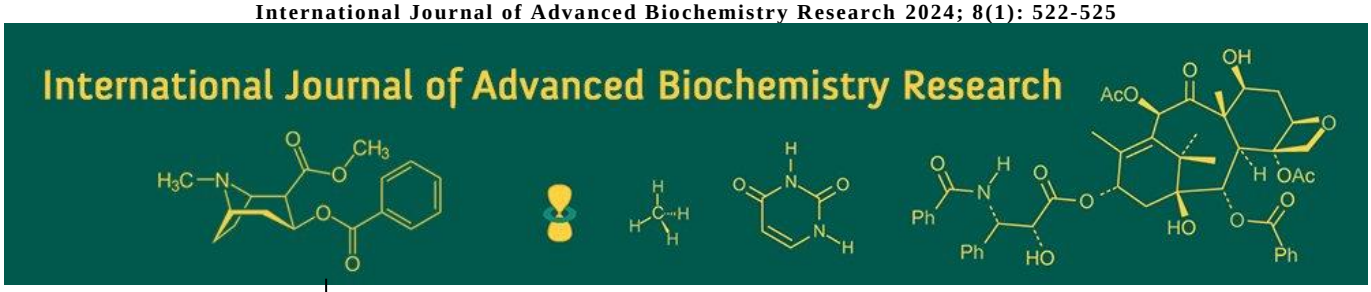

ISSN Print: 2617-4693
ISSN Online: 2617-4707
IJABR 2024; 8(1): 522-525
www.biochemjournal.com
Received: 18-10-2023
Accepted: 30-12-2023

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Characterization of *Pseudomonas aeruginosa* isolated from the chronic wound infection in camel

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DOI: https://doi.org/10.33545/26174693.2024.v8.i1g.432

Abstract
A case of chronic wound infection in camel was presented in the Veterinary clinics, RAJUVAS, Bikaner. The wound swab sample was cultured on BHI agar. Isolated bacteria was confirmed as *Pseudomonas aeruginosa* through cultural, biochemical and genotypic characterization. In ABST, bacteria were subjected to 15 antibiotics out of which bacteria was found resistant to 7, sensitive to 6 and moderately sensitive to 2. ABST result revealed that bacteria exhibit Multiple Drug Resistance (MDR). MAR-Index value was 0.46 (>0.2) which indicate high risk potential source of spread of MDR strains. Pathogenicity of bacteria was characterized by Analysing Haemolytic efficiency, antibiogram and presence of Quorum sensing genes. This report is an example of rising infection cases due to MDR strains which results in dodging antibacterial effects of many antibiotics. Eventually it results in difficulty to treat a patient and complicate the efforts to lower down the resistance.

Keywords: *Pseudomonas aeruginosa*, MDR strain, Quorum sensing, *lasI* gene, *rhlI* gene

1. Introduction
Pseudomonas aeruginosa is an opportunistic Gram-negative bacteria causing a wide range of acute to chronic infections in immune-compromised humans and animals. It leads to genital tract infections, otitis externa, cystitis, pneumonia and ulcerative keratitis in horses, dogs and cats. In birds of all ages it can lead to perihepatitis, pericarditis, congestion of internal organ, cheesy materials on the serous surfaces of peritoneum and airsacs. Infected birds may show ruffled feathers, drooping wings and pasted vents due to diarrhea. Recently, WHO has reported *P. aeruginosa* as an multiple antibiotic-resistant "priority pathogens". The pathogenesis of *P. aerugionosa* is controlled by various virulence factors that regulate bacteria-host cell interactions. These virulence factors can be cellular (flagella, pili, lectins, biofilm and lipopolysaccharide) and extracellular (proteases, hemolysins, cytotoxin, pyocyanin, siderophores, exotoxin A, exoenzyme S, and exoenzyme U), depending upon their secreation.
Quorum sensing (QS), a cell-to-cell signalling system organize a number of virulence factors in *P. aeruginosa*, is governed by several quorum sensing genes. These genes typically aid in the production of chemical signals, or auto-inducers, which promote the expression of many pathogenic genes. The Las, Rhl, quinolone-based QS systems and the recently discovered IQS (2-(2-hydroxylphenyl)-thiazole-4-carbaldehyde)-dependent system are the four QS systems that *P. aeruginosa* possesses. These systems work both individually and together (Lee and Zhang, 2015) [8]. The acyl-homoserine lactone (AHL) autoinducers N- (3-oxododecanoyl)-L-homoserine lactone (OdDHL) and N-butyryl-L-homoserine lactone (BHL), respectively, regulate the Las and Rhl systems.

2. Methods
2.1 Sample collection
Single use sterile cotton swabs sticks were used to collect sample from chronic infected wound in camel. The swabs were then returned aseptically into their container having phosphate buffered saline (PBS) and stored at 4 °C till further use.

2.2 Isolation, identification and biochemical characterization of *Pseudomonas aeruginosa*

Swab samples were cultured on Brain-Heart Infusion agar (BHI) and incubated at 37 °C overnight. The colonies were recultured on BHI to observe the cultural characteristics. Isolate was identified on the basis of standard bacteriological methods like morphology, colonial characteristics, pigment production on the media. Further identification were done by Gram staining, motility test by hanging drop method, odour and biochemical characterization.

2.3 Antimicrobial susceptibility Test (ABST)

The isolate was then subjected to antibacterial susceptibility test representing 8 classes of antibiotics tested using Kirby-Bauer disc diffusion method using disks on Muller-Hinton agar (MHA). Antibiotic susceptibility testing was performed as per the disc diffusion method on Muller-Hinton agar (MHA) against Gentamycin (10mcg), Norfloxacin (10 mcg), Imepenam (10 mcg), Cefepime (30mcg), Ceftriaxone (30 mcg), Ciprofloxacin (15 mcg), Oxytetracyclin (30 mcg), Azithromycin (15 mcg), Ofloxacin (1mcg), Nitrofurantoin (300 mcg), Tetracycline (30mcg), Penicillin (10 units), Ampicillin (10 mcg), Amoxicillin (30 mcg), Metronidazole (5 mcg). The inhibition zone around each disc was measured in millimeters, and the results were interpreted according to CLSI guidelines (CLSI, 2020).

2.4 Confirmation of Quorum-sensing genes

Primers for *lasI* and *rhlI* genes were designed using NCBI primer designing tool to conduct PCR for the confirmation of QS genes in the isolate (Table 1).

Table 1: Primers for validation of genes in *P. aeruginosa*

S. No.	Primer	Sequence (5'-3')	Product length (bp)
1.	LasI	F-GCGCGAAGAGTTCGATAAAA	177
		R-AAAACCTGGGTTCAGGAGTAT	
2.	RhlI	F-ATTCGACCAGTTCGACCATC	227
		R-ACCAGAATATCTTCATCGCCAG	

3. Results and Discussion

3.1 Isolation, identification and biochemical characterization of *Pseudomonas aeruginosa*

The isolated colonies were small, flat, 2-3mm in diameter, smooth with regular margins, greenish blue in color on BHI agar indicating the production of pigment. Bacteria failed to ferment lactose when cultured on McConkey Lactose agar (MLA). When cultured on 5% defibrinated sheep blood agar bacteria was able to show hemolysis. Further, bacteria was found to be gram negative after Gram staining when examined under microscope at 100X. The biochemical characterization was carried out using Vitek 2.0 Automated System by which bacteria was confirmed as *Pseudomonas aeruginosa*.

3.2 Antibiotic sensitivity test

Antibiotic sensitivity test was performed for 15 antibiotics out of which *P. aeruginosa* was found resistant to 7, sensitive to 6 and moderately sensitive to 2 antibiotics (Table 2). ABST result revealed that bacteria inhabit Multiple Drug Resistance (MDR). MAR-Index value was found to be 0.46 (>0.2) which indicate high risk potential source of spread of MDR strain.

Table 2: Antibiotic sensitivity test (ABST) against *P. aeruginosa*

Interpretation	Antibiotics
Sensitive	Gentamycin, Norfloxacin, Imepenam, Cefepime, Ceftriaxone, Ciprofloxacin
Intermediate	Oxytetracycline, Azithromycin
Resistant	Ofloxacin, Nitrofurantoin, Tetracycline, Penicillin, Ampicillin, Amoxicillin, Metronidazole

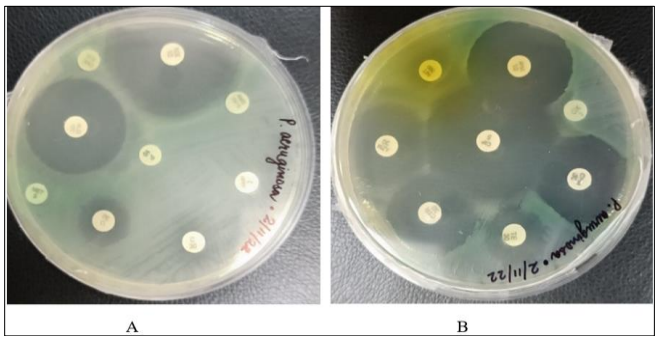

Fig 1: Inhition zone in Antibiotic sensitivity test (ABST) against *P. aeruginosa*

The inhibition zones of all drugs, were measured according to the CLSI guidelines for interpretation of antibiotic sensitivity against *P. aeruginosa* (Fig 1). Aminoglycosides like gentamycin and amikacin have been suggested against *P. aeruginosa* strains of veterinary origin, irrespective of species of animals and site of infection (Hariharm *et al.* 1995, Lin *et al.* 2012) [3, 11]. Similar to results of this study, Hariharam and coworker reported ≥85% of *P. aeruginosa* susceptible to gentamycin isolated from canine and feline otitis externa and all the isolates were reported resistant against amoxicillin-clavulanic acid, penicillin, ampicillin (Hariharam *et al.* 2006) [4]. *P. aeruginosa* has been reported notorious for its resistance to multiple antibiotics because of its low outer membrane permeability (Hancock 1998) [2], intrinsic or induced expression of efflux pumps (Li *et al.* 2000) [10], and β-lactamase production, *P. aeruginosa* is uniformly resistant to a wide range of antimicrobials including ampicillin, first-and second-generation cephalosporins, and erythromycin and is also often reported resistant to chloramphenicol and tetracycline (Li *et al.*,1994) [9]. In addition to these intrinsic resistances, *P. aeruginosa* can also acquire resistance through horizontal transfer of genetic elements that carry resistance genes (integrons, transposons, and plasmids) as well as mutational resistance (e.g. mutation in DNA gyrase and topoisomerase IV) (Livermore 2002; Linder *et al.*, 2005) [13, 12]. Owing to the highly variable resistance patterns, the empiric therapy of *P. aeruginosa* infection using penicillins, third- and fourth-generation cephalosporins, carbapenemes, aminoglycosides (AGs) and fluoroquinolone (FQs) may be ineffectual.

3.3 Confirmation of Quorum-sensing genes

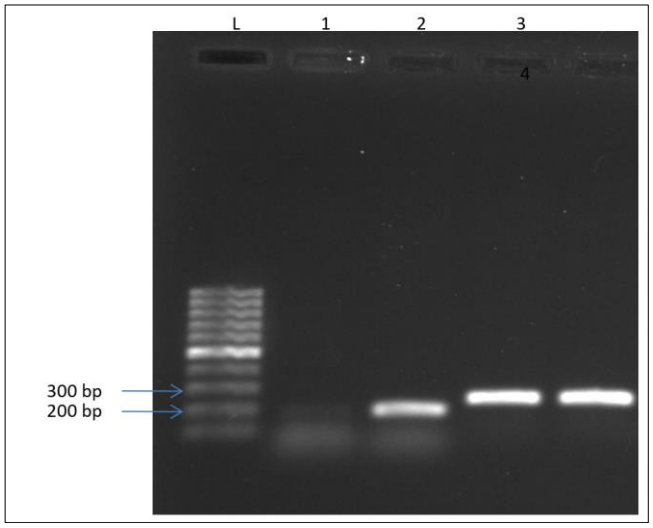

Fig 2: PCR of QS genes: Lane L: Ladder, Lane 1 & 2: 177bp of *las*I gene, Lane 3 & 4: 227bp of *Rhl*I gene.

Khadim and Ali (2014) [7] reported the role of QS in the pathogenesis of *P.aeruginosa* and the results showed high frequency of virulence factor genes in local isolates, suggesting that these isolates were QS proficient. The studies included screening of QS genes (lasI, lasR, rhlI, rhlR) by multiplex PCR technique and found 81.6% (49/60) isolates was positive for one or more QS genes while only 18.3% (11/60) was negative for all these genes. This ability of *P. aeruginosa* to coordinate the upregulation of virulence genes in a whole population translates into increased pathogenicity of QS capable strains compared to QS deficient mutants throughout a variety of animal models. Furthermore, several studies have shown that the autoinducer N-(3-oxododecanoyl)-L-homoserine-lactone is immunomodulatory and can depress host responses (Smith *et al.*, 2002 and 2001) [19, 18]. These roles of QS in virulence and immunomodulation make it a highly interesting candidate for pathogenesis targeting therapeutics to the extent that potential strategies involving each step of QS have been mapped out in an in-depth review (Smith *et al.*, 2003). Among such strategies, several have been explored experimentally. QS can be successfully inhibited by competitive antagonist analogs of acyl-homoserine-lactones (AHLs) leading to decreased elastase production and biofilm formation (Smith *et al.*, 2003) [17]. The signaling AHLs are degraded by AHL-lactonases. Plants naturally expressing these lactonases have increased resistance to QS capable pathogens (Dong *et al.*, 2001) [1]. Insertion of an AHL-lactonase gene into PAO1 led to decreased production of virulence factors (Reimmann *et al.*, 2002) [16]. In this search for QS inhibitors, following the discovery that antibacterial furanones produced by marine macroalga were capable of inhibiting QS, synthetic furanones were developed (Hentzer *et al.*, 2002) [5]. Synthetic furanones successfully inhibit QS and reduce virulence factor expression, increase bacterial clearance and survival in murine models of *P. aeruginosa* lung infection (Hentzer *et al.*, 2003 and Wu *et al.*, 2004) [6, 22]. Pursuing the quest for QS inhibitors has led to the development of screening strategies that have successfully shown QS inhibiting properties of novel compounds from garlic extracts (Rasmussen *et al.*, 2005) [15].

Closer to clinical practice, it has been found that azithromycin inhibits QS, decreasing virulence factor expression and biofilm production. This effect may account, in part, for the antipseudomonal effects of subinhibitory concentrations of macrolides (Tateda *et al.*, 2004 and Wozniak *et al.*, 2004) [20, 21].

4. Conclusion

In present study, the *P. aeruginosa* was isolated from chronic wound infection in camel. Molecular characterization confirmed the presence of *Las*I and *Rhl*I genes in the genome of bacteria. These genes are the part of quorum sensing system, which is responsible for the expression of major virulent genes of *P. aeruginosa*. Also, the bacteria showed multiple-antibiotic resistance with MAR index 0.42 (> 0.2) considered as an high risk contaminant.

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