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Prevalence β-lactam resistant *Pseudomonas aeruginosa* strains isolated from chronic suppurative otitis media infections: A single center survey in Eastern India

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ABSTRACT

Background: *Pseudomonas aeruginosa* is a pernicious opportunistic bacterium causing several human infections due to its ubiquitous presence and adaptability to various environmental conditions. Primarily, β-lactamase producing *P. aeruginosa* strains are the most prevalent ones, especially chronic suppurative otitis media (CSOM) infections.
Aim and Objective: This research was conducted to find the pervasiveness of β-lactam producing *P. aeruginosa* strains in CSOM patients attending a multispeciality hospital in Eastern India.
Materials and Methods: The 100 swabs were collected from CSOM patients, of which 76 were males (mean age 46) and 24 were females (mean age 41); *P. aeruginosa* strains were isolated and identified using routine microbiological methods. Kirby-Bauer method was employed to determine the antibiotic sensitivity of the isolated strains. Virulence tests like the swarming mortality test, biofilm formation assay, haemolysis assay, protease and pyocyanin production assay were conducted to detect the presence of various inherent virulence characters qualitatively. Both phenotypic and genotypic determination of β-lactamase-producing strains were confirmed by *in vitro* disc diffusion test and PCR methods.
Results: Out of 100 samples, 67 *P. aeruginosa* strains were isolated, and all were motile. All strains tested positive for pyocyanin and haemolysis enzymes. Most strains had the biofilm formation capacity and release of protease enzyme. Most strains were multidrug-resistant, particularly to the β-lactam group of antibiotics. Nearly 80% of strains harboured all four classes of β-lactamase genes, producing enzymes like ESBLs, MBLs, AmpC and carbapenemase.
Conclusion: The β-lactamase genes and the inherent virulence factors that increase the infectiousness and adaptability of *P. aeruginosa* strains are significantly correlated. Immediate modified antibiotic policy should be employed to overcome this issue of multidrug resistance.

1. Introduction

Among middle ear infections, chronic suppurative otitis media (CSOM) is one of the most prevalent, especially in youngsters. It occasionally results in acute otitis media but is often characterized by chronic middle ear drainage and tympanic membrane proportion.[1,2] In the present era of antibiotics, CSOM may be infrequent in developed countries; however, it is pervasive in third-world countries, where a need for rapid diagnosis and standard medical

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facilities are unavailable. Untreated CSOM infections may lead to petrositis, facial paralysis, labyrinthitis, lateral sinus thrombophylactic, meningitis and other complications such as hearing loss and intracranial absences.[3] The most frequently isolated bacterial organisms from CSOM patients are *Pseudomonas aeruginosa, Staphylococcus aureus*, *Escherichia coli*, *Proteus mirabilis*, *Klebsiella* species, and fungi like *Aspergillus* and *Candida*. Causative organisms may often vary depending on the climatic and geographical conditions of the patients.[4,5] Primarily, CSOM is treated with antibiotic drugs such as neomycin, gentamicin, tobramycin, and aminoglycosides. However, with the advent of multidrug-resistant (MDR) bacteria, particularly *P. aeruginosa*, *E. coli,* and *S. aureus*, treatment of CSOM infection has become difficult.[6–8]

P. aeruginosa is one of the most common Gram-negative bacteria isolated from CSOM patients. This bacterium generally damages the mucosal epithelium of the middle ear, resulting in acute otitis media and sometimes a form of biofilm in the middle ear.[6] The biofilm formation resists the topical antibiotic treatment, leading to complications in the treatment procedure.[7] Moreover, *P. aeruginosa* releases several virulence factors, such as toxins and enzymes like proteases and elastases, which facilitate tissue damage and the rapid spread of infection. Additional virulence factors that cause adherence and invasion of their host include mucoid exopolysaccharide, pili, exotoxin A, lipopolysaccharide, pigments, lipase, haemolysin, histamine, exoenzyme S, leucocidin, and rhamnolipids. These factors also weaken the host's immune responses and an antibiotic barrier formed by it.[9,10]Further, these strains harbour multiple mechanisms like antibiotic efflux, production of antibiotic resistance enzymes, and mutations in antibiotic target sides, making the antibiotic treatment ineffective.[11,12]

For millions of years, *P. aeruginosa* has lived in the soil alongside antibiotic-producing bacilli, actinomycetes, and moulds, allowing it to build resistance to various naturally occurring antibiotics. The main mechanisms behind beta-lactam resistance in *P. aeruginosa* strains include targeting beta-lactam antibiotics, such as cephalosporins, penicillin, and other similar drugs, by changing the outer membrane proteins and altering the penicillin-binding proteins.[11,12] Certain strains possess inherent resistance mechanisms, known as intrinsic mechanisms, or they can be acquired through mutations or horizontal gene transfer. Treatment of bacterial infections with beta-lactam antibiotics is made more difficult by disseminating resistance genes, primarily via plasmids and transposons. The commonly found β-lactamases-producing strains of *P. aeruginosa* are Extended-spectrum- β-lactamases (ESBLs) producing strains, Metallo-β-lactamases (MBLs) and AmpC-producing strains.[13,14]

β-lactamase-producing *P. aeruginosa* strains often lead to treatment failure as the β-lactamase present in the organisms breaks down the β-lactam antibiotics. These resistant bacteria require the use of more toxic antibiotics for treatment. They increase the health care cost and duration of hospitalization and increase the chances of higher morbidity and mortality rates.[15,16]Further complications, such as nosocomial infections like urinary tract infections and ventilator-associated pneumonia, become familiar and lead to outbreaks in nursing homes and hospitals. Resistant strains of *P. aeruginosa* can survive in several environmental conditions and serve as a reserve of antibiotic-resistant genes that can be transferred to other bacteria. Particularly in CSOM patients, β-lactamase, producing *P. aeruginosa*, limits the treatment choice, leading to resistance ear discharge and chronic middle ear inflammation. Further conductive hearing loss and sensorineural hearing loss are also seen in some patients. The infection may spread to the mastoid bone, leading to mastoiditis and other threatening infections, such as meningitis, brain abscess, and lateral sinus thrombosis.[17,18]Therefore, this study aims to determine the prevalence of β-Lactam resistant *P. aeruginosa* strains isolated from patients with CSOM infections attending a tertiary care hospital in eastern India.

2. Materials and Methods

2.1. Sample collection

Ear swabs from 100 CSOM patients, of which 76 were males (mean age 46), and 24 were females (mean age 41), who reported to the outpatient Department of Otorhinolaryngology, IMS, and Sum Hospital (a multispeciality hospital in Eastern India) were collected from April to June 2023. The collected swabs were aseptically transferred to the medical research laboratory of the same hospital for the isolation and identification of the causative organism. This study was conducted after the approval of the institutional ethical committee of IMS, and Sum Hospital vide letter no: IEC/IMS.SH/SOA/2022/403 dated 22^{nd} August 2022**.**

2.2. Bacterial identification and antibiotic sensitivity test

The bacterial identification was made by culturing the swab samples on the different culture media: Nutrient, McConkey, and Blood agar. The major isolated bacterial colonies were subjected to gram staining and biochemical tests as described elsewhere.[5,6] The Kirby Bauer method was employed to determine the antibiotic sensitivity pattern using the commonly prescribed antibiotics for CSOM patients per the previously described protocol.[5,6]

2.3. In vitro assays for determining virulence factors of isolated P. aeruginosa strains

2.3.1. Swarming motility test

The isolated *P. aeruginosa* strains were inoculated into 1.5% Luria-Bertani (LB) agar plates with the help of an inoculation needle and were incubated at 37°C for 24 hours. The swarming zone formation around the inoculation streak confirmed the motility of the isolated strains.[19]

2.3.2. Biofilm formation assay

The isolated *P. aeruginosa* strains were subjected to a biofilm development experiment utilising the microtiter plate technique. The strains of *P. aeruginosa* were diluted by LB broth to an optical density (OD) of 600nm (nearly 10^8 colony forming units/ml).200μl of the diluted *P. aeruginosa* culture was inoculated to each well of 96 wells of microtiter plate with proper levelling, and it was incubated for 48 hours at 37°C. After incubation, the planktonic cells were removed using a micropipette from each well. The cells were gently washed with sterilized buffer saline. Further, 1% crystal violet was used for 15 minutes to stain the adherent biofilm. The excess strains were washed gently in the water. The crystal violet strains were solubilized by adding 200μl of ethanol, and the absorbance was recorded for each well at 570nm using a microplate Elisa reader.[20]

2.3.3. Haemolysis assay

The isolated *P. aeruginosa* strains were inoculated in 5% sheep blood agar plates and incubated at 12 hours at 37°C.β-haemolysis was confirmed by a clear zone around the colonies.[21]

2.3.4. Protease production assay

In a shaker incubator, the isolated *P. aeruginosa* strains were incubated from LB broth at 37°C. 10μl of overnight *P. aeruginosa* cultures was spot inoculated onto skim milk agar plate and incubated at 37°C for 24 hours. Post incubation, the clear zone around the bacteria colonies indicates the protease activity.[22]

2.3.5. Pyocyanin production assay

The isolated *P. aeruginosa* strains were inoculated in LB broth at 37°C for 24 hours in a shaker incubator. Pyocyanin was extracted by adding an equal volume of chloroform to the culture, followed by vigorous vortexing and centrifuging to separate phases. The pyocyanin was collected with the chloroform layer mixed with the 2-molar hydrochloric acid. The absorbance of redissolved pyocyanin was taken at 520nm using a visible spectrophotometer. A reference absorbance of 700nm was taken to correct any background absorbance. The pyocyanin concentration was calculated using the formula: The pyocyanin concentration (μg/mL) = OD 520 × 17.072.[23]

2.4. Phenotypic determination of β-lactamase

The phenotypic detection of ESBLs, MBLs, AmpC and carbapenemase among the *P. aeruginosa* isolates was done by double disk synergic test as per CLSI guidelines 2023, meropenem-EDTA combined disc diffusion test, ceftazidime-boronic acid combined disc diffusion test and Modified Hodge test respectively.[24–28]

2.5. Genotypic determination of β-lactamase

The genotypic detection of different classes of β-lactamase (amber class A- bla$_{SHV}$ & bla$_{TEM}$, amber class B- bla$_{VIM}$, bla$_{IMP}$ & bla$_{NDM}$, amber class C- AmpC and amber class D- bla$_{OXA}$ & bla$_{KPC}$, genes were sequentially determined by PCR using specific primers. After incubation, the LB plate was lysed at 94°C, taken in 500μl of sterile water, and transferred to the ice for 10 minutes. After centrifuging the solution at 13,000 rpm for 1 minute at 4°C, 1.5 μl of the suspension was immediately employed as template DNA. 25μl master mix contains 5μl (5X) Promega PCR buffer, 2μl (25mM) $MgCl_2$, 2μl (2.5mM/dNTP) dNTP, 1.5μl (10μM) Forward primer, 1.5μl (10μM) reverse primer, 0.5 μl Taq polymerase, 1.5 μl template and volume maintained by Milli-Q water. The amplified PCR product was run in 1.2% agarose gel at 60V for 45 minutes and visualized in a Gel-Doc apparatus.[29–31]

3. Results

In this study *P. aeruginosa* strains were identified based on colony characteristics of Nutrient agar, McConkey agar, and Blood agar, as described in (Table 1), (Figure 1 a,b&c). It is a gram-negative bacillus, giving positive results for catalase, citrate, and nitrate reduction tests, whereas negative results for indole, methyl-red, Voges Proskauer and H_2S tests (Table 1).

In the *in vitro* virulence tests, all the isolated 67 strains had positive results for the swarming motility pyocyanin production and haemolysis assay. However, only 42 strains had biofilm formation and protease production capacity (Table 2, Figure 2a, b & c).

The antibiotic sensitivity results found that all the 67 strains isolated were multidrug resistant, of which nearly 97.01% were resistant to gentamicin. Amoxicillin/clavulanic was the antibiotic with the least resistance (47.76%) recorded. Resistance to β- lactamase antibiotics such as ceftriaxone, cefoperazone, cefuroxime imipenem and meropenem ranged between 72% to 88%, which signifies that these antibiotics are almost insignificant in the current treatment regimen of CSOM (Table 3).

In the phenotypic test done for the determination of β- lactamase-producing *P. aeruginosa* strains (Figure 3), out of 67 strains, 57 (85.07%) were ESBL, 59 (88.05%) were MBL, 52 (77.61%) were AmpC producers, and 54 (80.59%) were carbapenemase enzyme producers (Table 4).

Table 1: Microbiological and biochemical identification of isolated *P. aeruginosa* strains from CSOM swabs.

Total Samples	Samples with *P. aeruginosa* strains	Colony characters		Biochemical characters
100	67	Nutrient agar	large opaque and flat colonies with irregular margins and earthy odour.	Gram-negative; positive for catalase, citrate, and nitrate reduction. Negative for Indole, Methyl red, Voges Proskauer and H_2S test.
		McConkey agar	Non-lactose fermenting flat, circular, colourless colonies	
		Blood agar	β haemolytic mucoid colonies with a metallic sheen.	

Table 2: *In vitro* virulence tests of isolated *P. aeruginosa* strains.

Virulence tests	Number of strains responding to virulence test
Swarming Motility test	67 (100%)
Pyocyanin production assay	67(100%)
Biofilm formation assay	42(62.68%)
Protease	54 (80.59%)
Haemolysis assay	67(100%)

Table 3: Antibiotic sensitivity pattern of the isolated *P. aeruginosa* strains.

Antibiotics	Number of isolated *P. aeruginosa* strains resistant to prescribed antibiotics (n = 67)	Resistance percentage (%) to *P. aeruginosa* strains (n = 67)
Ampicillin	46	67.71
Amoxicillin/Clavulanic Acid	32	47.76
Amikacin	54	80.59
Ceftriaxone	59	88.08
Ciprofloxacin	54	80.59
Cotrimoxazole	47	70.14
Cefoperazone/Sulbactam	53	71.10
Cefuroxime	51	76.11
Gentamicin	65	97.01
Imipenem	52	77.61
Meropenem	47	70.14
Piperacillin/Tazobactam	43	64.17
Tigecycline	46	67.71

Table 4: Phenotypic tests for determination β-lactamase producing *P. aeruginosa* strains.

Phenotypic tests for β-lactamase producers	Number of strains [n=67 (100 %)]
ESBLs	57 (85.07%)
MBLs	59 (88.05%)
AmpC	52 (77.61%)
Carbapenemase	54 (80.59%)

From the above findings, it can be interpreted that isolated strains had multiple mechanisms for breaking down β-lactam antibiotics.

In the genotypic determination of *P. aeruginosa* strains harbouring β-lactamase genes, it was recorded that 54 (80.59%) strains harboured amber class A β-lactamase genes, 54 (80.59%) strains harboured amber class B gene, 52 (77.61%) strains contained amber class C gene, and 51(76.11%) strains contained amber class D β- lactamase gene. (Table 5). Hence, it can be inferred that the isolated strains from the CSOM patients harboured more than two types of β-lactamase-producing genes, which makes them MDR bacteria.

4. Discussion

The current study demonstrated the problem of multiple antibiotic resistance in *Pseudomonas* strains. The results obtained from the antibiotic sensitivity test following the conformation by phenotypic and genotypic tests were done for conforming β-lactamase producing *P. aeruginosa* strains. For instance, 52 strains conformed to harbouring

Table 5: Genotypic determination of *P. aeruginosa* strains harbouring β-lactamase genes.

β-lactamase genes	**Number of isolated strains harbouring β-lactamase genes [n=67 (100%)]**
amber class A bla$_{SHV}$ & bla$_{TEM}$	54 (80.59%)
amber class B bla$_{VIM}$, bla$_{IMP}$ & bla$_{NDM}$	54 (80.59%)
amber class C AmpC	52 (77.61%)
amber class D bla$_{OXA}$ & bla$_{KPC}$,	51 (76.11%)

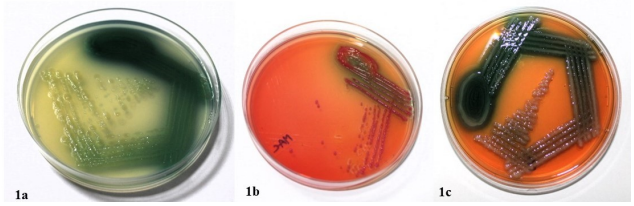

Figure 1: Growth of *Pseudomonas aeruginosa* on: **a:** Nutrient agar; **b:** McConkey agar; **c:** Blood agar.

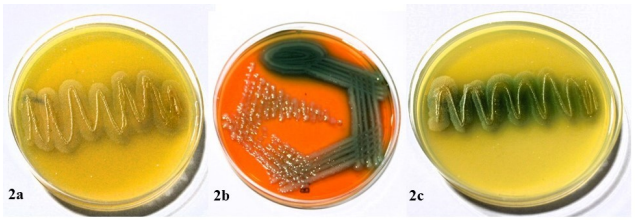

Figure 2: *In vitro* evaluation of inherent virulence factors the isolated *Pseudomonas aeruginosa* produces: **a:** Swarming motility test; **b:** Haemolysis assay; **c:** Protease production assay.

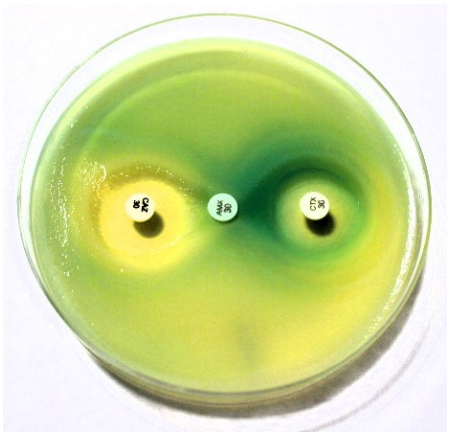

Figure 3: Double disk diffusion test for phenotypic detection of β lactamase enzyme production.

amber class C AmpC genes in phenotypic and genotypic tests. Likewise, there was a marginal difference in the genotypic test. Some strains of *P. aeruginosa* did not contain the β-lactamase genes, which initially identified the phenotypic test. Hence, based on the current findings, we can confirm that the CSOM patients were infected with *P. aeruginosa* strains, ESBL, MBL, AmpC producers, and carbapenem producers or, in other words, PAN drug resistance bacteria. A study from Odisha, India, isolated 371 MDR P. aeruginosa strains from CSOM patients who were resistant to antibiotics, particularly the β-lactam group.[32] Similarly, from South India, from 106 CSOM patients, 49 MDR *P. aeruginosa* strains are isolates highly resistant to β-lactam and carbapenem group of antibiotics.[33] ESBL *P. aeruginosa* strains were isolated from CSOM patients from a study in North India, which accounted for 36% of the total infections.[34] Similarly, in another Indian study, it was reported that ESBL-producing *P. aeruginosa* strains accounted for nearly 41.2% of the total infection, which was spread across all patients in each group.[35]

Earlier research studies have demonstrated the relationship between the β-lactamase-producing genes and virulence factors.[36,37] It is established that harbouring β-lactamase producing gene increases the degree of virulence and the organism's adaptability to different environmental conditions.[38] For example, the blaAmpC gene increases the proteolytic activity of the bacteria as the blaVIM and blaIMP genes are associated with the biofilm formation capacity of *Pseudomonas* strains.[39] Acquisition of the β-lactamase resistance gene also triggers the efflux plumps of *P. aeruginosa* strains, making the organisms resistant to other antibiotics like quinones and aminoglycosides. It initiates a pathway for cross-regulation of antibiotic resistance that develops new resistance genes in the genetic pool. This antibiotic resistance of genes can quickly transfer to other species of bacteria, particularly in clinical settings.[40] Therefore, doing more studies to determine the relationship between β-lactamase genes and other virulence factors of *P. aeruginosa* strains might be interesting.

Several studies have shown that *P. aeruginosa* was a leading causative organism in CSOM patients, which were β-lactamase producers. A study from Nigeria detected the FOX- AmpC β-lactamase gene in the *P. aeruginosa* strains isolated from CSOM patients.[41] Likewise, a study from Ethiopia detected ESBL-producing *Proteus mirabilis*, *P.*

aeruginosa, and *Klebsiella pneumoniae* strains in more than 238 patients with ear infections.[42] A study from Bangladesh reported a predominance of ESBL strains of *P. aeruginosa* over three years in a study conducted in two tertiary care hospitals. This study also reported that *P. aeruginosa* strains are resistant to aminoglycoside.[43] Similarly, ESBL *P. aeruginosa* strains have also been reported in poultry environments in Bangladesh.[44] From a Sri Lankan study, a high % of ESBL *P. aeruginosa* strains were isolated from ICU patients, majorly hospital-born infections.[45] Similarly, *P. aeruginosa* strains harbouring all kinds of ESBL genes were reported from diabetic food ulcer patients attending a medical university in Pakistan.[46] A recent study from Kathmandu, Nepal, also reported the prevalence of ESBL and MBL *P. aeruginosa* strains from two tertiary care hospitals conducted over a period of 2 years.[47] The above study shows the dominance of *P. aeruginosa* ESBL strains in CSOM patients and all other infections in hospitals and community settings, as seen in our study.

5. Conclusion

The present study gives a complete idea of the prevalence of β-lactamase producing *P. aeruginosa* strains in CSOM patients. As established, the resistance gene also enhances the virulence and pathogenicity of *P. aeruginos*a strains. Therefore, it decreases treatment options, creating a clinical concentration among the clinicians. Therefore, a modified antibiotic usage policy should be employed in hospitals to reduce the global burden of MDR *P. aeruginosa* strains and other MDR bacteria. As time progresses, this global burden of MDR bacteria will lead to a post-antibiotic era in which any majors may not control them. Hence, the immediate scientific major should be taken to reduce the exitance of the MDR bacteria.

6. Source of Funding

None.

7. Conflict of Interest

None.

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