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Original Article

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Multidrug Resistance Pattern of *Pseudomonas aeruginosa* Isolated from Patients with Nosocomial Infection

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Multidrug resistant (MDR) *Pseudomonas aeruginosa* is a threat to the patients having nosocomial infections as this pathogen increases the inpatients' morbidity and mortality by slowing down the whole treatment process. The aim of this study was to evaluate multidrug resistant phenotypes among nosocomial strains of *P. aeruginosa* for analyzing their antibiotic susceptibility pattern. A total of 108 *P. aeruginosa* clinical isolates were recovered from various samples (pus, wound swab, urine, sputum, blood and tracheal aspirate) of patients with nosocomial infections. Antibiogram was performed by disc diffusion according to Kirby-Bauer method to study the antibiotic sensitivity pattern of the pathogen against 14 regularly used antibiotics (amikacin, aztreonam, ceftazidime, ceftriaxone, co-trimoxazole, ciprofloxacin, gentamicin, levofloxacin, meropenem, netilmicin, doxycycline, amoxicillin/clavulanic acid, piperacillin/tazobactam, and tigecycline) in Bangladesh. The overall frequency of drug resistance was found to be very high (53.7% - 98.1%) to all of the anti-pseudomonal drugs tested. Resistance of *P. aeruginosa* strains against piperacillin-tazobactam was significantly less (53.7%) as compared to other thirteen antibiotics. However, isolates showed highest resistance (98.1%) to aztreonam. Next in order of resistance were doxycycline (95.4%), ceftriaxone (94.4%), amoxiclav (93.5%), and the others. The present study suggests that regularly used medications can no longer be utilized as first line therapies for suspected pseudomonad infections. This study claims for urgent epidemiological monitoring of the MDR *P. aeruginosa* strains in all hospitals of Bangladesh to prevent rapid dissemination of this opportunistic pathogen.

Keywords: Nosocomial infection, *Pseudomonas aeruginosa,* MDR, XDR

Introduction

Pseudomonas aeruginosa is an opportunistic and omnipresent pathogen having ability to adapt to a variety of settings, preferably humid environments. It is a typical nosocomial pathogen that causes severe hospital-acquired infections, particularly in patients who are critically unwell and immunocompromised. *P. aeruginosa* is the primary cause of infections such as urinary tract and respiratory infections, soft tissue and wound infections, and infections in individuals suffering from thermal traumas. It is also the main pathogen of cystic fibrosis [1,2]. Antibiotic resistance determinants can be easily acquired by *P. aeruginosa*, which is inherently resistant to several antibiotics. Additionally, *P. aeruginosa* has a significant potential for developing phenotypes of multidrug resistance. Different mechanisms of resistance have a substantial clinical impact since they reduce the range of therapeutic options available against *P. aeruginosa* infections, impair the effectiveness of drugs, and make *P. aeruginosa* infections exceedingly challenging to treat[3].

There have been numerous reports from around the world over the years indicating a surge in the rate of antibiotic resistance of *P. aeruginosa*, particularly to â-lactams, fluoroquinolones, and aminoglycosides[4]. Variations in the patterns of antimicrobial resistance for many organisms, including *P. aeruginosa*, also exist and may be caused by variations in the ways in which antibiotics are prescribed[5]. Antimicrobial drugs are frequently prescribed to the patients in Bangladesh; however, due to an increasing prevalence of multidrug resistant *P. aeruginosa*, hospitals currently have a scanty supply of medications for the treatment of their patients[6]. Dissemination of MDR strains of *P. aeruginosa* discharging from patients can impart in transferring the antibiotic resistance genes in the bacterial community.

To determine whether antibiotic-resistant pathogenic bacteria pose a risk to human health, it is necessary to monitor and characterize them. Local and regional surveillance studies help ~~to~~ better understanding of global trends in the organism's resistance to antibiotics. Periodic evaluation and analysis of multidrug resistance among microbial agents would allow doctors to spot trends in the MDR pattern to frequently prescribed antibiotics for a given organism. It might also help them choose the best antibiotic drug for empiric treatment in a specific situation. This study set out to assess the level of multidrug resistance to nosocomial strains of *P. aeruginosa* as well as the status of antimicrobial resistance to antipseudomonal agents.

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Materials and Methods

Sample Collection and Isolation of P. aeruginosa

This study was carried out during the period of November 2021 to August 2022. Samples were collected from the patients admitted to the hospital for at least 2-7 days. Samples from different sites (tracheal aspirate, blood, urine, pus, wound swab and sputum) were obtained from patients with Nosocomial Infections (NI) according to hospital records. The patients were independent of any epidemic outbreak and each isolate was taken from different individuals. Data on age, sex of the patients and source of the samples were also recorded. The preliminary works, i.e. sample collection, inoculation on primary plates were done in the laboratory of microbiology, Dhaka Medical College Hospital (DMCH), Bangladesh. Firstly, the patients' samples were inoculated onto MacConkey agar plate according to the protocol followed in DMCH laboratory. On the next day, the primary culture plates (MacConkey agar) onto which the raw samples were plated out, were collected and transported to the laboratory at the department, and suspected NLF colonies were picked and streaked onto Cetrimide agar (Oxoid Limited, England) to single out pure colonies of the *P. aeruginosa* isolates. All of the isolates were identified as *P. aeruginosa* by standard microbiological tests.

Biochemical Identification of Isolates

A series of biochemical tests (Gram staining, oxidase activity, catalase activity, lactose/dextrose/sucrose fermentation, indole production, MR-VP reactions, KIA, MIU, citrate reduction, urease activity, gelatin liquefication, starch hydrolysis and nitrate reduction) were performed according to the method described in 'Bergey's Manual of Determinative Bacteriology (2012)' for culture identification of the *P. aeruginosa* isolates.

PCR amplification of 16S rRNA gene amplification and sequencing

The isolates were identified presumptively according to the biochemical test results. For a confirmation, isolates were randomly selected for analysis of 16SrRNA gene sequence. For this, DNA was extracted by boiling method followed by amplification of the 16S rRNA gene using a universal primer set, 27F (52 -AGAGTTTGATCMTGGCTCAG-32) and 1492R (52 TACGGYTACCTTGTTACGACTT-32). The expected amplicon size is around 1450bp. In each setting, one positive and one negative control were included.

Antimicrobial Susceptibility Test

A standard disk diffusion (Kirby-Bauer) method was employed to determine the antimicrobial susceptibility profiles of *P. aeruginosa* isolates according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI)[7]. The isolates were classified as 'sensitive' (S), 'intermediate', and 'resistant' (R) accordingly. A twenty-four-hour old pure culture of isolates was standardized to 0.5 McFarland turbidity standards and swabbed on MHA media (Mueller-Hinton Agar, HiMedia, India) according to the standard working procedure. Incubation was performed at 37° C temperature for 18±2 hours. Antibiogram of the pathogens were determined using disc diffusion method onto MHA medium according to the CLSI guidelines to evaluate resistance to the following antimicrobials: amikacin (30 ìg), ceftazidime (30 ìg), co-trimoxazole (25 µg), ciprofloxacin (5 ìg), levofloxacin (5 ìg), amoxicillin / clavulanic acid (20/10 µg), piperacillin-tazobactam (100/10 ìg), doxycycline (30 µg), aztreonam (30 ìg), ceftriaxone (5 µg), colistin (10 ìg), gentamicin (10 ìg), meropenem (10 ìg), netilmicin (10 ìg), and tigecycline (30 µg). The isolates were defined as low-level drug resistant (LDR, non-susceptible to <3 antimicrobial classes), multidrug resistant (MDR, non-susceptible to e"3 antimicrobial classes), extensively drug-resistant (XDR, non-susceptible to all but d"2 classes), and pandrug-resistant (PDR, non-susceptible to all antimicrobial classes).

Phenotypic Detection of Carbapenemase activity

Modified Hodge test (MHT) was used to determine carbapenemase production. The presence of a 'cloverleaf shaped' zone of inhibition due to carbapenemase production by the test strain is considered as positive. An inoculum of *E. coli* ATCC 25922 was prepared and incubated for 2 hrs and adjusted to 0.5 McFarland standard and was inoculated on an MHA plate. After drying, 10 µg meropenem disk was placed at the centre of the plate and the test strain was streaked from the edge of the disk to the periphery of the plate in four different directions. The plate was incubated overnight at 35-37°C. The presence of a 'clover leaf' zone of inhibition due to carbapenamase production by the test strain was considered as positive.

Results

P. aeruginosa isolates in Clinical Samples

A total of 108 of the clinical isolates that were included each from a single patient, but from six different types of clinical samples. The isolates were presumptively identified as *Pseudomonas spp*. by biochemical tests (Table 1; Figure1) which were then confirmed to be *Pseudomonas aeruginosa* by 16S rRNA gene sequencing (Table 2). The frequency of *P. aeruginosa* isolates in different clinical samples is shown in Table 3. The highest occurrence of *P. aeruginosa* strains was spotted in wound swab samples (n = 52) followed by urine (n = 25), tracheal aspirate (n = 11), pus (n = 10), sputum (n = 9) and blood (n = 1).

Antibiotic Susceptibility Pattern of clinical P. aeruginosa isolates

Among the 108 clinical isolates of *P. aeruginosa*, the overall frequency of drug resistance was found to be very high (53.7% - 98.1%) to all of the anti-pseudomonal drugs tested. Resistance of *P. aeruginosa* isolates against piperacillin-tazobactam was significantly less (53.7%) as compared to 13 other antibiotics (ceftazidime, aztreonam, levofloxacin, ciprofloxacin, doxycycline, amoxiclav, tigecycline, netilmicin, amikacin, meropenem, co-trimoxazole, ceftriaxone and gentamycin). However, most highly resistant antibiotic was found to be aztreonam, 98.1% of the isolates showed high resistance to this antibiotic when compared

Table 1. *Results for Biochemical tests for the clinical isolates*

Biochemical Tests	Gram Staining	Catalase	Oxidase	KIA			Citrate use	MIU		NR	MR	VP	Indole	Presumptive Identification
				Slant	Butt	Gas		Motility	Urease					
Reaction Observed	Pink in color	Produced bubble	Blue	Red	Red	No crack	Prussian Blue	Turbid	Yellow	Red	Yellow	Yellow	No Red Ring	
Reaction Indicated	Gram Negative	Catalase Positive	Oxidase positive	Lactose Non-Fermenter	Glucose Non-Fermenter	No Gas	Citrate Positive	Motile	Urease Negative	Reduce nitrate	MR Negative	VP Negative	Indole negative	*Pseudomonas* spp.
No. of Isolates found positive	108/108	108/108	108/108	108/108	108/108	108/108	108/108	86/108	108/108	105/108	108/108	108/108	105/108	

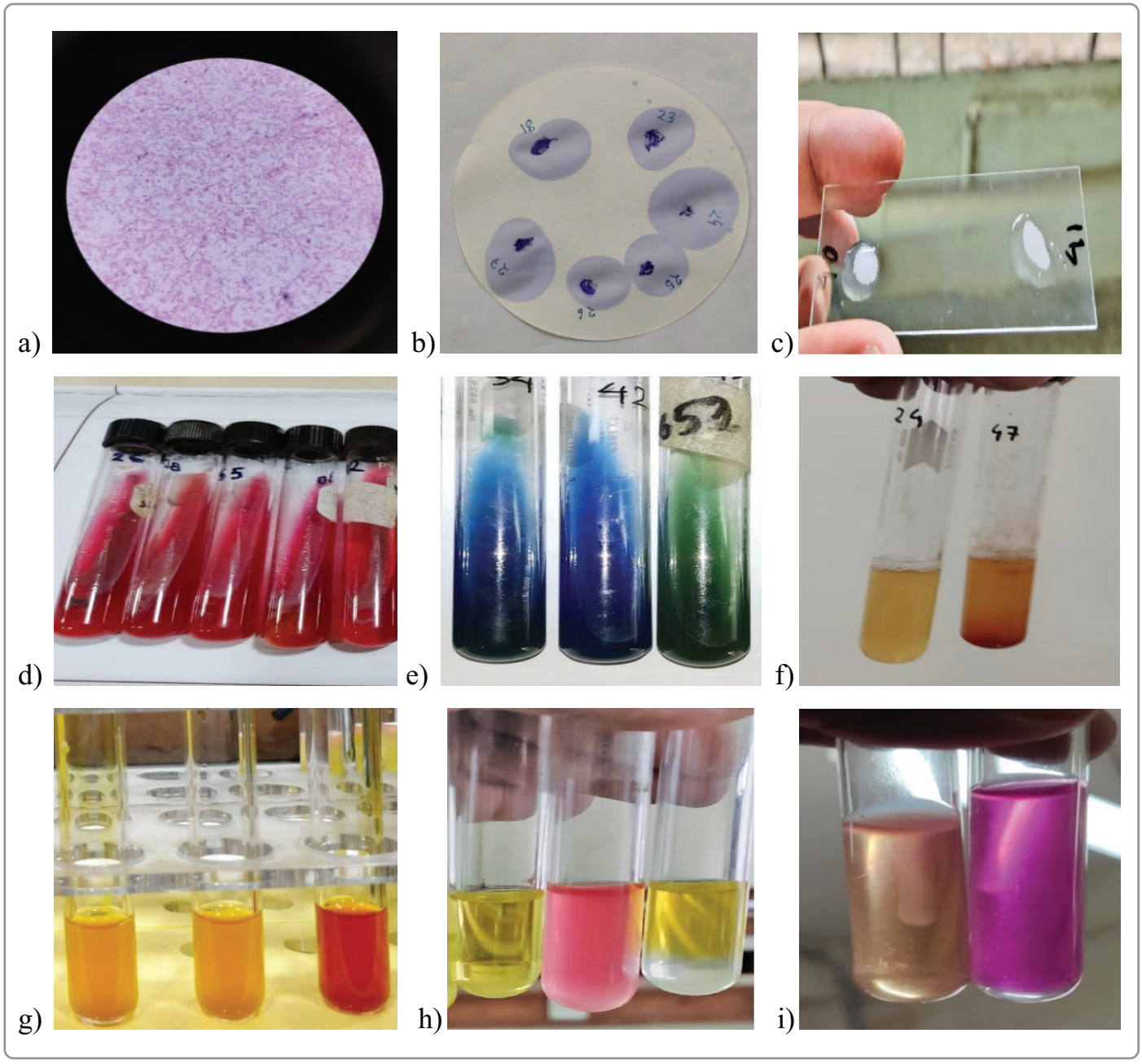

Fig. 1. *Representative figures of different biochemical tests performed on test isolates. (a) A Gram-negative isolate, (b) Oxidase positive isolates, (c) Catalase positive isolates, d) four Lactose non-fermenter isolates with a negative control (Right), (e) Citrase positive isolate (middle) with a positive control (Left) and a negative control (Right), (f) Nitrate reduction positive isolate (Right) with a negative control (Left), (g) two MR negative isolates with a positive control (Right), (h) two VP negative isolates with a positive control (Middle), (i) Urease-Indole negative isolate (Left) with a positive control (Right).*

Table 2. BLAST search results for 16S rRNA gene sequence similarity

Isolates	Closest blast match to NCBI Database	Query Coverage	E-value	PercentIdentity
MPY_0007	MK156466.1 *Pseudomonas aeruginosa* PF2	100%	0.0	98.00%
MPY_0018	MN490065.1 *Pseudomonas aeruginosa* BUYA-1	100%	0.0	99.87%
MPY_0026	OM534570.1 *Pseudomonas aeruginosa* BQ11	100%	0.0	99.93%
MPY_0034	OQ727070.1 *Pseudomonas aeruginosa* WS02	100%	0.0	99.93%
MPY_0045	OM818515.1 *Pseudomonas aeruginosa* GBWR9	100%	0.0	99.80%
MPY_0052	MW243044.1 *Pseudomonas aeruginosa* 39	100%	0.0	99.93%
MPY_0060	LT797517.1 *Pseudomonas aeruginosa* AT1RP4	100%	0.0	99.93%
MPY_0075	KX180920.1 *Pseudomonas aeruginosa* PBS	100%	0.0	99.91%
MPY_0082	MT373475.1 *Pseudomonas aeruginosa* NSJ008	100%	0.0	99.93%
MPY_0086	NR_026078.1 *Pseudomonas aeruginosa* DSM50071	100%	0.0	99.93%
MPY_0091	OQ568312.1 *Pseudomonas aeruginosa* M02	100%	0.0	99.93%
MPY_0097	MT300516.1 *Pseudomonas aeruginosa* NPP66	100%	0.0	99.93%
MPY_0100	OQ255854.1 *Pseudomonas aeruginosa* AC17	100%	0.0	99.93%
MPY_00105	MT771352.1 *Pseudomonas aeruginosa* PF-1	100%	0.0	99.93%
MPY_0108	OQ615324.1 *Pseudomonas aeruginosa* SI 1	100%	0.0	99.93%

Table 3. *Frequency of P. aeruginosa isolates in different clinical samples*

Sample Source	Frequency of P. aeruginosa isolates, no (%)
Wound Swab	52 (48.1)
Urine	25 (23.1)
Tracheal Aspirate	11 (10.2)
Pus	10 (9.3)
Sputum	9 (8.3)
Blood	1 (0.9)

to the others (Figure 2). Next in order of resistance were doxycycline (95.4%), ceftriaxone (94.4%), amoxiclav (93.5%), and the others.

Of 108 strains of *P. aeruginosa* obtained from various clinical sources, the majority 52 (48.15%) were isolated from the wound swab. Significantly high resistance to all antibiotics was shown by wound swab isolates and no significant difference was detected among the strains from other infections (Table 3). The resistance pattern of *P. aeruginosa* isolated from the lower respiratory tract (sputum sample) indicates that piperacillin-

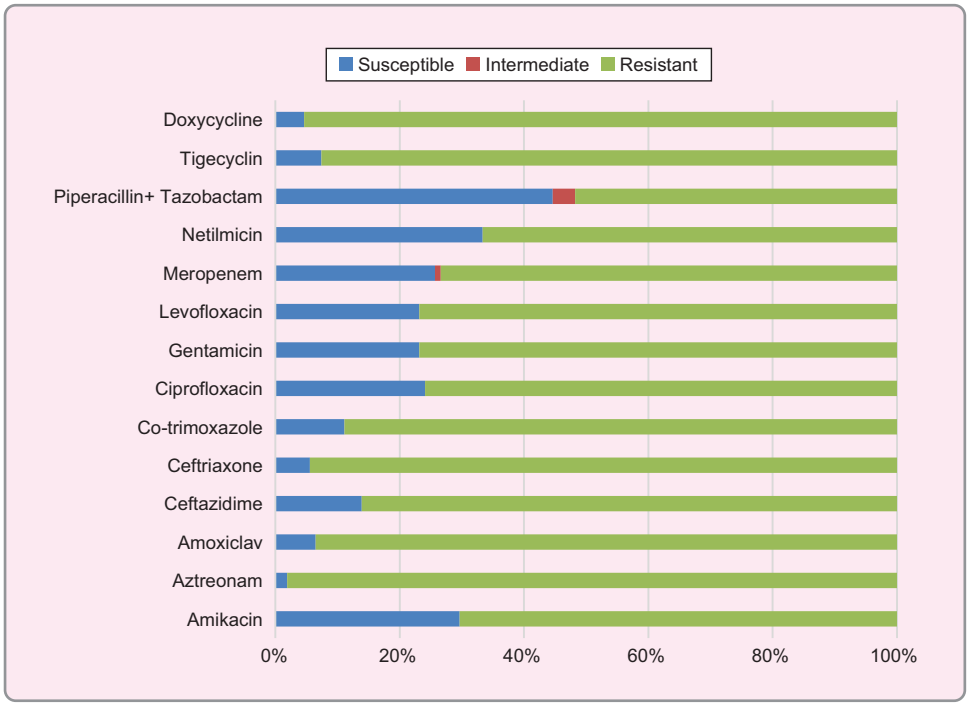

Fig. 2. *Overall drug resistance pattern of clinical isolates of Pseudomonas aeruginosa*

tazobactam occurs to be the most potent antibiotic (22.2% resistance rate). The strains were significantly less resistant (44.4%) to aminoglycosides (gentamicin, amikacin, and netilmicin) and meropenem (33.3%). There was resistance in around 90% of strains to ceftriaxone and ceftazidime (third-generation cephalosporins and antipseudomonal antibiotics). Subsequently, in order of resistance Fluoroquinolones exhibited the second-highest resistance among *Pseudomonas* isolates in this study . Highest resistance was observed against aztreonam and amoxiclav antibiotics. Significantly high resistance was shown to doxycycline by isolates from tracheal aspirate (100%). High levels of resistant isolates were spotted in wound swab and urine samples for all the antibiotics tested.

Only one of 108 isolates was found to be susceptible to all 14 antibiotics from 8 different classes. The other 107 strains were resistant to at least one of the antibiotics tested. Among them two strains were resistant to only one antibiotic and only one isolate was found to be resistant to two antibiotics of different classes. These *P. aeruginosa* strains (2.7%) can be categorized as LDR (Low-level Drug Resistant). Thirty isolates (27.8%) were resistant to at least three classes of antibiotics and thus categorized as MDR (Multidrug Resistant) strains. Sixty-three isolates (58.3%) were found to be extensively drug resistant (XDR) as they were non-susceptible to ≤2 classes of antibiotics. It is highly alarming that 11 of the clinical *P. aeruginosa* strains were resistant to all the antibiotics tested and so they can be classified as 'pandrug' resistant (PDR) strains.

Modified Hodge test was performed to detect the carbapenemase production ability among the carbapenem resistant isolates, since carbapenem are last resort for treating MDR *P. aeruginosa* and

Table 4. *Frequency of antibiotic resistant strains of P. aeruginosa in different clinical samples*

Antibiotics		Antibiotic ClassNumber of P. aeruginosa strains (n=108) %					
		Wound Swab n = 52	Urine n = 25	Tracheal Aspirate n = 11	Pus n = 10	Sputum n = 9	Blood n = 1
Amikacin	Aminoglycoside	41 (78.9)	19 (76.0)	7 (63.6)	4 (40.0)	4 (44.4)	0
Gentamicin		46 (88.4)	21 (84.0)	7 (63.6)	5 (50.0)	4 (44.4)	0
Netilmicin		35 (67.3)	20 (80.0)	7 (63.6)	5 (50.0)	4 (44.4)	1
Amoxiclav	Penicillin	52 (100.0)	23 (92.0)	8 (72.7)	9 (90.0)	8 (88.9)	1
Piperacillin-Tazobactam		32 (61.5)	13 (52.0)	6 (54.5)	4 (40.0)	2 (22.2)	1
Aztreonam	Monobactam	51 (98.1)	25 (100.0)	11 (100.0)	10 (100.0)	8 (88.9)	1
Ceftazidime	Cephalosporin	46 (88.4)	23 (92.0)	9 (81.8)	7 (70.0)	7 (77.8)	1
Ceftriaxone		50 (96.2)	24 (96.0)	7 (63.6)	9 (90.0)	8 (88.9)	1
Co-trimoxazole	Salfonamide	48 (92.3)	25 (100.0)	7 (63.6)	7 (70.0)	6 (66.7)	1
Ciprofloxacin	Quinolone	43 (82.6)	21 (84.0)	9 (81.8)	5 (50.0)	4 (44.4)	0
Levofloxacin		42 (80.7)	23 (92.0)	8 (72.7)	6 (60.0)	4 (44.4)	0
Doxycycline	Tetracycline	50 (96.2)	24 (96.0)	11 (100.0)	9 (90.0)	8 (88.9)	1
Tigecycline		50 (96.2)	23 (92.0)	9 (81.8)	9 (90.0)	8 (88.9)	1
Meropenem	Carbapenem	39 (75.0)	22 (88.0)	8 (72.7)	6 (60.0)	3 (33.3)	1

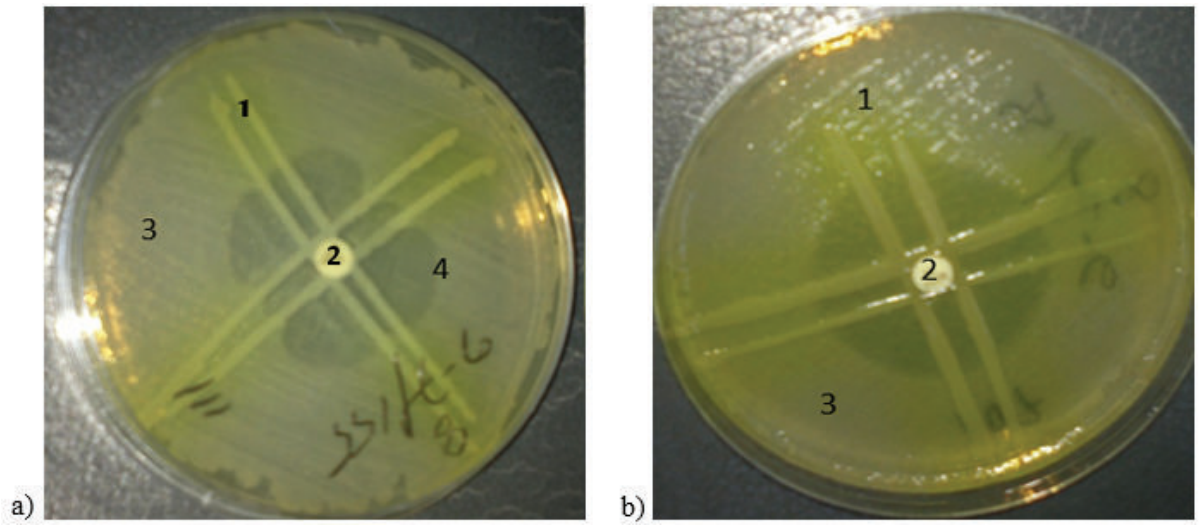

Fig. 3. *Determination of carbapenemase producing isolates by MHT; (a) MHT Positive; (b) MHT Negative; 1-Test strain, 2-Meropenem disk, 3- E. coli ATCC 25922, 4- Clover leaf shaped inhibition*

resistance against carbapenem has been emerged by producing this novel enzyme. We performed MH test for 74 carbapenem resistant isolates, among them 33 (45%) strains found to be carbapenemase positive. Isolates obtained from wound swab showed highest positivity (23/39).

Discussion

The multidrug-resistance pattern of clinical *P. aeruginosa* isolates collected in the present study indicates that the antibiotics which are the first line of therapy according to CLSI 2020 are now becoming obsolete, as they showed very high resistance to the drugs, including monobactams like aztreonam (97.2%) and tetracyclines such as doxycycline (95.4%) and tigecycline (92.6%). Resistance against amino glycosides tested in this study, gentamicin (76.9%), amikacin (70.4%), and netilmicin (66.7%), is also alarming whereas the scenario is different for aminoglycosides in India (30%)[8], Pakistan (20%)[9], and Nepal (25%)[10]. Determining the resistance pattern of antimicrobial agents may aid in selecting appropriate drug. The current study showed moderate-to-high antibiotic resistance (53.7% - 97.2%) in *P. aeruginosa* isolates, whereas *P. aeruginosa* showed low level drug resistance (5% - 30%) in studies from Saudi Arabia, Singapore, Malaysia, and Trinidad [6,11,12,13].

The rate of drug resistance against meropenem was 74.1% in this study, however, in a few studies a low rate of resistance to meropenem: (d"18%) [14] was reported. The reason behind the high resistance to meropenem in this study might be that, the drug is very commonly prescribed in the settings we studied. This claims a need to lessen the medication depending on cultures, as not just the *Pseudomonas* spp. will become resistant, but many other members from Enterobacteriaceae family would be resistant, for example, emergence of carbapenem-resistant Enterobacteriaceae.

Geographical variation in the susceptibility pattern of *P. aeruginosa* isolates may be related to antibiotic drug prescribing practices in discrete parts of the world. In our study, the lowest resistance rate of *P. aeruginosa* was against piperacillin-tazobactam (53.7%), which is still higher than the resistance (25%) reported in Jamaica[15]. However, researches from Iran and Saudi Arabia reported a high rate of resistance for piperacillin (66.4% and 54% respectively)[16], while a study showed low resistance (4-11%) to piperacillin in Dhahran[17]. These variations in the susceptibility rates may be associated with differences in the use of antibiotic in different selective pressure and settings.

Resistance of *P. aeruginosa* to fluoroquinolones is an emerging problem in many parts of the world. In this study, the resistance rates to ciprofloxacin and levofloxacin were 75.9% and 76.9%, respectively. Earlier studies from Saudi Arabia reported a much lower rate like the other parts of the world. Resistance to ciprofloxacin, in Saudi Arabia, was 50.9% [18], 42.8% [19] and 35% [14]. Similar rates were also found in India (49%)[20], Turkey (48.9%) [21], Iran (58%) [22]. In 2007, a study in Bangladesh showed resistance of clinical *P. aeruginosa* isolates towards ciprofloxacin being 75.5% [1]. However, researchers in Trinidad reported a lower rate (2.6%) of ciprofloxacin resistance[12]. The difference in the rate of ciprofloxacin resistance might be associated with the frequency of using fluoroquinolones and availability of oral doses.

In this study, resistance to ceftazidime was 86.1%, which is drastically higher than to the data showed in a study from Saudi Arabia (14%) [23]. However, a much higher rate of ceftazidime resistance was noticed in earlier studies from DMCH, Bangladesh (86.8%) [1]. Variable rates of resistance for ceftazidime were found in many parts of the world: Iran (68%) [22], India (40%) [24] and Singapore (23.4%)[11]. Resistance against other third generation cephalosporin drug (ceftriaxone) tested in this study was even higher (94.4%). The prescribing habits of each hospital and the selection pressure of particular antibiotics are thus typically correlated with the variations in the resistance rates. Ceftazidime should be regarded as a primary therapeutic agent for the treatment of severe pseudomonal infections or should be rotated with cefepime in order to prevent the establishment of resistance, either alone or in combination with aminoglycosides depending on the severity of the illness. However, resistance rate against co-trimoxazole has been lessened a little. In a study of 2007, the resistance rate of co-trimoxazole was 93.5% in Bangladesh [1], which, in the current study, is found to be 88.9%.

In the present study, the highest number of isolates were isolated from wound swab sample (48.1%), followed by from urine (23.1%), tracheal aspirate (10.2%) and pus (9.3%). Each hospital has a unique environment, so the distribution of *P. aeruginosa* samples may differ depending on the hospital. In our research, frequency of amoxiclav (100%) and ceftriaxone (96.2%) resistant microorganisms identified from surgical wound infections were larger than isolates collected from other sites. Similar resistance pattern was observed in a research by other groups in Bangladesh [25,26]. However, isolates obtained from urine were more resistant (100%) to aztreonam and co-trimoxazole than the strains from wound swab and others. Resistance against ciprofloxacin (96%) and ceftriaxone (84%) was also very alarming. On the contrary, earlier in a study carried out in Bangladesh showed 100% resistance to amoxicillin but a much lower resistance to ceftriaxone and ciprofloxacin (20.83% and 29.16%)[27]. All the isolates (100%) from lower respiratory infections (tracheal aspirate samples) were resistant to doxycycline, though less resistance was fond to piperacillin-tazobactam combination drug (22.2%) and meropenem (33.3%). The emerging rate of resistance to ciprofloxacin is frustrating (81.8%). On the contrary, in a study from Saudi Arabia, isolates from respiratory, urinary and wound infections showed high resistance to piperacillin[17]. It is encouraging that respiratory infections caused by *P. aeruginosa* can still be treated with these drugs.

According to the findings of this study, regularly used medications can no longer be utilized as first line therapies for suspected pseudomonad infections. In order to reduce infections,

routine microbiological surveillance should therefore be implemented as much as possible. A thorough investigation using more recent antimicrobials must also be conducted. Expectantly, this will lessen the resistance rate and thus the expense of treatment, and initiate high quality patientcare.

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