Journal of Animal Research: v.10 n.2, p. 269-273. April 2020

DOI: 10.30954/2277-940X.02.2020.15

# Isolation of *Pseudomonas aeruginosa* from Bovine Mastitic Milk Sample Along with Antibiogram Study

**Rahul Yadav[1*], Rajesh Chhabra[1], Garima Shrinet[2] and Mahavir Singh[1]**

*[1]College Central Laboratory, LUVAS, Hisar, INDIA*
*[2]Department of Veterinary Microbiology, LUVAS, Hisar, INDIA*

**Corresponding author: R Yadav; E-mail: drrahul16889@gmail.com*

**Received:** 10 Jan., 2020 **Revised:** 16 March, 2020 **Accepted:** 23 March, 2020

**ABSTRACT**

A total of 4378 cattle and buffalo were screened during period of study (July 2018-June 19). Out of which 27 *Pseudomonas aeruginosa* were isolated. The isolates were confirmed phenotypically based on pigment production on nutrient agar. These were then confirmed by PCR amplification of species specific oligonucleotide sequences. All the 27 isolates amplified 956bp amplicon 16srRNA *Pseudomonas aeruginosa* species specific nucleotide sequence. The isolates were also checked for *exo* and *agl*D virulence associated genes. All of them amplified 540bp and 313bp amplicon of *exo* gene and *agl*D gene. All the isolates were subjected to antibiotic sensitivity testing. Most of the isolates showed highest sensitivity for levofloxacin, streptomycin and enrofloxacin followed by gentamicin, moxifloxacin and amikacin. Neomycin, cefoperazone and ceftriaxone were intermediate in action.

**Keywords:** Mastitis, cattle, buffalo, *Pseudomonas aeruginosa*, virulence genes, antibiogram

*Pseudomonas aeruginosa* is a gram's negative opportunistic environmental pathogen and can cause severe clinical diseases in both animals and humans. Prevalence of pseudomonas mastitis is only sporadic (<10% prevalence) as reported previously, but it may be a serious herd problem due to persistence of high antibiotic resistance strains (Livermore, 2002; Sharma and Sindhu, 2007). It is typically found in soil, water, skin flora and most man-made environments as it requires minimal amounts of oxygen for growth. Thereby allowing it to colonize a multitude of both natural and artificial environments (Hameed *et al*., 2007). Contaminated teat dips, teat wipes, and dry-cow intramammary treatment tubes have also been implicated in outbreaks of acute and subacute mastitis in dairy cows, sheep, and goats. Contaminated dry-cow intramammary infusion tubes have been found to be the cause of *P. aeruginosa* mastitis following calving in a dairy herd in Australia with the outbreak resulting in the loss of almost one-third of the herd (death or culling) (Kelly and Wilson, 2016). The presence of unsanitary housing and bedding conditions can contribute to occasional outbreaks of *P. aeruginosa* infections.

When the host defences are decreased by stress, concomitant disease, or by nutritional imbalances, *P. aeruginosa* efficiently attacks weak or injured tissues of teats or mammary gland causing acute mastitis with systemic signs as well as subclinical chronic mastitis. In severe cases, the affected gland may be necrotic or even gangrenous and the blood stained milk. Most *P. aeruginosa* strains possess the type III secretion system (TTSS), which may increase somatic cell counts (SCCs) in milk from mastitis-affected cows. Moreover, most of *P. aeruginosa* bacterial cells can form biofilms, thereby reducing antibiotic efficacy (Park *et al*., 2014). The pathogen uses a wide range of virulence factors, including proteins capable of inducing toxicity, which effectively damage tissues (Lyczak *et al*., 2000). Some of these toxic effector proteins are injected into the

**How to cite this article:** Yadav, R., Chhabra, R., Shrinet, G. and Singh, M. (2020). Isolation of *Pseudomonas aeruginosa* from bovine mastitic milk sample along with antibiogram study. *J. Anim. Res.,* **10**(2): 269-273.

cytosol of host cells through the type III secretion system (TTSS) which are involved in the disruption of epithelial surface and cell death. These TTSS proteins include cytotoxin (*Exo*U), ADP-ribosylating enzymes (*Exo*S and *Exo*T) and adenylcyclase (*Exo*Y). These cytotoxinsare involved in adhesion, phagocytosis and systemic spread of the bacterial cells together (Vance *et al*., 2005; Engel and Balachandran, 2009; Hauser, 2009).

Outer membrane of *P. aeruginosa* bacterium creates permeability barrier, resultant became naturally resistant to many antibiotics. Moreover, *Pseudomonas* maintains antibiotic resistance plasmids, R-factors and resistance transfer fragment (RTFs) and it is able to transfer these genes by horizontal gene transfer (HGT), mainly transduction and conjugation (Livermore, 2002; Kobayashi *et al*., 2013). This high level of multidrug resistant *P. aeruginosa* strains are major concern to dairy industries. Although, incidence of *P. aeruginosa* infection are reported very few (less than 3%) if udder is already infected by *Staphylococcus* or *Streptococcus* etc. pathogens, but mastitis remained incurable due to presence of multidrug resistance strains of *P. aeruginosa* (Ama *et al*., 2016). On keeping above facts in mind the present study was designed to isolates and identify virulent *P. aeruginosa* from mastitic milk of cattle and buffalo along with their antibiogram.

## MATERIALS AND METHODS

### Sample collection, isolation and identification

Mastitic milk samples of 4378 cattle and buffalo during year 2018-19 were processed for isolation and identification of *Pseudomonas aeruginosa* isolates. All the samples were plated onto Nutrient agar and incubated at $37^0$C for 24 h. The presumptive isolates were phenotypically characterized on the basis of colony morphology, catalase, oxidase, pigmentation and gram's staining.

### Molecular confirmation and virulence gene detection of *P. aeruginosa* isolates

Presumptive *Pseudomonas aeruginosa* isolates were confirmed by genus and species specific 16S rRNA nucleotide sequence (Spilker *et al*. 2004). The virulence associated genes were also detected molecularly by *exo*S and *agl*D nucleotide sequence as shown in table 1. The reaction mixture (total volume 25µl) was prepared by mixing 12.5µl GoTaq® Green Master Mix (Promega), 1µl Primer-1 and 2 each (10 pM/µl), 3µl Template DNA (25ng/µl) and 7.5µl Nuclease free water (upto final volume 25µl). PCR Amplification was carried out in 'Thermal cycler gradient (BR Gradient Thermal Cycler)' as follows: initial cycle of denaturation at 95 °C for 5min, 30 cycle at (denaturation at 95 °C for 45 s, primer annealing at 55 °C for 45 s and primer extension at 72 °C for 60 s), and final extension at 72 °C for 5 min. The PCR products were analyzed by electrophoresis on 1-1.5% agarose gel with ethidium bromide (0.5 µg/ml) in 1X TAE buffer for 60 min at 100 V. The gel was then visualized by Azure C-15 UVP gel documentation system.

### Determination of antibiotic resistant strains of *P. aeruginosa* isolates

The antibiotic sensitivity testing was carried out as per

**Table 1:** Primer sequence for PCR amplification

| Gene description | Target gene | 5'<-----Sequence----->3' | Amplicon size (bp) | Reference |
|---|---|---|---|---|
| *Pseudomonas* genus specific | 16S rRNA | F-GACGGGTGAGTAATGCCTA<br>R-CACTGGTGTTCCTTCCTATA | 618 | Spilker *et al*. (2004) |
| *P. aeruginosa* specific | 16S rRNA | F- GGGGGATCTTCGGACCTCA<br>R- TCCTTAGAGTGCCCACCCG | 956 | Spilker *et al*. (2004) |
| **Virulence associated genes of *P. aeruginosa*** | | | | |
| *P. aeruginosa* exoenzyme S gene | *exo*S | F- CTTGAAGGGACTCGACAAGG<br>R- TTCAGGTCCGCGTAGTGAAT | 504 | Bogiel*et al*. (2017) |
| *P. aeruginosa* glycosyl transferase | *agl*D | F- GGTCTGCCGCGAGATCGGCT<br>R- GACCTCGACGGTCTTGCGGA | 313 | Saadoon, and Zghair (2019) |

standard protocol describe by Kirby *et al*. (1966) against 16 different antibiotics. Briefly, the isolates were inoculated in sterile BHI broth (5 ml) and incubated for 18-24 h at 37°C. The opacity of 0.5 McFarland opacity standards (Quinn *et al*., 2002) was well spread over the Muller Hinton agar with the help of sterilized swab and after that antibiotic discs were carefully placed, then incubated for 24 h at 37°C. The zone of inhibition was measured for each disc and interpretation was done as per Clinical and Laboratory Standards Institute (CLSI) guidelines (Wayne, 2009). All Multidrug resistant (MDR) isolates were evaluated for their multiple antibiotic resistances (MAR) index (Krumperman, 1983).

## RESULTS AND DISCUSSION

*Pseudomonas aeruginosa* is becoming a threat to dairy animals causing mastitis by drug resistance strain, since it requires few nutrients to multiply. It is the most commonly isolated microorganisms from clinical specimens, usually responsible for nosocomial infections (Chattergee *et al*., 2016). In the present study, 27 (17 from buffalo and 10 from cattle origin) *Pseudomonas aeruginosa* were isolated from mastitic milk samples of 4378 cattle and buffalo during year 2018-19. All the isolates were catalase and oxidase positive and produce green pigmentation on nutrient agar plate. Further, molecular confirmation was done by PCR amplification of genus and species specific oligonucleotide sequences (Fig. 1). All 27 isolates amplified 618bp amplicon and 956 bp amplicon of 16S rRNA *Pseudomonas* genus specific and 16S rRNA *P. aeruginosa* species specific nucleotide sequence. The prevalence of *P. aeruginosa* was detected as 0.61% in the present study. In agreement to our study, Nam *et al*. (2009) reported 5.6% prevalence of *P. aeruginosa* from bovine mastitis between 2003 and 2008 in Korea and Vasquez-Garcia *et al*. (2017) detected 9.6% prevalence of *P. aeruginosa* from bovine in Brazil. Banerjee *et al*. (2017) also detected 5.4% cases of bovine subclinical mastitis in South Bengal, India were associated with *P. aeruginosa*. Contrarily, Scaccabarozzi *et al*. (2015) detected high prevalence (nearly 27%) of *P. aeruginosa* as causative agent of mastitis in goats.

All the 27 *P. aeruginosa* isolates amplified 313bp amplicon of *agl*D virulence associated genes and 540bp amplicon of exoenzyme, *exo*S gene which helps in invasion. *exo*S and *exo*T are two homologous bifunctional Type III Secretion System (T3SS) virulence factors that induce apoptosis in target host cells (Kaminski *et al*. 2018).

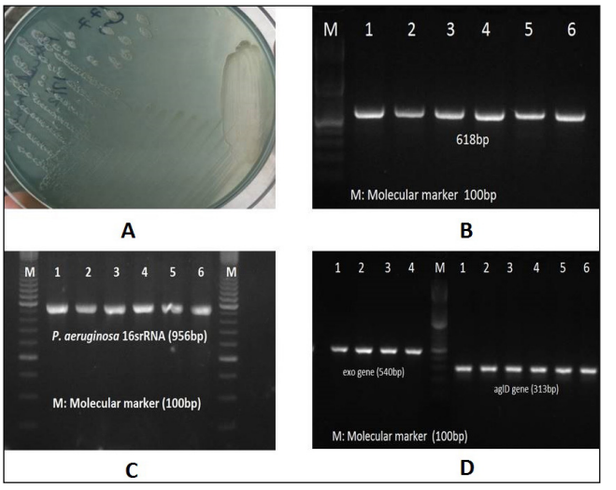


**Fig. 1:** Growth of *P. aeruginosa* on nutrient agar plate, PCR amplification of 16SrRNA genus specific (B), 16SrRNA *P. aeruginosa* specific specific (C) and virulence genes *exoS* and aglD gene (D)sequences

Similar to our observation Park *et al*. (2014) also detected TTSS-related genes (*exo*U and/or *exo*S genes) in 82.7% of the isolates including the invasive (*exo*U-/*exo*S+, 69.4%), cytotoxic (*exo*U+/*exo*S-, 8.3%) and cytotoxic/ invasive strains (*exo*U+/ *exo*S+, 5.0%). The *agl*D gene of *P. aeruginosa* is responsible for synthesis of the capsular polysaccharide alginate, an important virulence factor expressed in cystic fibrosis (CF) cases (Baynham *et al*. 1996). Similar to present study, Taee *et al*. (2014) 98% detection of *agl*D gene by PCR amplification from *P. aeruginosa* of clinical isolates.

All the isolates were tested against 16 antibiotics for antibiotic sensitivity. All the isolates showed absolute resistant to chloramphenicol, penicillin-G, cloxacillin, tetracycline, amoxycillin, kanamycin and ampicillin. The isolates were most sensitive to levofloxacin, streptomycin, enrofloxacin followed by gentamicin, moxifloxacin and amikacin (Fig. 2). Neomycin, cefoperazone and ceftriaxone were intermediate in action. Standard antibiotic regimes against *P. aeruginosa* are increasingly becoming ineffective due to the rise in drug resistance (Chattergee *et al*., 2016). Park *et al*. (2014) detected efficiency of antibiotic therapy against *P. aeruginosa* related bovine

mastitis and found that majority of isolates were sensitive to gentamicin, amikacin, meropenem and ciprofloxacin. They also suggested that determination of efficacy could also be improved by MIC analysis of the isolates.

In contrast to our observation, Smith *et al*. (2012) reported that all the 20 *P. aeruginosa* isolates from cystic fibrosis cases (humans) showed resistance to gentamicin (80%) and ciprofloxacin (70%). However, detection of resistance towards amoxycillin (100%), tetracycline (95%), augmentin (95%), ofloxacin (80%), nalidixic Acid (100%), was similar to observations in the present study. Imipenem was the drug of choice for treatment of cystic fibrosis previously but increasing trends in imipenem resistance leaves fluoroquinolones only in terms of susceptibility. The cephalosporins tested were showing emergence of resistance due to indiscriminate use.

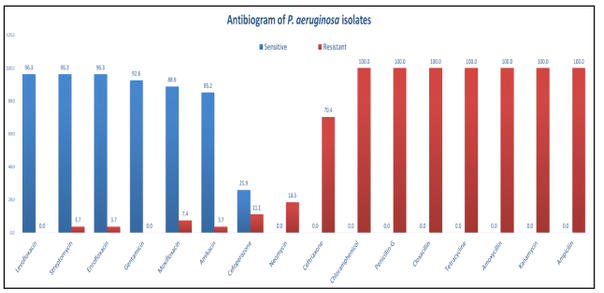


**Fig. 2:** Graphical representation of antibiotic sensitivity and resistance patterns of *P. aeruginosa* isolates

## CONCLUSION

From this study, majority of isolates were highly pathogenic on the basis of detection of *exo*S and *agl*D virulence genes and also resistant to majority of antibiotics (eight antibiotics) signifying constant antibiotic screening must be done before mastitis treatment for *Pseudomonas aeruginosa* infections.

## CONFLICT OF INTEREST

Authors don't have any conflict of interests.

## REFERENCES

Ama, A., El-Shafii, S.S.A., Elwahab A.M.O. and El-dayim, Z.A.A. 2016. Be detection of multidrug resistance genes in *Pseudomonas aeruginosa* isolated from bovine mastitic milk. *J. Dairy. Vet. Anim. Res.,* **3**(2): 43–49.

Banerjee, S., Batabyal, K., Joardar, S.N., Isore, D.P., Dey, S., Samanta, I., Samanta, T.K. and Murmu, S. 2017. Detection and characterization of pathogenic *Pseudomonas aeruginosa* from bovine subclinical mastitis in West Bengal, India. *Vet. World.,* **10**(7): 738.

Baynham, P.J. and Wozniak, D.J. 1996. Identification and characterization of AlgZ, an AlgT-dependent DNA-binding protein required for *Pseudomonas aeruginosa* algD transcription. *Mol. Microbiol.,* **22**(1): 97-108.

Bogiel, T., Deptula, A., Kwiecinska-pirog, J.O.A.N.N.A., Prażynska, M., Mikucka, A. and Gospodarek-Komkowska, E. 2017. The prevalence of exoenzyme S gene in multidrug-sensitive and multidrug-resistant *Pseudomonas aeruginosa* clinical strains. *Polish J. Microbial.,* **66**(4): 427-431.

Chatterjee, M., Anju, C.P., Biswas, L., Kumar, V.A., Mohan, C.G. and Biswas, R. 2016. Antibiotic resistance in *Pseudomonas aeruginosa* and alternative therapeutic options. *Int. J. Med. Microbiol.,* **306**(1): 48-58.

Engel, J. and Balachandran, P. 2009. Role of *Pseudomonas aeruginosa* type III effectors in disease. *Curr. Opin. Microbiol.,* **12**: 61–66.

Hameed, K.G.A., Sender, G. and Korwin-Kossakowska, A. 2007. Public health hazard due to mastitis in dairy cows. *Ani. Sci. Paper. Reports.,* **25**(2): 73-85.

Hauser, A.R. 2009. The type III secretion system of *Pseudomonas aeruginosa*: infection by injection. *Nature. Rev. Microbiol.,* **7**(9): 654.

Kaminski, A., Gupta, K.H., Goldufsky, J.W., Lee, H.W., Gupta, V. and Shafikhani, S.H. 2018. *Pseudomonas aeruginosa ExoS* induces intrinsic apoptosis in target host cells in a manner that is dependent on its GAP domain activity. *Sci. reports.,* **8**(1): 14047.

Kelly, E.J. and Wilson, D.J. 2016. *Pseudomonas aeruginosa* mastitis in two goats associated with an essential oil–based teat dip. *J. Vet. Diag. Invest.,* **28**(6):760-762.

Kirby, W.M., Bauer, A.W., Sherris, J.C. and Turck, M. 1966. Antibiotic susceptibility testing by a standardized single disk method. *Am. J. Clin. Pathol.,* **45**(4): 493-496.

Kobayashi, H., Isozaki, M., Fukuda, T., Anzai, Y. and Kato, F. 2013. Surveillance of fluoroquinolone-resistant clinical isolates of *Pseudomonas aeruginosa*. *Open J. Med. Microbiol.,* **3**(02): 144.

Krumperman, P.H. 1983. Multiple antibiotic resistance indexing of *Escherichia coli* to identify high-risk sources of fecal contamination of foods. *Appl. Environ. Microbiol.,* **46**(1): 165-170.

Livermore, D.M. 2002. Multiple mechanisms of antimicrobial resistance in *Pseudomonas aeruginosa*: our worst nightmare? *Clin. Infect. Dis.,* **34**(5): 634-40.

Lyczak, J.B., Cannon, C.L. and Pier, G.B. 2000. Establishment of *Pseudomonas aeruginosa* infection: lessons from a versatile opportunist. *Microbes. Infect.,* **2**: 1051–1060.

Nam, H.M., Lim, S.K., Kang, H.M., Kim, J.M., Moon, J.S., Jang, K.C., Joo, Y.S. and Jung, S.C. 2009. Prevalence and antimicrobial susceptibility of gram-negative bacteria isolated from bovine mastitis between 2003 and 2008 in Korea. *J. dairy. Sci.,* **92**(5): 2020-2026.

Park, H.R., Hong, M.K., Hwang, S.Y., Park, Y.K., Kwon, K.H., Yoon, J.W., Shin, S., Kim, J.H. and Park, Y.H. 2014. Characterisation of *Pseudomonas aeruginosa* related to bovine mastitis. *Acta. Vet. Hung.,* **62**(1): 1-12.

Quinn, P.J., Markey, B.K. and Carter, M.E. 2002. Veterinary microbiology and microbial disease. Ames, Iowa: Iowa State University Press.

Saadoon, Z.S. and Zghair, Z.R. 2019. Molecular Detection of *Pseudomonas aeruginosa* by Using Algd, Plch and Lasb Genes and Pathological Study of the Virulent Isolate from Human Blood. *Plant. Archives.,* **19**(2): 1633-1639.

Scaccabarozzi, L., Leoni, L., Ballarini, A., Barberio, A., Locatelli, C., Casula, A., Bronzo, V., Pisoni, G., Jousson, O., Morandi, S. and Rapetti, L. 2015. *Pseudomonas aeruginosa* in dairy goats: genotypic and phenotypic comparison of intramammary and environmental isolates. *PloS one*., **10**(11): p.e0142973.

Sharma, A. and Sindhu, N. 2007. Occurrence of clinical and subclinical mastitis in buffaloes in the State of Haryana (India). *Ital. J. Ani. Sci.,* **6**: 965-967.

Smith, S., Ganiyu, O., John, R., Fowora, M., Akinsinde, K. and Odeigah, P. 2012. Antimicrobial Resistance and Molecular Typing of *Pseudomonas aeruginosa* Isolated from Surgical Wounds in Lagos, Nigeria. *Acta. Med. Iran.,* **50**(6): 433-438.

Spilker, T., Coenye, T., Vandamme, P. and LiPuma, J.J. 2004. PCR-based assay for differentiation of *Pseudomonas aeruginosa* from other Pseudomonas species recovered from cystic fibrosis patients. *J. Clin. Microbiol.,* **42**(5): 2074-2079.

Taee, S.R., Khansarinejad, B., Abtahi, H., Najafimosleh, M. and Ghaznavi-Rad, E. 2014. Detection of *algD*, *oprL* and *exoA* genes by new specific primers as an efficient, rapid and accurate procedure for direct diagnosis of *Pseudomonas aeruginosa* strains in clinical samples. *Jundishapur. J. Microbiol.,* **7**(10): e13583.

Vance, R.E., Rietsch, A. and Mekalanos, J.J. 2005. Role of the type III secreted exoenzymes S, T, and Y in systemic spread of *Pseudomonas aeruginosa* PAO1 *in vivo*. *Infect. Immun.,* **73**: 1706–1713.

Vasquez-Garcia, A., Silva, T.D.S., Almeida-Queiroz, S.R.D., Godoy, S.H., Fernandes, A.M., Sousa, R.L. and Franzolin, R. 2017. Species identification and antimicrobial susceptibility profile of bacteria causing subclinical mastitis in buffalo. *Pes. Vet. Brasil.,* **37**(5): 447-452.

Wayne, P. 2009. Clinical and Laboratory Standards Institute (CLSI) performance standards for antimicrobial disk diffusion susceptibility tests 19th ed. approved standard. CLSI document M100-S19, 29 (2011), M100-S21.