Original article:

Isolates of *Pseudomonas aeruginosa* from clinical specimens and their susceptibility pattern to Amikacin, Ceftazidime, and Ciprofloxacin

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Abstract

Introduction: *Pseudomonas aeruginosa* is a leading cause of hospital-acquired infections and has innate resistance to many disinfectants and antibiotics. Multiple drug resistance in this organism has made treatment options limited.

Aim: To identify and characterise*Pseudomonas aeruginosa* isolated from clinical specimens and to analyse the antibiotic susceptibility pattern to amikacin, ceftazidime, and ciprofloxacin.

Materials &Methods: This was a hospital-based study done for a period of 6 months.176 nonfermenters were isolated during this period of which 135 isolates were identified as *Pseudomonas aeruginosa.*Identification was done by the characteristic odour, colony morphology, pigment production, gram staining, motility, and biochemical tests. Pigment production was studied in detail. Antibioticsusceptibility testing was performed on Mueller-Hinton agar by Kirby-Bauer disc diffusion method. Susceptibility results for amikacin, ceftazidime, and ciprofloxacin were analysed.

Results: *Pseudomonas aeruginosa* was the most frequently isolated nonfermenter in the study (135 isolates, 76.70%).37.04%of the *Pseudomonas aeruginosa* strains were isolated from pus samples,31.11% from miscellaneous specimens which included catheter tips, drain tips, bronchial wash and tissues, 21.48% from sputa,8.15%from urine and 2.22% from body fluids.70.37% of *P.aeruginosa*strainsproduced pigment on nutrient agar at 30^0C.71.85% of isolates produced pigment on gelatin agar.Of these, 38.14% produced pyocyanin, 32.98% produced pyomelanin, 28.87% produced pyorubrin. 28.15% strains were nonpigmented. The isolates were most susceptible to ceftazidime (50.37%), followed by amikacin (49.63%), and ciprofloxacin (40.74%).

Conclusion: Proper infection control practices and regular dissemination of microbiology data can limit the emergence and spread of this nonfermenter.

Key words: *Pseudomonas aeruginosa*, identification, antibiotics

Introduction

Pseudomonas aeruginosa(*P.aeruginosa*) belongs to the group of nonfermentative gram-negative bacilli, and is the major pathogenic species in the family Pseudomonadaceae.The organism flourishes as a saprophyte in warm moist environmentand is often isolated from water in sinks and drains,respirators, humidifiers, disinfectants, soaps, irrigation fluids and medical equipment.[1,2,3,4]It has innate resistance to many antibiotics.[3]Inspite of possessing almost all major classes of bacterial virulence factors,[1]*P.aeruginosa* still remains an opportunistic pathogen[5]. It is primarily a nosocomial pathogen and can cause pneumonia, urinary tract infection (UTI), skin and soft tissue infections, bone and joint infections, eye and ear infections, bacteraemia, meningitis etc.[6,7]Respiratory tract is a major site of predilection for this organism. Itcauses chronic infections of the airways in patients with predisposing conditions, the most common being

cystic fibrosis.[1]*P.aeruginosa* is also an established agent causing outbreaks in hospitals. Several reports of outbreaks of colonization and infection with this bacterium have been published.[8,9,10,11]Biofilms play a very significant role in the pathogenesis of certain infections by *P.aeruginosa*, and the molecular aspects of biofilm formation have been very well studied in this pathogen.[12,13]

The antibiotics effective against *P.aeruginosa* infections are antipseudomonal penicillins, cephalosporins, aminoglycosides, fluoroquinolones, and carbapenems. Amikacin, ceftazidime, and ciprofloxacin are commonly used to treat *P.aeruginosa* infections. Aminoglycosides are usually used as combination regimens and are generally not advised as monotherapy.[14]They are bactericidal and are often given in combination with beta lactam antibiotics. Amikacin is not affected by many modifying enzymes that inactivate gentamicin and tobramycin.[15]Of the cephalosporins, ceftazidime is a suitable alternative to aminoglycosides and can be used as monotherapy to treat serious hospital-acquired infections due to *P.aeruginosa*,[16] but resistance is an issue.Ciprofloxacin, reported to be the most active quinolone against *P.aeruginosa*,[17] exhibits good activity against the organism and has excellent tissue distribution[16]. Unlike other antipseudomonal antibiotics, this drug can be given orally, but resistance may develop quickly during the course of therapy.There are various mechanisms of resistance in *P.aeruginosa* such as production of extended spectrum β-lactamases (ESBL), Amp C β-lactamases,metallo β-lactamases (MBL),amin-oglycoside modifying enzymes, outer membrane impermeability due to porin loss, and active efflux pumps.[18,19]

P.aeruginosa can be identified on the basis of colony morphology, production of bluish-green diffusible phenazine pigment, a positive oxidase test, motility, and a triple sugar iron agar reaction of alkaline over no change[2].But problem arises when nonpigmented strains are isolated in culture. Many laboratories fail to identify nonpigmented *P.aeruginosa* strains. This leads to underdiagnosis of *P.aeruginosa* infections. In such cases, additional reactions have to be done inorder to confirm the identity of the isolate as *P.aeruginosa*.

This study was done to identify and characterise *Pseudomonas aeruginosa* isolated from clinical specimensand analyse the susceptibility for amikacin, ceftazidime, and ciprofloxacin.

Materials & methods

The study was a hospital-based one carried out for a period of 6 months during which, 176 nonfer-mentative gram-negative bacilli isolated from various clinical specimens were further characterised.Of these,135 isolates were identified as *P.aeruginosa*.An organism was considered to be a nonfermenter, if it failed to produce acid in routine glucose medium or utilized glucose oxidatively.[20] Growth on triple sugar iron (TSI) agar slant with no growth extending into the butt and no acidification of the butt was taken as that of a nonfermenter. Identification of *P.aeruginosa* was done by the characteristic odour, colony morphology, pigment production, gram staining, motility, and biochemical properties. Cultural characteristics were determined on blood agar, MacConkey agar, nutrient agar, milk agar, and gelatin agar.The biochemical tests used in the study were oxidase test, indole production, TSI agar, urea hydrolysis, citrate utilization, nitrate reduction, arginine dihydrolase, lysine and ornithine decarboxylases, phenylalanine deaminase, Hugh-

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Leifson oxidation-fermentation test, acetamide utilization, aesculin hydrolysis and DNAse tests.Carbenicillin (100mcg), Polymyxin B (300 units/dic), and Kanamycin (30mcg) discs were also used to support the identification of *P.aeruginosa*.[20]Antibioticsusceptibility testing was performed on Mueller-Hinton agar by the Kirby-Bauer disc diffusion method.

Results

135 of the 176nonfermentative isolates were identified as *P.aeruginosa* (76.70%), which was the most commonly encountered nonfermenter in the study.*P.aeruginosa* was isolated from pus samples (50 isolates, 37.04%),miscellaneous specimens (42 isolates, 31.11%)which included catheter tips, drain tips, bronchial wash and tissues, sputum samples (29 isolates,21.48%),urine (11 isolates,8.15%), and body fluids (3 isolates, 2.22%)(**TABLE1**).

Pigment production by*P.aeruginosa* can be enhanced by growing them on milk-, gelatin-, or potato-containing media and also by incubating them at 25 to 30^0C.[20] Nutrient agar, gelatin agar, and milk agar were used for this purpose.TABLE 3 compares pigment production of *P.aeruginosa* on nutrient agar at 30^0C and at 37^0C.On nutrient agar, 95 isolates (70.37%) of *P.aeruginosa* produced pigment when grown at 30^0C when compared to 90 pigment producing strains (66.67%) grown at 37^0C (**TABLE 2**). Of the 3 culture media used for pigment enhancement, gelatin agar could identify the maximum number of pigment-producing strains of *P.aeruginosa* (97 isolates, 71.85%) (**TABLE3**).Among the 97 pigment-producing strains of *P.aeruginosa*, 37 (38.14%) produced pyocyanin, 32 (32.98%) produced pyomelanin, and 28 (28.87%) produced pyorubrin (**TABLE 4**). 38 (28.15%) strains were nonpigmented.

Susceptibility test results were analysed for amikacin, ceftazidime, and ciprofloxacin (**TABLE 5**). Isolates of *P.aeruginosa* were most susceptible to ceftazidime (50.37%), followed by amikacin (49.63%), and ciprofloxacin (40.74%).

TABLE 1- ISOLATION OF PSEUDOMONAS AERUGINOSA FROM VARIOUS CLINICAL SPECIMENS

SPECIMEN	NO: OF ISOLATES	PERCENTAGE (%)
Pus	50	37.04
Miscellaneous	42	31.11
Sputum	29	21.48
Urine	11	8.15
Body fluids	3	2.22
Blood	0	0

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TABLE 2- PIGMENT PRODUCTION BY PSEUDOMONAS AERUGINOSA ON NUTRIENT AGAR AT DIFFERENT TEMPERATURES

INCUBATION TEMPERATURE	PIGMENT PRODUCTION			
	POSITIVE	%	NEGATIVE	%
25-30^{0}C	95	70.37	40	29.62
37^{0}C	90	67.11	45	33.33

TABLE 3- COMPARATIVE STUDY ON PIGMENT PRODUCTION BY PSEUDOMONAS AERUGINOSA USING DIFFERENT MEDIA

	PIGMENT PRODUCTION			
	POSITIVE	%	NEGATIVE	%
NUTRIENT AGAR	95	70.37	40	29.63
GELATIN AGAR	97	71.85	38	28.15
MILK AGAR	92	68.15	43	31.85

TABLE 4- PIGMENTS PRODUCED BY PSEUDOMONAS AERUGINOSA

	NO.	%
PIGMENT POSITIVE	97	71.85
PYOCYANIN PRODUCERS	37	38.14
PYOMELANIN PRODUCERS	32	32.98
PYORUBRIN PRODUCERS	28	28.87

TABLE 5- ANTIBIOTIC SUSCEPTIBILITY PATTERN OF PSEUDOMONAS AERUGINOSA TO AMIKACIN, CEFTAZIDIME, AND CIPROFLOXACIN

NO. OF ISOLATES	AMIKACIN		CEFTAZIDIME		CIPROFLOXACIN	
	NO.	%	NO.	%	NO.	%
135	67	49.63	68	50.37	55	40.74

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Discussion

P.aeruginosa was the most frequently isolated nonfermenter in our study (76.70%). This finding is supported by several studies.[21,22,23,24,25]Javiya etal[26]have reported a 20.28% isolation rate of *P.aeruginosa* from clinical specimens. The organism was isolated predominantly from the ulcers of diabetic patients.Selvi etal[27]have commented in their study that *P.aeruginosa* isolated from diabetic wound infections should never be considered insignificant. Resmi et al[28]have reported a 72.5% isolation rate from pus samples. 29 isolates (21.48%) of *P.aeruginosa*were recovered from sputum samples. It is the leading cause of nosocomial respiratory tract infections, particularly in the intensive care units.[29]Colonisation of the upper respiratory tract and endotracheal tubes are common and must be differentiated from true infection.[2] All the 11 nonfermenters isolated from urine samples were identified as *P.aeruginosa*.Majority of these urinary isolates were from catheterized patients.*P.aeruginosa* UTI often occur in patients on urinary catheters, stents, with stones, or in whom surgery or instrumentation was undergone.[1]17 out of the 135 isolates (12.6%) produced mucoid colonies. Pier GB et al[6]state that *P.aeruginosa* strains isolated from environment and nosocomial infections are usually nonmucoid.Mucoid strains are very common in patients with cystic fibrosis, and less frequently in bronchiectasis.[30]

P.aeruginosa produces several diffusible pigments of which pyocyanin production is of diagnostic significance. Pigment production depends on a dynamic metabolic equilibrium provided by the medium constituents such as peptones, minerals, and various ions. We noted that pigment production by *P.aeruginosa* was enhanced at room temperature than at 37^0C.Nutrient agar, milk agar, and gelatin agar were selected for pigment enhancement as they were cheap, easily available, and easy to prepare. In our study, majority of the isolates produced pyocyanin (38.14%).Pigment production was also observed on nutrient agar butts and they were examined weekly for 3 weeks. It was found that pigment production was enhanced after 5 or 6 subcultures.38 (28.25%) isolates did not produce any pigment.Resmi et al[29]had in their study, isolated 54% of pyocyanin-producing strains and 18.1% of nonpigment-producing strains of *P.aeruginosa*.

About 10-15% of *P.aeruginosa* strains produce pigmentonly when grown on pigment enhancement media.[16]Eventhough the identification of pigment-producing strains is not difficult, nonpigment-producing strains need additional tests.

It has been hypothesized that antibiotic usage may favourthe occurrence of nonpigment-producing strains.[31]Majority of the *P.aeruginosa* colonies were flat, spreading with serrated edges (Type 1) and the remaining were coliform-like colonies (Type 2) and mucoid colonies (Type 5).Regarding the denitrification feature, 130 (97.19%) strains reduced nitrate and 5 (3.93%) did not reduce nitrate. A study conducted by Hartingsveldt etal[32]showed that mutants of *P.aeruginosa* lacking nitrate reductase A have been isolated in many ways.

For identification purpose, 3 antibiotic discs were used- carbenicillin, polymyxin B, and kanamycin.Except for 1 isolate, all the strains were resistant to kanamycin. All the strains were sensitive to polymyxin B, and 4 strains were resistant to carbenicillin.

The*P.aeruginosa*in our study were most susceptible to ceftazidime (50.37%), followed by amikacin (49.63%), and ciprofloxacin (40.74%).Javiya et

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al[27]had reported resistance rate of 67.86% for ceftazidime, 50% for amikacin, and 69.64% for ciprofloxacin. They suggest that the use of amikacin should be limited to serious nosocomial infections inorder to prevent further resistance.In a study by Selvi et al,[28] 66.7% of *P.aeruginosa* were resistant to ciprofloxacin and 66.6% were resistant to amikacin and ceftazidime each.The percentage resistance to ciprofloxacin was 42% and to ceftazidime was 46% in a work done by Shampa et al.[33]Meharwal et al[34]had reported a high resistance rate for ciprofloxacin (76.3%) followed by 65.8% for ceftazidime, and 50% for amikacin. According to Bhatnagar et al[35]in a study, 44% of *P.aeruginosa* strains were resistant to ceftazidime, whereas 41% were resistant to ciprofloxacin and only 14.5% to amikacin.Juyal et al[36]report a high resistance rate to ciprofloxacin (73.77%), 68.85% resistance to ceftazidime, whereas only 27.87% resistance to amikacin.The percentage resistance reported by Krishnaprakash et al[37]was 75% for ciprofloxacin, 67% for ceftazidime, and 66% for amikacin. Most of these studies have amikacin as the most effective antipseudomonal antibiotic in vitro.But many strains do not respond to these antibiotics in vivo, although effective in vitro.[3]

An aminoglycoside combined with a beta-lactam antibiotic is often used in clinical practice.[31] Resistance can occur during treatment of infections caused by *P.aeruginosa* and multidrug resistance makes therapeutic options limited. The misuse of antipseudomonal antibiotics have also favoured the onset of multidrug resistance.

Conclusion

A total of 135 strains of *P.aeruginosa* were identified from 176 nonfermenters isolated from clinical specimens.*P.aeruginosa* was the most frequently isolated nonfermenter. Majority were pigment producers. Of the three antipseudomonal antibiotics (ceftazidime, amikacin, and ciprofloxacin) analysed, ceftazidime was the most effective in vitro and ciprofloxacin the least.The nonfermenters, especially *P.aeruginosa* are a continuing threat to effective therapeutic strategies because of their intrinsic and acquired resistance to many antibiotics. Improved antibiotic stewardship and infection control measures are the pressing needs of this hour to contain the emergence and spread of these multiply drug resistant pathogens.

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