 hour were again reinoculated in the fresh medium at least for two attempts before being adjudged as negative for the presence *Pseudomonas aeruginosa*.

Phenotypic and cultural characteristics on selective media

Cultural characteristics on other laboratory media viz. Nutrient agar, Blood agar and MacConkey Lactose agar were also recorded. Bacterial colonies showing the blue-green or brown pigmentation were presumptively identified as *Pseudomonas aeruginosa* isolates (Goto and Enomoto, 1970) [7]. The identified bacterial colonies were subjected to Gram staining and biochemical reactions for further confirmation.

Gram staining

Smear prepared from each of the isolate was stained by Gram staining and morphology of organism was recorded for phenotypic confirmation (Collins and Lyne, 1998) [4]. *Pseudomonas aeruginosa* appears as Gram negative pleomorphic bacilli.

Biochemical Tests

Further identification of each bacterial isolates was carried out based on biochemical characterization by performing pigmentation test, catalase test, citrate test, oxidase test, lecithinase activity on egg yolk agar, DNAse activity, indole test, methyl red test, Voges-Proskauer test as per procedure described by Collins and Lyne (1998) [4]. Interpretation was done based on standard biochemical characteristics of *Pseudomonas aeruginosa* ATCC 27853.

Genotypic characterization

The individual bacterial colonies presumptively identified based on cultural and biochemical and phenotypic characteristics were further processed for species identification by molecular tests *i.e*, PCR amplification of 16S rRNA using species specific primers (Spilker *et al.*, 2004) [21].

Extraction of Genomic DNA

Genomic DNA of phenotypically identified isolates were isolated using commercial bacterial genomic DNA isolation kit by following the manufacturer`s instructions (Qiagen, Germany). Bacterial genomic DNA of each isolate was used as template for PCR amplification of *Pseudomonas aeruginosa* specific 16S rRNA. PCR amplification reaction was optimized using positive and negative controls.

Quantification of DNA

DNA was quantified spectrophotometrically in Nano Drop and the concentration was expressed as ng/µl. Purity of DNA was determined from the ratio of absorbance at 260 and 280 nm. Ratio of A260/280 between 1.8 and 2.0 was considered as pure (Nicklas and Buel, 2003).

Polymerase Chain Reaction for 16S rRNA

PCR reactions were performed with a total volume of 25µl containing 12.5 µl PCR master mix (Dream Taq), 0.5 µl each of 0.5mM gene-specific forward and reverse primer, 1 µl of bacterial DNA and 10.5 µl nuclease free water. PCR amplification was performed in thermocycler. Initial denaturation was performed at 94˚C for 2 min followed by 25 amplification cycles including denaturation at 94˚C for 20 sec, annealing at 58˚C temperature for 20 sec and elongation at 72˚C for 40 sec. The PCR reaction was ended with a final elongation step at 72˚C.

Visualization of PCR product

Amplified products were electrophoresed on 1.5% agarose gel stained with ethidium bromide in 1X TBE buffer at 100 V for 5 min initially followed by 80V for 30-45min. Stained DNA bands were visualized and recorded using a Gel Documentation System. 16S rRNA gene amplified product was compared with 100bp DNA ladder.

Virulence determination of *Pseudomonas aeruginosa*

Detection of haemolysin

Pseudomonas aeruginosa isolates were inoculated on the on 5% sheep blood agar medium by streak plate method and incubated aerobically at 37ºC for 24hr and observed for zone of haemolysis. Subsequently, cold treatment was given at 4ºC in refrigerator for at least 4 hr. Based on zones of haemolysis,

results were interpreted as α-haemolysis (greenish zones), β-haemolysis (clear zone) or γ-haemolysis (no visible haemolysis), (Pramodhini *et al.,* 2016) [16].

Detection of phospholipase/ lecithinase

Pseudomonas aeruginosa isolates were inoculated on Modified egg yolk agar base supplemented with 10% egg yolk emulsion and incubated at 35-37ºC for 24-48hr for assessment of phospholipase activity. Appearance of a milky white opaque halo around the colonies indicated phospholipase C production (Mahmood and Aljobori, 2015) [9].

Detection of DNAse activity

Pseudomonas aeruginosa isolates were inoculated on DNAse test agar plates by making a single circular spot and incubating at 37°C for 24hr (Fazlul *et al.*, 2018) [5]. Then plates were flooded with 1N hydrochloric acid (HCl). A clear zone formation around bacterial colonies was recorded and interpreted as strong (++) and weak (+) DNAse positive. The colour of the medium remained unchanged, if the test is negative.

Detection of gelatinase

Gelatinase production was evaluated by inoculating the *Pseudomonas aeruginosa* colonies in tubes containing nutrient gelatin medium. The tubes were incubated at 37ºC for 48 hr. Liquefaction of the culture medium by placing the culture tube at 4°C overnight indicated gelatinase production by bacteria (Stehling *et al.*, 2008) [22].

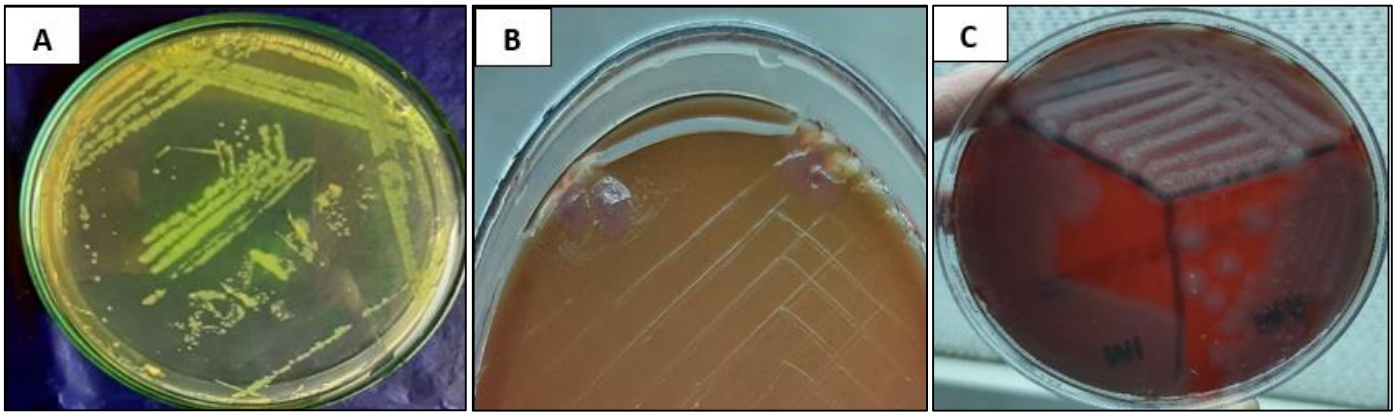
Results and Discussion

Incidence of *Pseudomonas aeruginosa* in mastitis milk

Out of total 72 mastitis milk samples screened for *Pseudomonas aeruginosa*, 17 (23.61%) isolates were confirmed positive phenotypically (Fig. 2). Several studies have reported prevalence rate of nearly 26% (Neamah, 2017) [14] and 27% (Scaccabarozzi *et al.,* 2015) [18]. Simultaneously contradictory reports of lower prevalence of *Pseudomonas aeruginosa* from milk samples has been reported as 0.61% (Yadav *et al.,* 2020) [26], 5.15% (Sekhri *et al.,* 2021) [19], 7.4 % (Ama *et al.,* 2016) [2] and 9.6% (Vasquez-Garcia *et al.,* 2017) from milk samples. The lower prevalence in these studies might be due to collection of milk samples from both healthy and clinical cases in those studies. The variable reporting of presence of *Pseudomonas aeruginosa* may be attributed to change in locality, hygienic measure and farm practices followed.

Banerjee *et al.* (2017) [3] also screened milk samples from different cattle farms and isolated *P Aurogenosa* using selective media, *viz.*, cetrimide and Pseudomonas agar to obtain circular mucoid smooth colonies with grape like odour and confirmed the presence of the bacteria by fluorescent technique.

Biochemical characterization of *P. aeruginosa* isolates revealed positive reaction for catalase, oxidase, lecithinase, DNAase test and negative for indole, methyl red and Voges-Proskauer test.

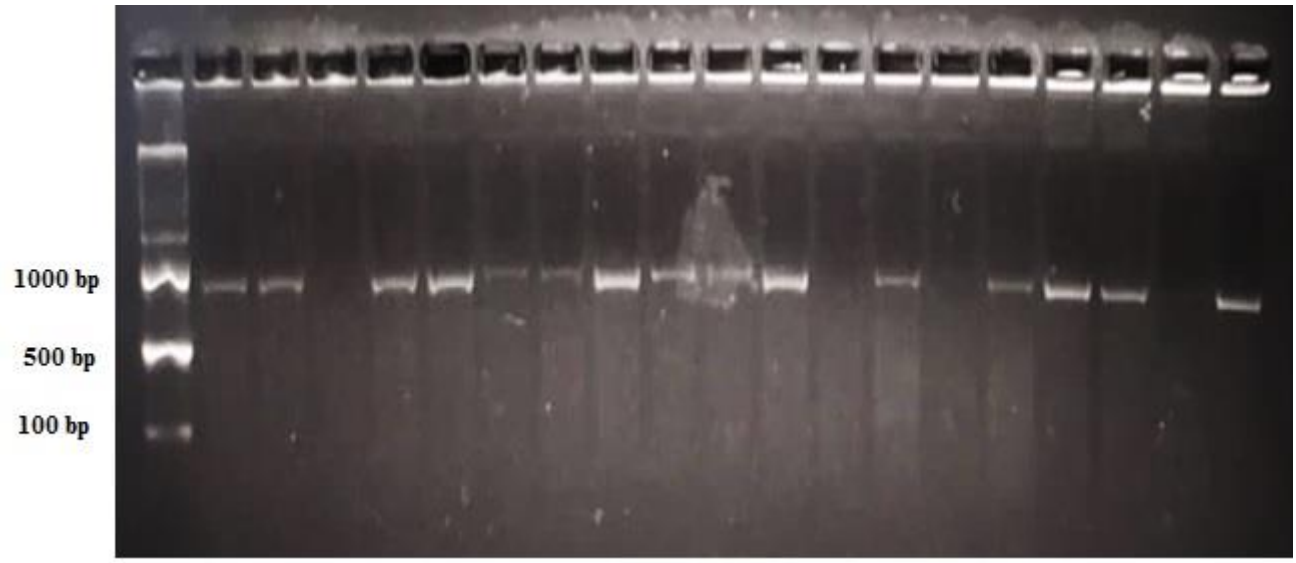


A. Green fluorescent colonies on Pseudomonas agar, B. Lactose non-fermenting colourless colonies on Mac Conkey Lactose Agar, C. Haemolytic colonies on Blood Agar

Fig 2: Selective isolation and culture of *Pseudomonas aeruginosa*

Genotypic characterization of *Pseudomonas aeruginosa*

Genotypic screening for species specific 16S rRNA gene by PCR assay of the 17 presumptively positive *Pseudomonas aeruginosa* isolates revealed 14 (82.35%) positive samples amplifying 16S rRNA gene of 956 bp by polymerase chain reaction (Fig. 3). Universal primers targeting conserved sequence of the 16S rRNA genes were used to specifically identify the ATCC reference strains and all the clinical isolates of *P. aeruginosa* (Tyler *et al.,* 1995). Our results indicate that PCR assay based on 16S rRNA gene provides rapid, simple, and reliable identification of *P. aeruginosa*. It can be easily employed for screening of large number of samples. Spilker *et al.* (2004) [21] have designed two PCR assays; one specific for the genus *Pseudomonas*, while the other specific for *Pseudomonas aeruginosa*. Both assays showed 100% sensitivity and specificity and thus could be used for specific identification of *Pseudomonas aeruginosa* from cultured isolates. High specificity and sensitivity of test for the intended target make it more useful for validation trials. Yadav *et al.* (2020) [26] screened 4378 mastitic cattle and buffalo to recover 27 *Pseudomonas aeruginosa* isolates and phenotypic identification was based on pigment production on nutrient agar and confirmed by PCR amplification of species-specific oligonucleotide sequencing. Molecular characterization is more precise than phenotypic methods in view of the variable phenotypic characters of *Pseudomonas aeruginosa* and extensively laborious and time taking biochemical tests.



A. Lane 1: 100 bp ladder Lane 2,3,5-12,14,16-18: 954bp amplified product, Lane 4,13,15: no amplification, Lane 19: Negative control, Lane 20: Positive control (ATCC27853 strain)

Fig 3: Polymerase chain reaction amplification of 16S RNA gene

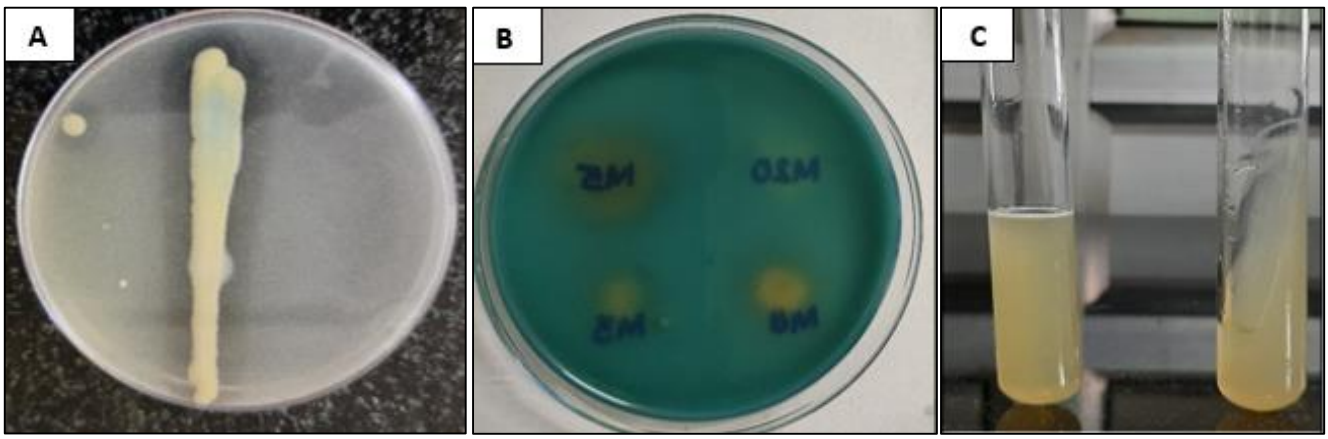
Virulence determinants in *Pseudomonas aeruginosa*

In the present study, nearly 85.71% of isolates exhibited hemolysis on blood agar and in majority cases there was complete haemolysis. Hemolysin is known to be important virulence factors of *P. aeruginosa* and hemolysin production in *Pseudomonas aeruginosa* infection is dependent on the isolates. It helps bacteria for bloodstream invasion, diffusion, and tissue damage after colonization. Ama *et al.* (2016) [2] have also reported β-hemolysis in 90% isolates of *P. aeruginosa* isolated from mastitis milk. The high percent of β-hemolytic isolates in present study indicates their virulence in clinical mastitis.

Among all the isolates from clinical mastitis milk specimens, 85.71% were positive for phospholipase C production (Fig. 4). Our results are in accordance with earlier report of 87% (Mohammad 2013 [12]) and 81% (Khalil *et al.,* 2015 [8]) of phospholipase production in clinical specimens from human with *Pseudomonas aeruginosa* infections. Phospholipase C and more specifically hemolytic phospholipase C targets eukaryotic membrane phospholipids and has been shown to participate in the pathogenesis of *Pseudomonas aeruginosa*. Existence of these virulent properties in majority of isolates indicates their importance in pathogenic strains. Pramodhini *et al.* (2016) also observed 71.4% positivity for phospholipase in isolates from clinical samples collected at tertiary care teaching hospital in Puducherry.

A total of 78.57% isolates from mastitis were positive for DNAse activity (Fig. 4). In contrast to our findings, Pramodhini *et al.* (2016) revealed DNAse activity in 44.8% of isolates from clinical samples while Finlayson and Brown (2011) reported DNAse positive isolates from wound swabs as 41%, sputum as 54% and ear swabs as 64% respectively.

In mastitis milk samples under study 92.86% of *Pseudomonas aeruginosa* isolates were positive for gelatinase activity (Fig. 6). Our results are in accordance with earlier report indicating 81.25% (Mohammad, 2013) and 78% (Khalil *et al.,* 2015 [8]) of gelatinase production in human *Pseudomonas aeruginosa* from various clinical specimens. In contrast to our findings, Fazlul *et al.* (2019) [5] reported gelatinase activity in only 31.66% of isolates.



A. Phopholipase C activity (Representative isolates showing opaque halo clear zone, B. DNAse activity (Representative isolates showing variable clear zones), C. Gelatinase activity (Representative isolates showing liquefaction at 4°C)

Fig 4: Virulence determinants of Pseudomonas aeruginosa

Conclusion

Pseudomonas aeruginosa is one of the potent microorganisms responsible for bovine mastitis and causes economic loss to dairy farmers. The molecular detection method proves to be

easy and rapid method for screening and confirmation of bacteria. Nearly all the clinical isolates exhibits virulence factors such as Lecithinase activity, DNAse activity, Gelatinase production and Hemolytic activity.

References

1. Abril D, Marquez-Ortiz RA, Castro-Cardozo B, Moncayo-Ortiz JI, Olarte Escobar NM, Corredor Rozo ZL, *et al.* Genome plasticity favours double chromosomal Tn4401b-bla KPC-2 transposon insertion in the *Pseudomonas aeruginosa* ST235 clone. BMC Microbiol. 2019;19:45.
2. Ama A, El-Shafii SSA, Elwahab AMO, El-dayim ZAA. Be detection of multidrug resistance genes in *Pseudomonas aeruginosa* isolated from bovine mastitis milk. J. Dairy. Vet. Anim. Res. 2016;3(2):43–49.
3. Banerjee S, Batabyal K, Joardar SN, Isore DP, Dey S, Samanta I, *et al.* Detection and characterization of pathogenic *Pseudomonas aeruginosa* from bovine subclinical mastitis in West Bengal, India. Vet. World. 2017;10(7):738-742.
4. Collins Lyne. Microbiological Methods edited by Collins CH, Lyne PM, Grange JM, Falkinham JO. 8th edition Oxford University Press Inc., New York, 1998.
5. Fazlul MKK, Najnin A, Farzana Y, Rashid MA, Deepthi S, Srikumar C, *et al.* Detection of virulence factors and β lactamase encoding genes among the clinical isolates of *Pseudomonas aeruginosa*. J. Inter. Pharm. Res. 2018;45:190-202.
6. Finlayson EA, Brown PD. Comparison of antibiotic resistance and virulence factors in pigmented and non-pigmented *Pseudomonas aeruginosa* West Indian Med. J. 2011;60: 24-32.
7. Goto S, Enomoto S. Nalidixic acid cetrimide agar. A new selective plating medium for the selective isolation of *Pseudomonas aeruginosa* JPN. J. Microbiol 1970;14(1):65-72.
8. Khalil MA, Ibrahim FS, Mohamed AF, Ali SS. Comparative study of virulence factors among ESBL-producing and nonproducing *Pseudomonas aeruginosa* clinical isolates. Turk. J. Med. Sci. 2015;45(1):60-69.
9. Mahmood AN, Aljobori AH. Isolation and identification of *Pseudomonas aeruginosa* from infected sheep and detection of phosolipase C (lecithinase) Iraqi J. Vet. Med. 2015;39(1):28-32.
10. Barrasa MJL, Gomez LP, Gonzalez LZ, Tejedor JMT. Antibacterial susceptibility patterns of *Pseudomonas* strains isolated from chronic canine otitis externa. J. Vet. Med., Series B. 2000;47:191–196.
11. Mathee K, Narasimhan G, Valdes C, Qiu X, Matewish JM, Koehrsen M, *et al.* Dynamics of Pseudomonas aeruginosa genome evolution. Proc. Natl. Acad. Sci. 2008;105:3100-3105.
12. Mohammad HH. Phenotypic investigation for virulence factors of Pyocine producing *Pseudomonas aeruginosa* isolated from burn wounds. Iraq. Int. J. Eng. Res. 2013;4(7):2114-2120.
13. Moroni P, Nydam D, Ospina P, Scillieri-Smith J, Virkler P, Watters R, *et al.* Diseases of the teats and udder. In: Rebhun's Diseases of Dairy Cattle (3rd Edition), 2018.
14. Neamah AA. Molecular Detection of virulence factor genes in *Pseudomonas aeruginosa* isolated from human and animals in Diwaniya province. Kufa J. Vet. Med. Sci. 2017;8(1):218-230.
15. Nicklas JA, Buel E. Quantification of DNA in forensic samples. Anal Bioanal Chem. 2003;376:1160-1167.
16. Pramodhini S, Umadevi S, Seetha KS. Detection of virulence determinants and its association with drug resistance in clinical isolates of *Pseudomonas aeruginosa* Int. J. Res Med Sci. 2016;4(9):3917-3923.
17. Reshi AA, Husain I, Bhat SA, Rehman MU, Razak R, Bilal S, *et al.* Bovine mastitis as an evolving disease and its impact on the dairy industry. Int. J. Curr. Res. Rev. 2015;7(5):48-55.
18. Scaccabarozzi L, Leoni L, Ballarini A, Barberio A, Locatelli C, Casula A, *et al. Pseudomonas aeruginosa* in dairy goats: genotypic and phenotypic comparison of intramammary and environmental isolates. PloS one. 2015;10(11): e0142973.
19. Sekhri I, Chandra M, Kaur G, Narang D, Gupta DK, Arora AK. Prevalence of *Pseudomonas aeruginosa* and other microorganisms from mastitis milk and their antimicrobial resistance pattern. Indian J Anim. Res. 2021;55:716-721.
20. Shahid M, Malik A. Plasmid mediated Amikacin resistance in clinical isolates of *Pseudomonas aeruginosa* Indian J. Med. Microbiol. 2004;22:182-184.
21. Spilker T, Coenye T, Vandamme P, LiPuma JJ. PCR-based assay for differentiation of *Pseudomonas aeruginosa* from other *Pseudomonas* species recovered from cystic fibrosis patients. J. Clinical Microbiol. 2004;42(5):2074-2079.
22. Stehling EG, Da Silveira WD, Da Silva DL. Characteristics of *Pseudomonas aeruginosa* strains isolated from patients with cystic fibrosis and from patients with extra-pulmonary infections. Brazilian J. Infectious Diseases. 2008;12(1):86-88.
23. Szmolka A, Cramer N, Nagy B. Comparative genomic analysis of bovine, environmental, and human strains of Pseudomonas aeruginosa. FEMS Microbiol. Lett. 2012;335:113-122.
24. Tyler SD, Strathdee CA, Rozee KR, Johnson WM. Oligonucleotide primers designed to differentiate pathogenic pseudomonads on the basis of the sequencing of genes coding for 16S-23S rRNA internal transcribed spacer. Clin. Diagn. Lab. Immun. 1995;2:448–453.
25. Vasquez-Garcia A, Silva TDS, Almeida-Queiroz SRD, Godoy SH, Fernandes AM, Sousa RL, *et al.* Species identification and antimicrobial susceptibility profile of bacteria causing subclinical mastitis in buffalo. Pes. Vet. Brasil. 2017;37(5):447-452.
26. Yadav R, Chhabra R, Shrinet G, Singh M. Isolation of *Pseudomonas aeruginosa* from bovine mastitic milk sample along with antibiogram study. J. Anim. Res. 2020;10(2):269-273.