Virulence Characterization of *exo-S*, *exo-U* and *algD* Genes and Antibiogram Study of *Pseudomonas aeruginosa* Isolated from Goat Mastitis Milk in India

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ABSTRACT

The present study was conducted to investigate the multiple-drug resistance potential *vis-a-vis* virulence characterization of *Pseudomonas aeruginosa,* isolated from goat mastitis milk samples and also to elucidate the threat posed by *P. aeruginosa,* an important opportunistic pathogen, to public health. A total of 50 mastititic milk samples were collected aseptically from the affected quarter(s) of goats and enriched in Malachite Green Broth and plated on Nutrient Agar and MacConkey Agar. Suspected colonies were confirmed by species-specific PCR. Out of 50 samples, 18 isolates of *P. aeruginosa* were obtained (36% isolation rate). Subsequently, all the isolates were subjected to antibiotic sensitivity against 13 antibiotics of different classes. Isolates showed maximum susceptibility to Piperacillin-Tazobactam (100%), Meropenam (100%) and colistin (100%) followed by Gentamicin and Levofloxacin (88.8%). Maximum resistance was found for Cefazolin (88.8%) followed by Kanamycin (66.6%). Further, isolates were investigated for presence of three virulence genes viz. *exo-S*, *exo-U* and *algD* gene. Mutually exclusive virulence genes e*xo-S* and *exo-U* were detected in 12 and 6 isolates respectively whereas biofilm associated *algD* gene was found in all 18 isolates. The study revealed a high occurrence of virulent and multidrug resistant *P. aeruginosa* from goat milk, which can lead to an intractable mastitic condition. The study concludes that goat milk consumed by people without proper knowledge, hygiene and pasteurization can have serious outcomes, as the bacteria is known to posses various antibiotic resistant determinants which can be transferred to other microbial flora of humans.

Keywords: *Pseudomonas aeruginosa*; Mastitis; Antibiogram; *exo S*; *exo U*; *algD*; Biofilm.

INTRODUCTION

Pseudomonas aeruginosa is a well-known pathogen in humans causing a number of nosocomial infections (1). In animals, it can also cause a number of infections including otitis and urinary tract infections in dogs, mastitis, endometritis in horses and hemorrhagic pneumoniae in fur-bearing animals such as mink or foxes (2-4). Mastitis is an important concern particularly in caprine population which involve severe clinical mastitis with systemic involvement and subclinical chronic mastitis (5, 6) and in extreme cases necrotic or even gangrenous mastitis (7). Available literature suggests very limited work has been carried to study the occurrence and evaluate the antibiotic resistance profile of *P. aeruginosa* from mastitis cases, especially from small ruminants like goats, which can pose serious threat to the human population (8, 9).

One of the unique features of *P. aeruginosa* is its ability

to produce thick biofilms inside host tissue along with other bacteria like *Staphylococcus aureus*, which lead to complicated mastitis. Such cases are difficult to treat as thick biofilm impedes the penetration of antibiotics to the target site. *P. aeruginosa* makes biofilms by producing three distinct exopolysaccharides: alginate, PEL and PSL (10). Alginate is the major component of the exopolysaccharide of *P. aeruginosa*. Moreover, *Pseudomonas* possesses antibiotic resistance plasmids, R-factors and resistance transfer fragment (RTFs), and it can transfer these genes by horizontal gene transfer (HGT), transduction and conjugation (11, 12).

The bacterium is well-known for the production of many exo-enzymes which confers virulence to the bacterium. So far, four effector toxins of *P. aeruginosa* have been identified: ExoS, ExoT, ExoU and ExoY (13). These four effector toxins are rarely all present in one strain. More commonly, strains have either the *exoS* or the *exoU* gene (14). ExoU is a potent cytotoxin–present in cytotoxic *P. aeruginosa* strains–with phospholipase A_2 activity, capable of inducing rapid necrotic cytotoxicity in various eukaryotic cells (15) while ExoS is present in invasive *P. aeruginosa* strains, which works to rearrange the actin cytoskeleton and induce apoptosis in target host cells (16).

P. aeruginosa possess various antibiotic resistant determinants genes owing to high chromosomal mutation or horizontal transmission. The bacterium is found in a wide range of ecological niches where it can interact with various bacterial species and can pass these resistance determinants on to them. This has lead to the emergence of multidrug resistant strains worldwide. The main challenges faced with the emergence of antibiotic resistance are difficulty of treatment, severity of infection and increased mortality rates (17). Moreover, the bacteria and their genes can be transmitted to humans through consumption of non-pasteurized milk, wild animals, contaminated waterways and food chain (18).

MATERIALS AND METHODS

Sample collection and Isolation

A total of 50 milk samples were collected from goats over a period of one year from different marginal farms in Bikaner, Rajasthan, India. Proper physical examination of both udder and teats was done by visual observation and manual palpation. The animals showed variable degree inflammation of the udder with the consistency of milk varying from normal to watery and in few animals, flakes were present in the milk. Milk samples were collected from does with clinical and sub-clinical mastitis. The udder was thoroughly disinfected with 70% ethanol before collection of milk sample. The milk sample from both udder was collected in one sterile bottle after discarding the first 3-4 streams of milk. The samples were immediately brought to the laboratory on ice for further processing. All the samples were enriched in Malachite Green Broth (Himedia, Mumbai India) and plating was done on Nutrient Agar and MacConkey Agar (Himedia, Mumbai, India). Presumptive colonies were cultured on Cetrimide Agar (Himedia, Mumbai, India) to study colony characteristics and pigment production.

Biochemical characterization

A set of 14 biochemical tests were conducted in the study. Out of the 14 biochemical tests, 12 tests viz. ortho-Nitrophenyl-β-galactoside (ONPG), Lysine utilization, Ornithine decarboxylase, Urease, Phenylalanine deaminase, Nitrate reduction, Esculin Hydrolysis, H_2S production, Citrate Utilisation, Voges Proskaeur, Methyl red, indole, Malonate test were done on Hi Enterobacteriaceae Kit (Himedia, Mumbai, India). Aesculin hydrolysis test was done in Esculin broth (Himedia, Mumbai, India) and Oxidase test was conducted on Oxidase discs (Himedia, Mumbai, India).

Pellicle formation assay

Pellicle formation was checked on the walls of test tube. Strains of *P. aeruginosa* were inoculated in Luria Bertini broth (Himedia, Mumbai, India) supplemented with 1% glucose and inoculated at 37°C for 24hr. A thin pellicle formed at the air-liquid interface visible to the naked eye was considered as biofilm producers.

Molecular identification

The suspected colonies were confirmed by *16SrRNA* species-specific PCR using protocol of Spilker *et al* (19). The DNA extraction was made by taking two to three purified colonies in 500 µl of sterile nuclease free water in 1ml eppendorff. The eppendorff tubes were heated at 100°C for 15 min and chilled rapidly. The supernatant was used as template. Primers used were in the study included *16SrRNA*(F) – 5' GGGGGATCTTCGGACCTCA 3' and *16SrRNA* (R) 5' TCCTTAGAGTGCCCACCCG 3'.

Table 1: List of Antibiotic discs used in the study with concentration and cut-off values for *P. aeruginosa:*

Class of Antimicrobial	Antibiotic	Concentration	Inhibition Zone Cut-off		
			Sensitive	Intermediate	Resistant
Quinolone	Nalidixic acid	30 µg	≥19	14-18	≤13
Flouroquinolones	Levofloxacin	5 µg	≥17	14-16	≤13
	Gemifloxacin	5 µg	≥20	16-19	≤15
Aminoglycosides	Gentamicin	10 µg	≥15	13-14	≤12
	Amikacin	30 µg	≥17	15-16	≤14
	Kanamycin	30 µg	≥18	14-17	≤13
Cephalosporins	Cefazolin	30 µg	≥23	20-22	≤19
Semi-synthetic Penicillin	Ampicillin	10 µg	≥17	14-16	≤13
Combination Penicillin	Piperacillin-tazobactum	100/10 µg	≥21	18-20	≤17
Carbapenems	Meropenam	10 µg	≥19	16-18	≤15
Polypeptides	Colistin	10 µg	≥17	12-16	≤11
Carboxypenicillin	Ticarcillin	75 µg	≥18	–	≤18
Anti-folate	Trimethoprim	5 µg	≥16	11-15	≤10

Virulence characterization

Molecular detection of virulence associated genes (viz *exo-S* and *exo-U)* and biofilm associated *algD* gene was carried out using previously established protocols of Shaver and Hauser (20) with the following primer sequences:

Gene	Primer sequence 5'-3'	Product size (bp)
exo-S	Forward: CTT GAA GGG ACT CGA CAA GG	504
	Reverse: TAA GTG ATG CGC CTG GAC	
exoU	Forward: GCA GCC TAT CGT GCA AG	1500
	Reverse: GCG TGC AGT GAT TGC GA	
algD	Forward: GGT CTG CCG CGA GAT CGG CT	313
	Reverse: GAC CTC GAC GGT CTT GCG GA	

Antibiogram

All confirmed isolates were subjected to *in vitro* antibiotic susceptibility testing against 13 different antibiotics (Table 1), using Disk diffusion method (21) on Mueller Hinton Agar (Himedia, Mumbai, India), in accordance with the recommended procedures of Clinical Laboratory Standard Institute protocol. The inhibition zones were interpreted for SIR classification using CLSI guidelines (CLSI, 2019).

RESULTS AND DISCUSSION

The isolation frequency of *P. aeruginosa* was 36% (n=18) in the present study. *P. aeruginosa* produced large, mucoid, opaque colonies with irregular margins, had sweet grape odor in the nutrient agar. On Cetrimide Agar, *P. aeruginosa* produced blue-green colonies due to pigment production (Figure 1), but did not ferment lactose sugar on MacConkey agar. Although, mastitis associated with *P. aeruginosa* is occasionally been described in small ruminants (22, 23) and cows (24) but it is an important opportunistic pathogen, which can complicate mastitis and also pose threat to public health. Different researchers have reported variable frequencies of isolation of this bacterium from mastitic milk. The recovery

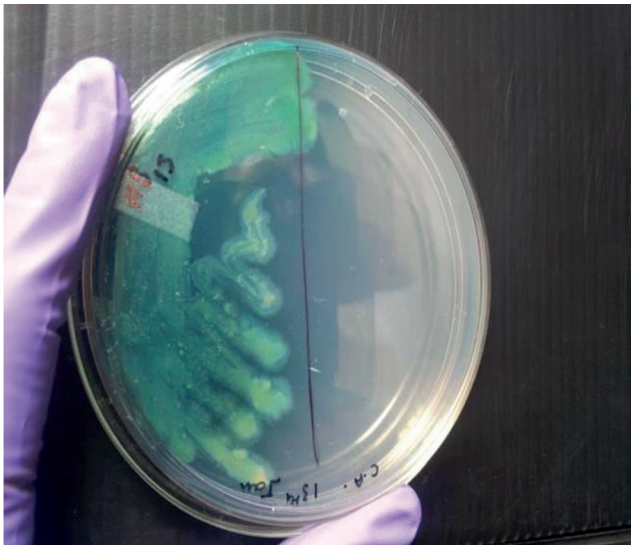

Figure 1: Pigment production on Cetrimide agar

Table 2: Biochemical test results

Biochemical Test	Positive	Negative
ONPG	8	10
Lysine utilization	0	18
Ornithine	12	6
Urease	8	10
Phenylalanine	0	18
Nitrate	8	10
Esculin Hydrolysis	6	12
H2S production	18	0
Citrate Utilization	18	0
VP test	0	18
MR test	0	18
Indole	0	18
Malonate	18	0
Oxidase	18	0

Table 3: Antibiotic-disc susceptibility test result for 13 antimicrobial agents tested for the 18 *P. aeruginosa* isolates:

Class of Antimicrobial	Antibiotic	Sensitive isolates%	Resistant isolates%
Quinolone	Nalidixic acid	66.7%	33.3%
Flouroquinolones	Levofloxacin	88.8%	11.1%
	Gemifloxacin	88.8%	11.1%
Aminoglycosides	Gentamicin	88.8%	11.1%
	Amikacin	77.7%	22.23%
	Kanamycin	33.4%	66.6%
B-lactams (Penicillins & Cephalosporins)	Cefazolin	11.2%	88.8%
Semi-synthetic Penicillin	Ampicillin	11.2%	88.8%
Combination Penicillin	Piperacillin-tazobactum	100%	0%
Carbapenems	Meropenam	100%	0%
Polypeptides	Colistin	100%	0%
Carboxypenicillin	Ticarcillin	100%	0%
Anti-folate	Trimethoprim	11.2%	88.8%

rate of *P. aeruginosa* was on higher side (40% and 42%) from goat mastitis in a study conducted by Sori *et al* (25) and Ebrahimi and Taheri (26) respectively. However, Codkin and Leises reported an isolation rate of 7.9% (27) and in other study by Estein and Denis (28) the rate of isolation was as low as 1%.

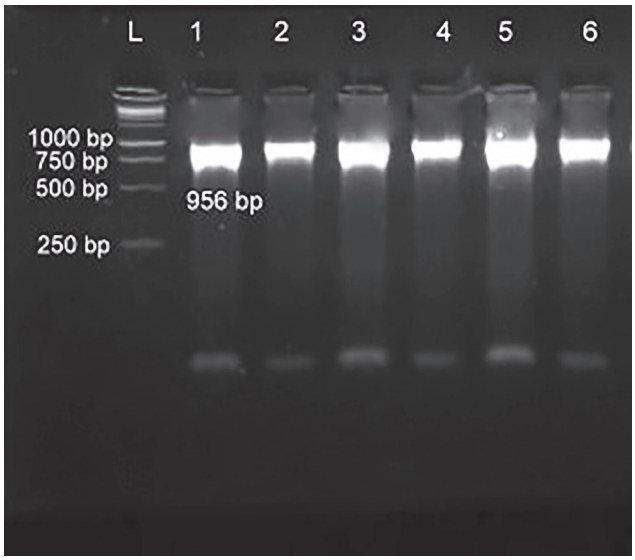

Figure 2: Gel Image of PCR product showing species-specific 956 bp amplified product. Lane L: 250 bp ladder, L1: Positive control, L2 to L6: isolates showing PCR amplified species-specific product of 956 bp.

These differences may be attributed to hygienic measures where the bacterium has been detected in contaminated wash hoses in milking parlors, in water, spray nozzle and in contaminated antibiotic preparations.

Biochemical characteristics of all isolates is shown in table 2 which revealed that all isolates were oxidase positive and gave IMViC pattern as -/-/-/+ . A total of eight positive isolates reduced nitrate. Interestingly, six isolates were found positive for Esculin hydrolysis which has been rarely reported and indicates presence of different biovars among the isolates (29).

All isolates produced H_2S, which is also a novel finding. All isolates produced thick biofilm on walls of test tubes known as pellicle. Some recent studies have reported that phenotypic resistance associated to biofilm formation or to the emergence of small-colony variants, may be important in the antibiotic treatment response of *P. aeruginosa* populations (30).

All the 18 isolates were confirmed molecularly by PCR amplification product of 956 bp specific for *P. aeruginosa* using species-specific PCR (Figure 2).

The detailed antibiogram results of 18 isolates of *P. aeruginosa* are presented in the Table 3. Resistance against β-lactam antibiotics viz. cefazolin and ampicillin was most prevalent. This resistance may be attributed due to produc-

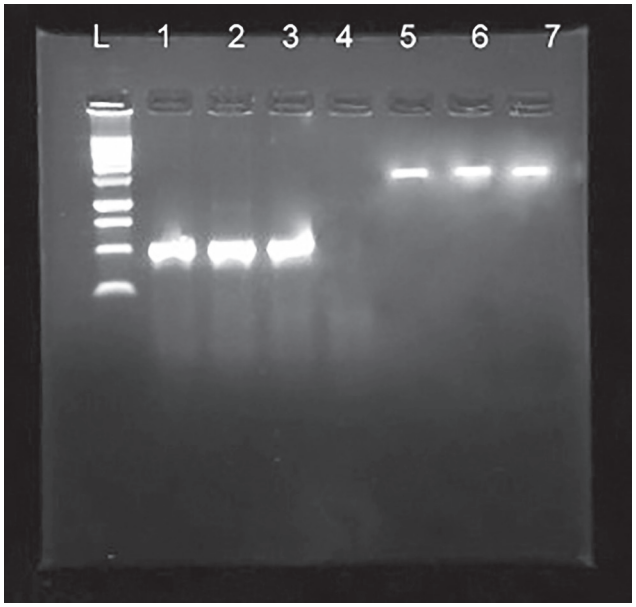

Figure 3: Gel image of PCR showing amplification products of 504bp and 1500bp confirming *exoS* and *exoU* genes respectively. Lane L: 250 bp ladder. Lane 1 to 3: Isolates positive for *exoS*. Lane 5 to 6: isolates positive for *exoU*

tion of extended-spectrum-β-lactamases (ESBLs) which confer a high degree of resistance to broad spectrum β-lactam antibiotics, cephalosporins (31). When a combination of piperacillin and tazobactum was used, all isolates were found susceptible as tazobactum acts as an inhibitor of the action of bacterial beta-lactamases.

Among aminoglycoside group, maximum resistance (66.6%) was found against kanamycin. Aminoglycoside resistance in *P. aeruginosa* can be due to multiple factors, such as reduced cell membrane permeability, increased efflux, ribosomal changes and enzyme modification. Among these mechanisms, the enzymatic modification of amino and glycoside groups in the aminoglycoside molecular structure plays a predominant role in resistance to this class of antibiotics (32). Three types of aminoglycoside-modifying enzymes have been discovered in bacteria: aminoglycoside phosphotransferase (APH), aminoglycoside acetyltransferase (AAC) and aminoglycoside nucleotidyltransferase (ANT) (33). *P. aeruginosa* APHs have been found to transfer a phosphoryl group to the 3'-hydroxyl of aminoglycosides such as kanamycin, neomycin and streptomycin, thereby inactivating these antibiotics (34).

A total of 88.8% isolates were also found resistant to trimethoprim, a synthetic anti-folate antibiotic. The reason

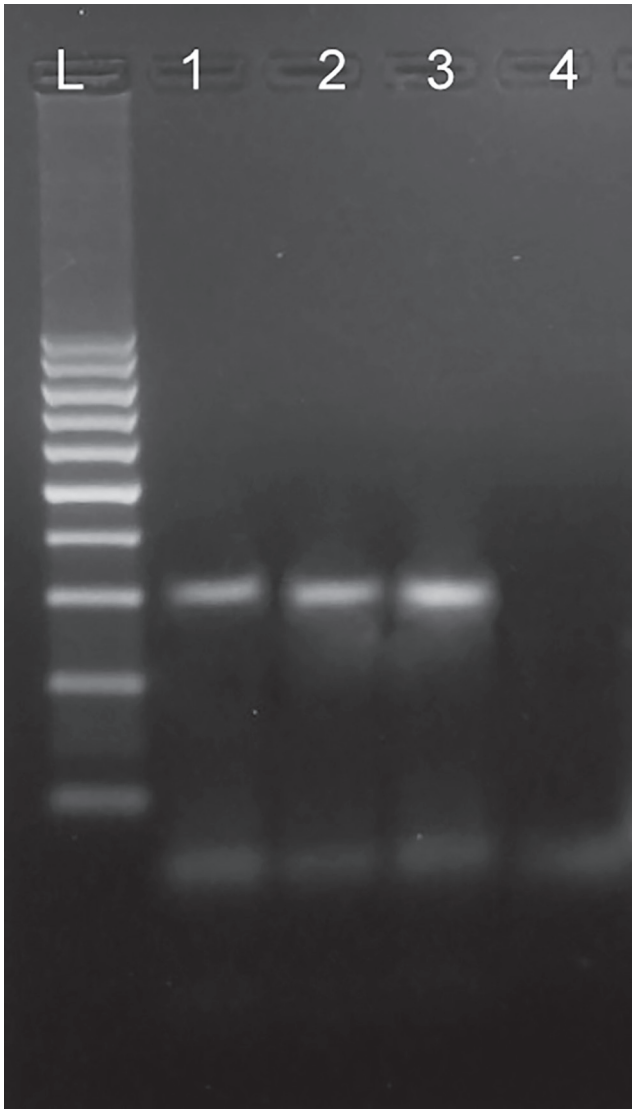

Figure 4: Gel image of PCR for a*lgD* gene. Lane L: 100 bp ladder, Lane 1 to 3: *P. aeruginosa* isolates showing a 313 bp product specific for the *algD* gene. Lane 4: negative control.

for this resistance is unknown, but poor affinity for the target enzymes and low outer membrane permeability have been suggested. Recently, it has been shown that OprM (35,36) and OprK, which is identical to OprJ (37), define two genetically distinct multidrug efflux systems in *P. aeruginosa* (38). It has been found that OprM- and OprJ-over expressing strains display increased resistance to TMP and was also demonstrated that the mexABoprM efflux system is mainly responsible for the intrinsic resistance of *P. aeruginosa* to trimethoprim and suplhinamides (39). In general, various adaptive mechanisms contribute to the antimicrobial resis-

tance of this pathogen, of which formation of biofilm is best characterized (40).

The pathogenicity of *P. aeruginosa* strains is associated with different virulence factors, that include proteases, elastases, exotoxins, or phenazine pigments. The major virulence determinants of this microorganism are the Type 3 Secretion System (T3SS) and its toxins, termed effectors (ExoU, ExoS, ExoT, ExoY). In the present study, among 18 isolates, 12 and 6 isolates showed presence of mutually exclusive virulence genes e*xo-S* and *exo-U* respectively, as they amplified to produce a 540bp and 1500bp PCR product respectively (Figure 3). Virulence genes *exo S* and *exo U* cause strains to be highly invasive and pathogenic. ExoS is a major cytotoxin involved in stages of colonization, invasion and dissemination of infection (41). ExoU is a potent cytotoxin with phospholipase activity, capable of killing a variety of eukaryotic cells *in vitro* (42, 43). Virulence genes *exo S* and *exo U* make strains highly invasive and pathogenic.

Biofilm associated gene *algD* was detected in all the isolates (Figure 4). This gene is responsible for the production of alginate exopolysaccharide, which protects the bacterium from host immunity and makes an effective barrier for drugs to penetrate. This biofilm building block is responsible for various intractable infections resulting in poor disease prognosis (44).

P. aeruginosa is a ubiquitous bacteria found in diverse ecological niches owing to its capacity to metabolize a wide range of metabolites. *P. aeruginosa* has a high relevance in humans for nosocomial infections particularly cystic fibrosis and burn patients. The association of this bacterium with mastitis in small ruminants has been only occasionally reported (22). This study throws light on the threat that may be posed by this opportunistic pathogen to a vulnerable population. In India, goats are reared by many small marginal farmers and considered as the "poorman's cow". Goat are hardy animals, as they are resistant to many diseases and thrive well on small amounts of feed. However, they are kept under intensive units with poor sanitation and hygienic environment. This can influence their health paving the way for opportunistic pathogens like *P. aeruginosa* to affect their health. The bacterium can multiply and produce biofilms in the udder of the animal to cause severe mastitis. The bacteria can easily spread from one animal to other during milking through hands of farmers and other environmental sources. Animals exhibit overt clinical signs in clinical mastitis but in case of sub-clinical mastitis, disease symptoms are not apparent. Animals showing subclinical mastitis can shed the bacterium through milk for a prolonged period of time. This can cause cross contamination and infection in apparently healthy individuals. Poorer farmers may not practice regular pasteurization of milk which can pose high risk to possible infections or intoxications through goat milk and milk products (45).

We hypothesise that *P. aeruginosa* poses a serious health risk to humans as the bacterium can interact with bacterial flora present in the gut of milk consumers to transmit their resistance genes. Epidemiologically, this is an important means of transmission of resistance genes during interaction with other bacterial community in the gut leading to emergence of new multidrug resistant pathogens.

There is a need to conduct studies in this area to test the viability and impact of horizontal transfer of antibiotic resistance determinants among different strains of bacteria in gut. As the studies from animal sector are scarce particularly from India (46), our effort represent an initiative towards studying the incidence level, antimicrobial resistance potential, virulence profile and biofilm characteristics of *P. aeruginosa* isolated from goats of India.

CONFLICT OF INTEREST STATEMENT

The authors declare that they have no conflict of interest.

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