Egyptian Journal of Medical Microbiology Volume 34 / No.1 / January 2025 1-9 Online ISSN: 2537-0979

ORIGINAL ARTICLE

Detection of *OprD* Porin Gene in Multidrug Resistant *Pseudomonas Aeruginosa* Isolates in Patients with Chronic Liver Disease

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Key words:
MDR, P. aeruginosa, Antibiotic susceptibility, OprD.

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ABSTRACT

Background: *Pseudomonas aeruginosa is a gram-negative opportunistic organism that causes severe global healthcare-associated infections such as respiratory tract infections, sepsis, urinary tract and surgical site infections. A key determinant of P. aeruginosa is its remarkable resistance to antibiotics especially in immunocompromised patients and many of the isolates are multiple drug resistant.* ***Objectives:*** *The aim of the study is to investigate the existence of multidrug-resistant Pseudomonas aeruginosa among chronic hepatic patients in National Liver Institute, Menoufia university, to evaluate the multidrug resistance profile of isolated P. aeruginosa and detect oprD gene expression role in multidrug-resistant strains.* ***Methodology:*** *From 230 patients admitted in NLI, samples were taken after 48 hours from admission, and cultured on cetrimide agar, the GN-ID cards of VITEK-2 system was used to confirm Pseudomonal isolation and species identification. Antibiotic susceptibility was done using VITEK2 AST-GN73 cards and conventional-PCR was used for identification of oprD gene.* ***Results:*** *P. aeruginosa was the most common type of pseudomonal species, it represents (78.72%) of Pseudomonas species isolated mainly from urine samples. (81.08%) of the tested strains of P. aeruginosa were MDR. Parenteral nutrition was the significant risk factor, also Pseudomonas species were the most common cause of peritonitis and wound infections. 90% of multidrug-resistant P. aeruginosa strains showed oprD gene, while 10% of the isolates were oprD negative. The highest percentage of oprD gene was detected in P.aeruginosa isolates resistant to Carbapenems, Cephalosporins, and Quinolones.* ***Conclusion:*** *Identifying the resistance mechanism of bacteria is very complicated. Although oprD gene plays an important role in the resistance of antibiotic in P.aeruginosa because the action of oprD gene in a nonspecific manner. It is elusive to consider or describe an antibiotic resistance on the presence or absence of oprD gene.*

INTRODUCTION

Pseudomonas aeruginosa is an opportunistic human organism which causes acute life-threatening infections in elderly, critically ill and immunocompromised patients worldwide[1].

The development of multidrug-resistant (MDR) strains that display resistance to nearly all antibiotics except for one or two classes is becoming an uppermost problem of public health, increasing morbidity, mortality and length of hospital stay. Unfortunately, *P.aeruginosa* harbors a wide array of MDR mechanisms, including the loss of outer membrane barriers (porin *OprD*), over-expression of multidrug efflux pumps and endogenous antimicrobial inactivation[2].

Among gram negative bacteria, a well-known system conferring resistance of antibiotic in *P. aeruginosa* is *oprD* protein. The *oprD* is a purine located on the cytoplasmic membrane of *P. aeruginosa*. Due to extensive resistance of antibiotic in *P. aeruginosa*, only a few antibiotic classes can be utilized for treatment of infections. Therefore, according to previous studies, carbapenems antibiotic classes are the most important antibiotics used for this purpose. In fact, the carbapenem drugs are considered as one of the last alternatives for treating gram negative bacterial infections, particularly *P. aeruginosa* after failure of many antibiotics[3]. It has been known that the main reason for resistance to carbapenems is reduced diffusion of these antibiotics into bacterial cell caused by alteration in *oprD* gene expression. In fact, *P. aeruginosa* by reducing *oprD* gene expression leads to resistance of carbapenems antibiotic classes[3].

Due to seriousness and significant clinical implications associated with MDR *Pseudomonas*

isolates, this study aimed to isolate, identify, and characterize *Pseudomonas* organisms obtained from hepatic patients admitted in Gastrohepatology Department at National Liver Institute (NLI) Hospital, Menoufia University. By determining their prevalence in various specimens, evaluating antimicrobial susceptibility and detecting expression of *oprD* gene in multidrug-resistant *P.aeruginosa* .

METHODOLOGY

From December 2020 to January 2022, our study had been conducted involving 230 patients (143 males and 87 females), their ages ranged from 18 to 80 years admitted to Gastrohepatology Department in National Liver Institute (NLI) Hospital, Menoufia University, which got approval from Ethical Committee Board from NLI, Menoufia University by number NLI IRB 0003413/00362/2022.

We collected the patients' data including demographics, comorbidities, cause of hospital admission, duration of ICU stay, previous hospitalization and ICU stay, use of corticosteroids or chemotherapy, antibiotics intake, and use of invasive medical devices.

Samples collection:

Samples were taken from patients who were admitted for more than 48 hours developing clinical infection signs.

Samples involved urine, blood, ascetic fluid, tracheal tube aspirate, throat and nasal swabs**,** drain, urinary catheter, central venous catheter, sputum and wound.

Identification of the isolates:

Pseudomonas aeruginosa were identified by morphology of the colony, Negative gram stain, positive catalase test, positive oxidase test and green exopigment on nutrient agar and on cetrimide agar[4] then confirmed by VITEK-2 compact system GN-ID cards (bioMerieux,France)Fig (1).

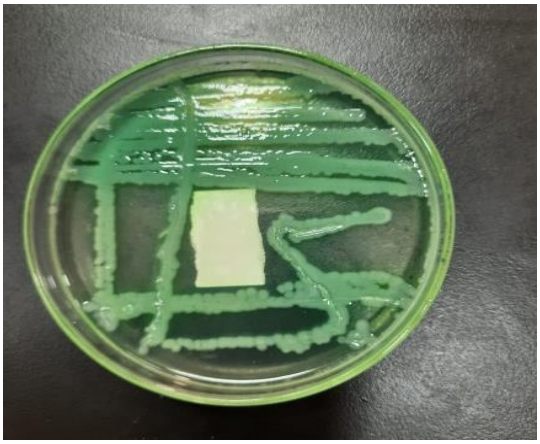

Fig. 1: *Pseudomonas aeruginosa* colonies on cetrimide agar producing characteristic greenish pigment.

Testing of antibiotic susceptibility:

The sensitivity of tested pseudomonal isolates to antibiotics was done by disk diffusion method and confirmed by VITEK2 AST-GN73 cards **(bioMérieux, France)** following the manufacturer's instructions. Antibiotic disks tests these antibiotic drugs: piperacillin (PPL, 100µg), piperacillin/ tazobactam (TZP, 100/10 µg), ceftazidime (CAZ,30 µg), cefepime (FEP, 30 µg), azetronam (ATM,30 µg), imipenem (IPM,10 µg), meropenem (MEM,10 µg), gentamicin (CN,10 µg), tobramycin (TOB,10 µg), amikacin (AK,30 µg), ciprofloxacin (CIP,5 µg), levofloxacin (LEV,5 µg), ofloxacin (OFX,5 µg).

Genotypic identification of virulence gene:

Detection of *oprD* gene in the pseudomonal strains was done by using conventional PCR and the following primers[5]:

oprDF 5'-ATGAAAGTGATGAAGTGGAG-3'
oprDR 5'-CAGGATCGACAGCGGATAGT-3'

Extraction and purification of DNA:

Thermo Scientific gene JET^{TM} genomic DNA Purification Kit was used for purification of DNA according to Manufacturers' instructions.

DNA amplification:

DNA amplification was done using the gene Primers purchased from **(**Thermo Fisher Scientific, USA). Mixtures of PCR contain Master Mix (2x) PCR of DreamTaq green, 10 µl from DNA Extract, 0.25 µl from each gene forward primer, 0.25 µl from each gene reverse primer.

The PCR program was carried out in a thermal condition as follows: initial denaturation at 94^{o}C for 3 min, 40 denaturation cycles at 94^{o}C for 40 sec, temperature of annealing at 55^{o}C for 45 sec, then extension at 72^{o}C for 1 min, and final extensions at 72^{o}C for 10 min.

Amplified products detection:

The amplified products size was visualized using (2%) agarose gels after ethidium bromide staining (Sigma, USA). *OprD* (1329bp) had been determined in comparison to a DNA ladder (500-3000bp) (Fermentas, Germany). Following electrophoresis, visualization was conducted through a UV trans-illuminator and photographed by digital camera.

Statistical analysis:

Statistical analysis has been calculated by the SPSS- version 20. Quantitative variables described as mean, SD, range with using Student t-test. Qualitative variables described as percentage, and Fisher's exact test or Chi-square test were used. Statistically significant result was adjusted at p value <0.05 *(version 20; Inc., Chicago. IL).*

RESULTS

This analytical cross sectional study involved 230 patients (143 females and 87 males), their mean age was (45.30 ± 18.75) years of whom (62.17%) were males.

Samples (230) were taken from the patients. Of them, 47 pseudomonal isolates (20.43%) were taken from different clinical samples; 11from urine, 10 from drain, 9 from blood, 4 from sputum, 4 from ascetic fluid, 3 from wound, 2 from CVC and T.T and one from throat and stent.

Pseudomonas aeruginosa represents (78.72%) of *Pseudomonas* species isolated mainly from urine samples, *Pseudomonas stutzeri* represents (12.76%) of *Pseudomonas* species isolated mainly from blood samples, *pseudomonas fluroscens* represents (6.38%) of *Pseudomonas* species isolated mainly from blood samples, the least percent among samples was *Pseudomonas putida* (2.13%) isolated from ascetic fluid samples with no significant relation (p value > 0.05) (table 1).

Table 1: Distribution of the isolated *Pseudomonas* species in different samples by Vitek-2 compact system.

Sample among patients	*Pseudomonas* species (n=47)					Test of Sig.	P value
	Total No. (%)	*Pseudomonas aeruginosa*	*Pseudomonas stutzeri*	*Pseudomonas fluroscens*	*Pseudomonas putida*		
Total	**(n=47)**	**N=37(78.72)**	**N=6(12.76)**	**N=3(6.38)**	**N=1(2.13)**		
Ascetic	4 (8.51)	2 (5.4)	1 (16.67)	-	1 (100)	Fisher's Exact Test	>0.999
Blood	9 (19.15)	3 (8.11)	4 (66.67)	2 (66.67)	-		
Urine	11 (23.04)	11 (29.73)	-	-	-		
Drain	10 (21.28)	10 (27.03)	-	-	-		
Throat swab	1(2.13)	1 (2.7)	-	-	-		
Wound	3 (6.38)	2 (5.4)	1 (16.67)	-	-		
Sputum	4 (8.51)	4 (10.81)	-	-	-		
Stent	1(2.13)	1 (2.7)	-	-	-		
central venous catheter (CVC)	2 (4.25)	1 (2.7)	-	1 (33.33)	-		
Tracheal tube	2 (4.25)	2 (5.4)	-	-	-		

A significant relationship was detected between pseudomonal infection, wound infections and peritonitis (p value <.05) as shown in table 2.

Table 2: Distribution of the isolated Pseudomonas in different types of infections.

	Samples taken	All infection N = 164	Pseudomonal infection (n =47)	Non-Pseudomonal infection (n =117)	Test of Sig.	P value
Respiratory tract infection	Throat, Sputum, Tracheal tube	40 (24.39)	7 (14.89)	33 (28.21)	X2 = 2.540	0.111
Urinary tract infection(UTI)	urine	36 (21.95)	11 (23.4)	25 (21.37)	X2 = 0.006	0.939
peritonitis	Ascetic, drain	29 (17.68)	14 (29.79)	15 (12.82)	X2 = 5.517	0.018*
Wound infection	wound	3 (1.83)	3 (6.38)	0 (0.00)	Fisher's Exact Test	0.022*
Sepsis	Blood, cvc, stent	56 (34.15)	12 (25.53)	44 (37.61)	X2 = 1.670	0.196

A significant relationship was detected between *pseudomonas* infection and parenteral nutrition (p value <0.05) (Figure 2).

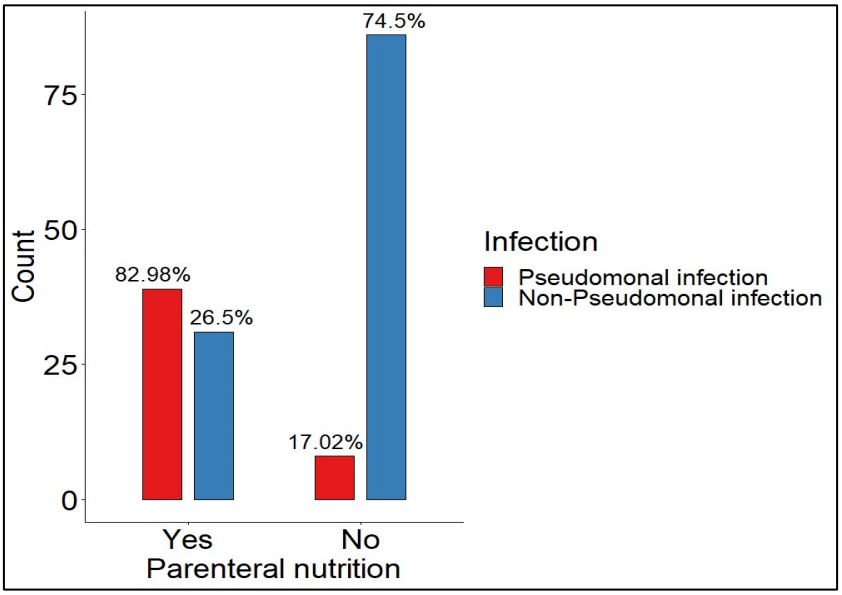

Fig. 2: Frequency of *Pseudomonas* Infection among patients on parenteral nutrition.

Antibiotic sensitivity of *pseudomonas* isolates:

Testing of *pseudomonas* isolates sensitivity to antibiotics showed that *Pseudomonas* exhibited high resistance rates for most tested antibiotics. The highest resistance rates were for Imipenem (81.08%), Meropenem (81.08%), Cefotaxime (81.08%) Ceftazidime (81.08%), Ciprofloxacin (81.08%), followed by Norfloxacin (78.38%) and finally Cefepime and Gentamycin (75.68%) as shown in table 3.

Table 3: Pattern of antibiotic sensitivity of *pseudomonas* by VITEK 2 compact system

Antimicrobial groups	Antimicrobial agent MIC	Antibiotic concentration (µg)	*Pseudomonas aeruginosa* (no=37)		
			Sensitive	Intermediate	Resistant
			No (%)	No (%)	No (%)
Carbapenems	**Imipenem** (IPM)	10	7(18.92)	-	30 (81.08)
	Meropenem (MEM)	10	7(18.92)	-	30 (81.08)
Cephalosporins	**Cefotaxime (cfx)**	30	7(18.92)	-	30 (81.08)
	Ceftazidime (FEP)	30	7(18.92)	-	30 (81.08)
	Cefepime (CEF)	30	8 (21.62)	1 (2.70)	28 (75.68)
Aminoglycosides	**Amikacin** (AK)	30	8 (21.62)	2 (5.40)	27 (72.98)
	Gentamycin (GEN)	10	8 (21.62)	1 (2.70)	28 (75.68)
Penicillin	**Piperacillin-Tazobactam** (TZP)	100/10	10 (27.03)	-	27 (72.98)
Quinolones	**Ciprofloxacin** (CIP)	5	7(18.92)	-	30 (81.08)
	Norfloxacin (NOR)	10	8 (21.62)	-	29 (78.38)

The antibiotic sensitivity pattern of *P. aeruginosa* isolates revealed that (81.08%) were MDR while (18.92%) were resistant to one or two groups of antibiotics as described in (table 4).

Table 4: Drug resistance pattern in the isolated strains by Vitek-2 compact system.

Variables	isolates cases (No=37)	
	No.	%
MDR	30	81.08
Non MDR	7	18.92

According to virulence gene frequency, *oprD* gene had been detected in 90 of multidrug-resistant *Pseudomonas aeruginosa* isolates and absent in 10% of *Pseudomonas aeruginosa* isolates as shown in Figure 3.

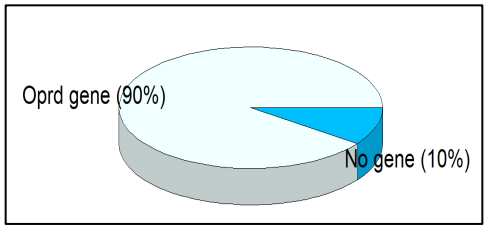

Fig. 3: Frequency of *oprD* gene among studied isolate.

Identification of oprD gene among studied Multidrug resistant Pseudomonas aeruginosa isolates by using conventional PCR:

Lanes from 1,2,3,4,5,6,7,9,11,12,13,14,15 were positive for *oprD* gene, Lanes 8,10,28 were negative for *oprD* gene Fig (4,5).

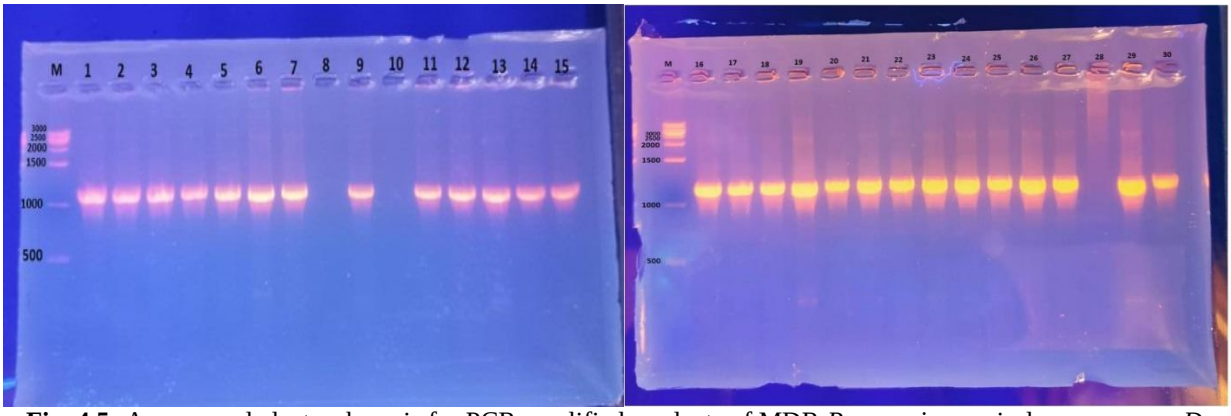

Fig. 4,5: Agarose gel electrophoresis for PCR amplified products of MDR *P. aeruginosa* virulence gene *oprD*.

Relationship between antibiotic susceptibility and virulence gene:

No significant relation between antibiotic resistance patterns among MDR *P. aeruginosa* isolates and presence of *oprD* gene. The highest frequency of *oprD* gene detected in *P. aeruginosa* isolates resistance against Carbapenems, Cephalosporins, and Quinolones Fig (6).

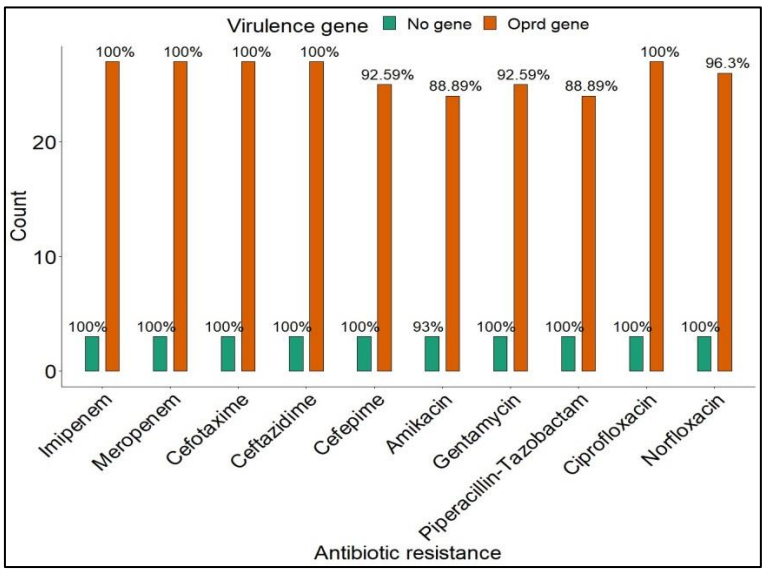

Fig. 6: Antimicrobial- resistance pattern of MDR *P. aeruginosa* in relation to *oprD* gene.

DISCUSSION

Multidrug-resistant *P. aeruginosa* has a significant public health concern among hospitalized patients. Pseudomonal infections can be fatal and managing them is difficult and associated with high morbidity and mortality rates[6].

Two hundred and thirty patients participated in our study; their mean age was (45.3) years of whom (62.17%) were males. Our findings agreed with results of *Yang et al.,*[7] in China and *Matta et al.,*[8] in Lebanon where 58% and 54.5% of included patients were males. In contrast to our results, *Ibrahim et al.,*[9] in Sudan and *Al Rahmany et al.,*[10] in Oman found that most of the involved patients were females (59%) in Sudan and (61.6%) in Oman.

Pseudomonas strains were isolated in our study at a rate of (20.43%). Similar to our results, *El-Badawy et al.,*[11] in Egypt reported that *P. aeruginosa* represent (13%) of the gram-negative isolates at Ain Shams University Hospital, Egypt. Also, *Tabah et al.*[12] recorded that most isolated gram- negative organisms were *Klebsiella* spp. (27.9%), *E. coli* (15.8%) and *Pseudomonas* spp. (14.3%).

Our study stated that *P. aeruginosa* was the predominant *Pseudomonas* spp. representing (78.72%) (37/47) followed by *P. stutzeri, P. flutoscens and P. putida* (12.76%,6.38% and 2.13%, respectively). Similarly, in concurrence with our study, *Addis et al.,*[13] in Ethiopia found that (98%) of *Pseudomonas* spp. were *P. aeruginosa* followed by *P. putida* that represent (2%). *Kumari et al.,*[14] in India also reported that *P. aeruginosa* (95%) was the prevalent species and *P. putida* was (2.5%). Also, *Bitew,*[15] in Ethiopia reported that *P. aeruginosa* was the prevalent species while *P. putida* represented only (7.7%).

In our study we found that *Pseudomonas* species were the most prevalent cause of wound infections and peritonitis, the results were significant (p value < 0.05). This was in agreement with *Carmeli et al.,*[16] who reported that *P.aeruginosa* is accountable for about 10% -20% of hospital acquired infections as sepsis and bacteremia in ICU, cystic fibrosis, pneumonia, urinary tract infections, burn infection and wound infection.

In our study (81.08%) of the tested strains of *P. aeruginosa* were MDR and showed resistance to at least one antibiotic in the three classes of antibiotics, so considered as MDR[17]. Higher levels of MDR (96%) of *Pseudomonas* species were recorded by *Abd El-Baky et al.,*[18] in Egypt. Also, *Adesoji et al.,*[19] in Nigeria and *Bitew,*[15] in Ethiopia found that among *Pseudomonas* isolate, (100%) and (69%) were MDR. Lower rates were recorded in Ethiopia where the overall MDR total tested *Pseudomonas* isolates rates were recorded to be (23%)[13]. These could be due to difference in the nature of infection with different associated risk factor[20]. In addition, the epidemiology of MDR microorganisms fluctuates from year to year between hospitals, wards, departments and the geographical zone. Also due to implementation and applying infection control measures[21].

Regarding the antibiotic sensitivity in our study, we found that *Pseudomonas* species exhibited high resistance rates for most of the tested antibiotics. The highest rates of resistance were for Imipenem, Meropenem, Cefotaxime, Ceftazidime, Ciprofloxacin (81.08% for each), followed by Norfloxacin (78.38%) and finally Cefepime and Gentamycin (75.68% for each). These findings emphasize the importance of continuous surveillance and appropriate antimicrobial stewardship to treat the emergence and spread of multidrug-resistant bacteria.

Our results were aligned with studies reported by *Fahim,*[22] in Egypt, who reported resistance rates of (79.8%, 76.3%, 80.7% and 71%) for ciprofloxacin, levofloxacin, ceftazidime and gentamycin, respectively. *Abd El-Baky et al.,*[23] in Egypt reported comparable rates for ceftazidime (63%) and azetreonam (60%) and tobramycin (50%). Also, *Shebl and Mosaad,*[24] in Egypt, found rates of resistance (43.5%, 66.7%, 64% and 76.5%) for piperacillin/tazobactam, ceftazidime, norfloxacin and ofloxacin, respectively. *Ibrahim,*[25] in Saudi stated that resistance to piperacillin/tazobactam and cefepime were (46.3%) and (53.3%), respectively. *Kumari et al.,*[14] in India reported higher resistance levels, with rates of (69%) for levofloxacin, (68%) for gentamycin, (67%) for ciprofloxacin, (66%) for ceftazidime, (63%) for cefepime, (58%) for amikacin, and (56%) for piperacillin., *Bitew,*[15] in Ethiopia reported the drug resistance of *Pseudomonas* isolates with tobramycin was (6.6%), gentamycin (13.1%), piperacillin/tazobactam combination (16.4%), cefepime (19.7%), ciprofloxacin (19.7%), levofloxacin (23.0%), and ceftazidime (27.9%) demonstrating reduced resistance. Conversely, lower ceftazidime resistance rate (43.8%) was reported by *Wassef et al.,*[26] in Egypt.

P. aeruginosa is a common pathogen isolated from patients with hospital acquired infections. Due to its antimicrobial resistance, limited classes of antibiotics can be used for cure of infection with *P. aeruginosa*. Of these, the carbapenems are very important; however, the occurrence of carbapenem-resistant strains is gradually increasing over time. Deficiency or loss of the outer membrane protein *OprD* confers *P. aeruginosa* a basal level of resistance to carbapenems, especially to imipenem. Functional studies have revealed that loops 3and 2 in the *OprD* protein contain the entrance and/or binding sites for imipenem. So, any mutation in these loops causes conformational changes could result in carbapenem resistance. *OprD* is also a common channel for some amino acids and peptides, and competition with carbapenems through the channel may also occur.

Furthermore, *OprD* is a highly regulated protein at transcriptional and post-transcriptional levels by some metals, small bioactive molecules, amino acids, and efflux pump regulators. Because of its hypermutability and highly regulated properties, *OprD* is thought to be the most prevalent mechanism for carbapenem resistance in *P. aeruginosa*. Developing new strategies to combat infection with carbapenem-resistant *P. aeruginosa* lacking *OprD* is an ongoing challenge.

our study revealed that *oprD* gene was present in (90%) of multidrug-resistant *Pseudomonas aeruginosa* strains and absent in (10%) of them. The highest frequency of *oprD* gene was detected in *P. aeruginosa* isolates resistant to Carbapenems, Cephalosporins, and Quinolones. This was in agreement with *Nmema et al.,*[5] who reported that o*prD* gene was detected in (90%) of the isolates. (75%) of Carbapenem resistant strains (CR) were among the strains showing *oprD* gene. (25%) CR strain was *oprD* negative and *Li et al.,*[27] who reported that none of the strains showed low expression of *oprD*. Also, *Terzi et al.,*[28] reported that increased *oprD* levels were detected in 2 of the 18 imipenem-resistant *P. aeruginosa* clinical isolates, although the data support the idea that the basic mechanism of imipenem resistance could be via the loss of *oprD*, they do not fully explain the role of *oprD* and indicate that other mechanisms may play an important role. Also, *Hassuna NA, et al.,*[2] reported efflux pumps were overexpressed in 21.8% and 18.7% of the isolates, with no down-regulation of *oprD*. Also, *Arabestani, et al.,*[3] reported that several strains of resistant *P. aeruginosa* were found with significant *oprD* gene expression. Also, *Elmosallamy et al.,*[29] reported that the different levels of concurrence between phenotypic and genotypic outcomes of carbapenem resistance in P. aeruginosa isolates based on the expression of MexA, MexE, and MexX efflux pump systems, can be explained by considering that other factors beyond efflux pump expression can influence carbapenem resistance. For instance, mutations in other genes, like those involved in outer membrane permeability or carbapenemase production, can impact resistance levels and contribute to variations in agreement.

We could not find a significant correlation between *oprD* gene expression and carbapenems resistance; thus, it is thought that other mechanisms such as carbapenemases have caused this resistance pattern. The roles of other involved agents and mechanisms in resistance development should not be ignored. Finally, the clinical context should not be ignored since the isolates used in this study was obtained from different clinical sources and patient populations. Variations in clinical context, such as patient demographics, comorbidities, and prior antibiotic exposure, can influence resistance mechanisms and expression levels.

Further studies will be important to understand the mechanisms underlying this apparent discordance between *oprD* levels and multidrug resistance. While *OprD* porin proteins exhibit an important role in carbapenem resistance in *P. aeruginosa*, this resistance cannot be explained by *OprD* levels alone, and other important interactions may influence carbapenem susceptibility. Characterization of mechanisms of carbapenem resistance could provide additional therapeutic targets or allow for alternative strategies to enhance the carbapenem efficacy.

CONCLUSION

The high prevalence of MDR among *P. aeruginosa* isolates was alarming leaving only few therapeutic options available for management of *p. aeruginosa* infections. Considering the present study and also other similar studies, concluded that *P. aeruginosa* use various mechanisms to avoid other toxic molecules and antibiotics. *OprD* is a house-keeping gene. However, the expression level of this gene has not always the role in decreasing the susceptibility to various antibiotics, the roles of other involved agents and mechanisms in resistance development should not be ignored. Therefore, surveillance of the antimicrobial susceptibility patterns of *P. aeruginosa* is of critical importance in understanding new and emerging resistance trends, reviewing antibiotic policies and informing therapeutic options. Increasing Carbapenem resistant *P. aeruginosa* strains from hospital patients calls for greater commitment in drug development and research.

Declarations:

Consent for publication: Not applicable

Availability of data and material: Data are available upon request.

Competing interests: The author(s) declare no potential conflicts of interest with respect to the research, authorship and/or publication of this article.

This manuscript has not been previously published and is not under consideration in another journal.

Funding: Authors did not receive any grants from funding agencies.

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