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Original article

Molecular detection of efflux pump and virulence factors genes in *Pseudomonas aeruginosa*

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ABSTRACT

Background: *Pseudomonas aeruginosa (P. aeruginosa)* is an opportunistic virulent bacterium with natural resistance to several antibiotics. It is an important causative agent in respiratory tract infections, surgical wound infections, burn wounds, and urinary tract infections. It has many virulent factors enhancing its pathogenesis e.g., tox A, type III secretion system that play a crucial role in tissue destruction. *Pseudomonas aeruginosa* is resistant to many antibiotics through different mechanisms including the availability of an efflux pump that extrudes antibiotics outside the bacteria. Numerous efflux genes are discovered e.g., MexAB-OprM, MexCD-OprJ, or MexXY (-OprA). **Aim of work:** To investigate some of the virulence genes and efflux pump genes in *P. aeruginosa* and to estimate their impact on antibiotic resistance. **Methods:** This current study was conducted on 250 different samples (sputum, urine, surgical wound, and burns) culture and sensitivity were done to the isolated bacteria, then molecular detection of efflux pump and some virulence genes by PCR. **Results:** Out of 250 samples, 46 yielded *P. aeruginosa*. These 46 samples were collected from 22 males and 24 females. Forty percent of the isolates were resistant to ceftazidime and cefepime followed by 37% resistance to ofloxacin and 36% resistance to gatifloxacin. Imipenem was the most susceptible antibiotic. Of all the isolated *Pseudomonas* 87% were multidrug-resistant. Exotoxin S (Exo S) gene was found predominantly in surgical wound samples 10 (21.7%), followed by burn samples, and sputum samples. **Conclusion:** There was a significant association between the availability of efflux genes and antibiotic resistance in *Pseudomonas*. *Pseudomonas aeruginosa* carries multiple mechanisms to destroy human cells and to compete with antibiotics, knowing these factors may be helpful to counteract them by targeting virulence or inhibiting efflux mechanisms.

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Introduction

Pseudomonas aeruginosa (*P. aeruginosa*) is an opportunistic virulent bacterium with natural resistance to several antibiotics. For humans, *P. aeruginosa* is highly important in several disorders such as burns and cancer and immunocompromised patients [1]. *Pseudomonas aeruginosa* is also highly prevalent in burns and surgical wound infections, as well as pneumonia, endocarditis, endophthalmitis, meningitis, septicemia, and conjunctivitis [2].

Several virulence factors, such as exotoxins, proteases, pigments, secretion systems, alginate capsules, flagella, and pili, are linked to the virulence of *P. aeruginosa* [3]. Exotoxin A, which is produced by the tox A gene, is one of them. It is an extracellular enzyme that prevents host cells from synthesizing proteins by turning off elongation factor 2 [4]. The type III secretion system (T3SS) exotoxins (exo S, exo T, exo U, and exo Y) are thought to be crucial virulence factors for *P. aeruginosa* [5]. Actin cytoskeleton disruption is caused by the type III cytotoxins (exoS, exoT, and exo Y), which have adenylate cyclase activity. Cell lysis is caused by exoU, a type III cytotoxic phospholipase, which breaks the host cytoplasmic membrane [6].

Antibiotic resistance pathways in *P. aeruginosa* might be innate or acquired [7]. One of the most crucial pathways that result in multidrug-resistant (MDR) phenotypes is the up-regulation of multidrug efflux systems. Antibiotic resistance is caused by transport proteins called efflux pumps, which help remove hazardous chemicals from cells [8]. Gram-negative bacteria frequently have resistance nodulation cell division (RND) efflux pumps, which are almost invariably chromosomally encoded [9]. In *P. aeruginosa*, several RND family systems have been identified [10]. MexAB-OprM, MexCD-OprJ, MexEF-OprN, or MexXY (-OprA) overexpression among these systems has been observed to play a clear role in decreased sensitivity to antibiotics [11].

Numerous antimicrobials, such as meropenem, chloramphenicol, tetracycline, ciprofloxacin, and nalidixic acid, as well as cephalosporines or penicillins, are pumped out via the MexAB-OprM system [12]. So, this study aimed to investigate some of the virulence determinants of *P. aeruginosa* and the presence of MexAB-OprM efflux genes and their relation to antibiotic resistance.

Patients and methods

This cross-sectional study was constructed at the Department of Medical Microbiology and Immunology and Clinical Pathology Departments, Faculty of Medicine Sohag University from June 2022 to February 2023. After obtaining ethical committee approval and institutional review board IRB number: Soh-Med-22-11-18.

Sample collection, identification, and antibiotic sensitivity testing

The current study was conducted on 250 different samples (sputum, urine, surgical wound, and burns). Out of them, 46 samples yielded *P. aeruginosa* which were taken under aseptic conditions from different departments(plastic, tropical medicine, ICU, chest, general and vascular surgery).

Samples were cultured on cetrimide agar (Himedia, India), and incubated at 37 ^{0}C for 24 hrs, growth appears as green colonies on cetrimide agar that were confirmed by oxidase test. Bacteria were preserved on trypticase soy broth with 20% glycerol and stored at -60 ^{0}C for further molecular examination.

Disc diffusion method was done to all of the isolates using the following discs: piperacillin 100 µg, ceftazidime 30g, cefepime 30µg, aztreonam 30 µg, imipenem10 µg, colistin sulfate 10µg, polymyxin B300 units, gentamycin10µg, tobramycin 10 µg, amikacin 30 µg, netilmycin 30 µg, ciprofloxacin 5µg, levofloxacin 5 µg, lomefloxacin 10 µg, norfloxacin 10 µg, ofloxacin 5µg, gatifloxacin 5 µg. According to CLSI 2016, inhibitory zone diameters were classified as being sensitive, moderate, or resistant.

We choosed CLSI 2016 as it has a diameter zones of both colistin and polymyxin used in this sudy but CLSI 2022 has no readings for diameters of disc diffusion method, also by revising the diameters of CLSI 2022 and 2016 there was no change except for ciprofloxacin and also the diameters are cosistant with our readings

DNA extraction and molecular identification of the virulence and efflux genes

DNA was extracted from freshly subcultured bacteria according to the method [13]. Extracted DNA was stored at -20 for further use. Conventional PCR (Biometra, Germany) was done to detect genes of virulence(tox A, type III secretion system genes(exo U, exo S) and efflux genes (mex A, mex B, opr M, opr D), primers sequences (Invitrogen by

Thermo Fischer), cycling condition and amplicon size are summarized in **table (1).** The following reaction mixture was used in each PCR reaction to adjust the volume to a final value of 25 µL: 12.5 µL of cosmo red PCR Master Mix [2x] (Willowfort, Uk), 1 µL of forward primer, 1 µl of reverse primer, 3 µl of template DNA then the reaction was adjusted to 25 µL with nuclease-free water Additionally, negative control tubes without a DNA template were added. Following amplification, agarose gel electrophoresis was used to analyze 10 µL of the PCR mixture (2% agarose in Trisborate-EDTA stained with ethidium bromide). The Gene Ruler 50 bp DNA ladder (Invitrogen, Thermo Fisher) was used as a DNA size marker visualization of bands was done using a DNA documentation system [13].

Statistical analysis

Statistical analysis was done by SPSS version 16 (SPSS Inc., Chicago, USA) to analyze the data. Categorical data are expressed as numbers and percentages, whereas continuous data are expressed as mean (standard deviation). The Chi-square test was used to analyze categorical data. Graphswere produced by using the Excel program.

Results

Forty-six *P. aeruginosa* were collected from 22 males (47.8%), and 24 females, (52.2%). The patient's mean age was 50.5 years **(Table 2).**

Forty percent of the isolate were resistant to ceftazidime and cefepime followed by 37% resistance to ofloxacin and 36% resistance to gatifloxacin, imipenem was the most susceptible antibiotic 31(67.3%) as illustrated in **table (3).**

Of all the isolated pseudomonas 40(87%) were MDR (The European Centre for Disease Prevention and Control (ECDC) criteria define MDR as acquired non-susceptibility to at least one agent in three or more antimicrobial categories).

Virulence genes in *Pseudomonas* Figure (1)

ExoU, exo S were found in 18(39.13%) of the isolates but toxA was found in 4(8.7% **.**

Distribution of virulence genes and efflux genes according to the type of infection

Tox A gene was equally present in sputum and diabetic foot samples 2(4.3%). While, the exo S gene was found predominantly in surgical wound samples 10 (21.7%), followed by a burn, sputum samples 4(8.7%), but mex A gene was significantly present in surgical wound samples 8(17.4%) as in **table (4).**

Relation of Presence of virulence genes and multi drug resistance

There was no significant association between the presence of Exo S. ExoU and tox A and presence of MDR **(Table 5).**

The association between efflux genes and MDR isolates was significant (*p*-value <0.001). the presence of 2 efflux genes was present in 55% of MDR-resistant strains **(Table 6)**.

We deleted cycling conditions in response to comment that **table (1)** is over crowded.

In respone to comment, Table 2 is better to select the most important data only as (age, sex, type of infection,....) and remove less important data.

Table 1. Different gene sequences with their cyclic conditions used in the PCR reaction.

The gene	Primers sequence	Amplicon size(bp)	References
Tox A	F:CTGCGCGGGTCTATGTGCC R:GATGCTGGACGGGTCGAG	270	**(14)**
Exo U	F: CTAGAAGAGAAAGGCATGCTCG R:CTATGCGTGGGAGTACATTGAG	274	**(15)**
Exo S	F: CCATCACTTCGGCGTCACT R: GAGAGCGAGGTCAGCAGAG	129	**(16)**
Mex A	F: CTCGACCCGATCTACGTC R: GTCTTCACCTCGACACCC	503	**(17)**
MEX B	F: TGTCGAAGTTTTTCATTGAG R: AAGGTCAC GGTGATGGT	280	**(17)**
OPR M	F: GATCCCCGACTACCAGCGCCCCG R: ATGCGGTACTGCGCCCGGAAGGC	247	**(17)**
OPR D	F: ATCTACCGCACAAACGATGAG R: GCCGAAGCCGATATAATCAAAC	156	**(16)**

Table 2. Clinical and admission characteristics of the studied population(underlying medical conditions were deleted).

Parameters	**N (%)**
Age, Mean±SD, Range (years)	50.5±19, (1:80)
Male/Female	22(47.8%)/24(52.2%)
Ward distribution • Chest department • General surgery • ICU • Plastic surgery • Vascular surgery • Hepatology department	 10(21.7%) 11(23.9%) 12(26.1%) 6(13%) 5(10.9%) 2(4.3%)
Type of infection Surgical wound infection Urinary tract infection Acute exacerbation in COPD Diabetic foot infection Burn infection Ventilator-associated pneumonia Community-acquired pneumonia Infection in cystic fibrosis	 12 (26%) 7 (15.2%) 7 (15.2%) 6 (13%) 6 (13%) 3 (6.5%) 4(8.5%) 1 (2.1%)
Prolonged hospital admission	30 (65.5%)

Table 3. Pattern of *P. aeruginosa* antibiotic sensitivity.

Name of Antibiotic	**Sensitive**	**Intermediate**	**Resistance**
Piperacillin	18 (39%)	8 (17.4%)	20 (43.5%)
Ceftazidime	4 (8.7%)	2 (4.3%)	40 (87%)
Cefepime	4 (8.7%)	2 (4.3%)	40 (87%)
Aztreonam	12(26%)	8 (17.4%)	26(56.5%)
Imipenem	31(67.3%)	0 (0%)	15(32.8%)
Colistin sulphate	28(70%)	0(0%)	18 (39%)
Polymyxin B	25(55%)	0(0%)	21 (45%)
Gentamycin	22(47.8%)	0(0%)	24(52.2%)
Tobramycin	16(34.8%)	0(0%)	30 (65.2%)
Amikacin	16(34.8%)	2(4.3%)	28(60.9%)
Netilmycin	14 (30.4%)	4 (8.7%)	28(60.9%)
Ciprofloxacin	14 (30.4%)	0(0%)	32(69.6%)
Levofloxacin	14 (30.4%)	2(4.3%)	30 (65.2%)
Lomefloxacin	10 (21.7%)	4 (8.7%)	32(69.6%)
Norfloxacin	12(26%)	0(0%)	34(73.9%)
Ofloxacin	9(19%)	0(0%)	37(81%)
Gatifloxacin	10 (21.7%)	0(0%)	36 (87.3%)

Table 4. Frequency of virulence factors of *P. aeruginosa* according to the type of sample.

	Sputum N=15	**Surgical Wound N=12**	**Urine N=7**	**Diabetic foot N=6**	**Burn N=6**	***P*-value**
Tox A	2(4.3%)	0(0%)	0(0%)	2(4.3%)	0(0%)	0.1
Exo U	3 (6.5%)	4 (8.7%)	5 (10.9%)	4 (8.7%)	2(4.3%)	0.1
Exo S	4 (8.7%)	10 (21.7%)	0(0%)	0(0%)	4 (8.7%)	0.000*
MEX A	7(15.2%)	8(17.4%)	1(2.2%)	0(0%)	2(4.3%)	0.042*
MEX B	5(10.9%)	4(8.7%)	3(6.5%)	6(13%)	2(4.3%)	0.057
OPR M	3(6.5%)	3(6.5%)	1(2.2%)	0(0%)	2(4.3%)	0.63
OPR D	13(28.3%)	10(21.7%)	7(15.2%)	6(13%)	5(10.9%)	0.68

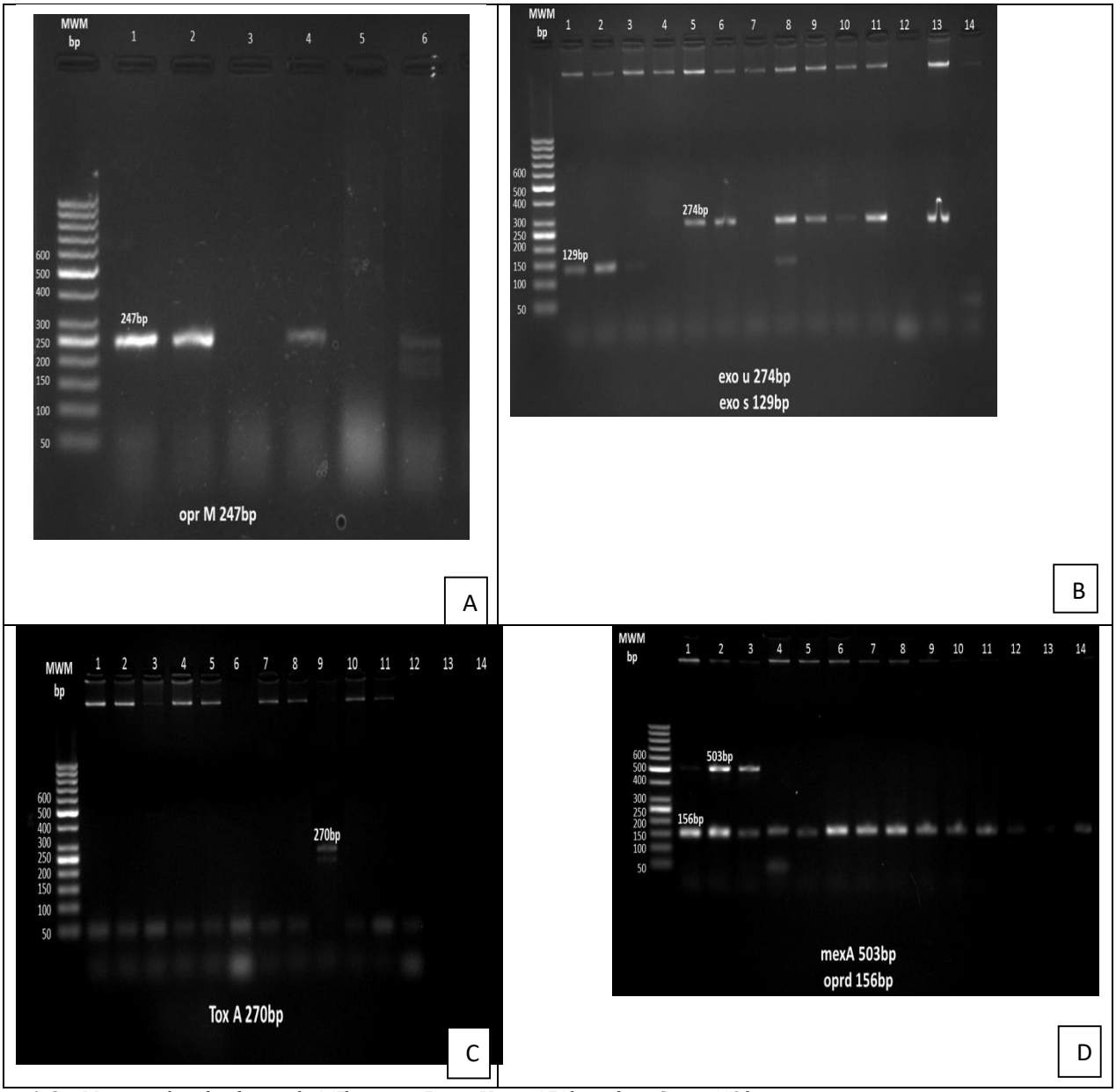
Table 5. Virulence gene and MDR.

	Tox A		***P*-value**	**Exo S**		***P*-value**	**Exo U**		***P*-value**
MDR	**+ve N=4**	**-ve N=42**		**+ve N=18**	**-ve N=28**		**+ve N=18**	**-ve N=28**	
Yes (N=40)	4 (100%)	36 (85.7%)	0.97	16 (88.9%)	24 (85.7%)	1.0	16 (88.9%)	24 (85.7%)	1.0
No (N=6)	0(0%)	6 (14.3%)		2 (11.1%)	4 (14.3%)		2 (11.1%)	4 (14.3%)	

Table 6. Association between the presence of efflux genes and MDR *P. aeruginosa*.

Multi-drug resistance			**Efflux genes**					***P*-value**
			0	1	2	3	4	
		Negative 6 (13%)	0	0	1	4	1	
			(0%)	(0%)	(16.7%)	(66.7%)	(16.7%)	
		Positive 40(87%)	3	10	22	5	0	0.001*
			(7.5%)	(25%)	(55%)	(12.5%)	(0%)	

Figure 1. Distribution of various studied genes with their molecular size.



A:Opr M gene with molecular weight 247bp B: exo U gene 274 bp and exo S gene 129 bp.

C: TOX A 270 bp. D: mex A GENE 503 bp and OPR D 156 bp.

Discussion

Pseudomonas aeruginosa is one of the ESKAPE organisms with a complicated structure that facilitates the excretion of virulence factors in the cytoplasm of target cells as a type III secretion system. This system is a contact-dependent protein secretion pathway secreting exotoxins. It has been linked to major host cell apoptosis [18]. This bacterium produces exotoxin A, a toxic virulence factor that prevents protein synthesis and is encoded by the tox A gene. It causes tissues to necrotize and speeds up the colonization process [19]. In individuals infected with those microorganisms, these virulence characteristics typically correlate with worse death results. Another problem in this organism is its antibiotic resistance by multiple mechanisms e.g. exclusion of antibiotics through multi-drug resistance (MDR) efflux systems, especially those belonging to the RND family. MexAB-OprM, Mex XY, and Mex CD-OprJ are considered the main cause of intrinsic and acquired multi-drug resistance [20]. So, this study aimed to

determine some of these virulence factors of *P. aeruginosa* and detect some of the efflux genes as a mechanism of antibiotic resistance.

In this study, 46 *P. aeruginosa* were collected from 22 males (47.8%), and 24 females (52.2%) from different departments in Sohag university hospital. The highest number of our isolates were from ICU 12 (26.1%), General surgery 11(23.9%), and Chest departments 10 (21.7%). This was explained by the presence of co-morbidities and the immunocompromized state of the admitted patients, also *P. aeruginosa* can survive well in the surrounding environment. Patients included in this study were suffering from anemia 40(87.4%) and diabetes mellitus 28 (60.8%). Both factors impair immune response and enhance infection by this organism.

When assessing the antibiotic profile of the isolated bacteria, 40% of the isolates were resistant to ceftazidime and cefepime which was inconvenient [21] who reported (98%) resistance to cefepime and (91%) ceftazidime. Imipenem was the most susceptible antibiotic 31 (67.3%). Our results were different from that by **Elnagar et al.** who reported that piperacillin/tazobactam 33 (73.33%) was the most sensitive antibiotic followed by imipenem 28 (62.22%)[22].

The frequency of MDR *P. aeruginosa* in our study was 87% which was higher than the previous studies that reported 51.11% *of P. aeruginosa* species were MDR [22,23]. The capacity of this bacteria to develop antibiotic resistance by horizontal gene transfer and also spontaneous mutation can be used to explain the emergence of various MDR *P. aeruginosa* [24].

P. aeruginosa harbors multiple virulence genes enhancing its ability for adhesion and tissue destruction. In our study, exo U, exo S were equally found in 18 (39.13%) of the isolates. However, Fazeli and Momtaz documented that exo S (67.64%) is more prevalent compared to exo U [25]. Furthermore, **Mitov et al.** reported that exo U (18.8%) was less prevalent than exo S (51%) [26]. Tox A was found in 4 (8.7%) of our isolates. Our result was lower than those reported by previous studies [27,28] who reported (50%) , (95.7%) for tox A gene.

Non significant association between the presence of virulence genes and MDR was inconvenient with elnagaar et al., who detected significant association between presence of exo S ,exo u genes with MDR, *p* value was 0.001 and 0.024 for both genes. This could be explained by the small sample size of our study.

Distribution of virulence genes according to the type of sample, tox A gene was equally present in sputum and diabetic food samples 2 (4.3%). ExoS gene was found predominantly in surgical wound samples 10(21.7%), followed by burn, and sputum samples 4 (8.7%). The likelihood that some *P. aeruginosa* strains are better suited to the particular conditions found in particular infectious sites is increased by variation in the prevalence of virulence factor genes in the populations [29].

Efflux genes of *P. aeruginosa* in the studied samples, opr D was the most prevalent in the present study 41(89.13%) and oprM was the least prevalent 9(19.56%), mexB 20(43.47%), mexA 18(39.13%). These results were convenient with **Zehadi et al.** who detected 61 isolates (70.9%) opr D ,59 isolates (68.6%) opr M, 44 isolates (51.6%) mexA , 33 isolates (38.37%) mex B [30].

Pseudomonas aeruginosa RND efflux pumps (MexAB-OprM and MexCD-OprJ) are transport proteins that are essential for the extrusion of all kinds of antimicrobial drugs from inside the cell into the surrounding environment [31]. Here we assessed the efflux pump genes and their relation to antibiotic resistance. Among MDR strains, MexA, MexB, oprM, and oprD genes were substantially more prevalent. These results were similar to those of **Zehadi et al.** [30].

Conclusion

Pseudomonas aeruginosa is a serious organism containing multiple virulence genes facilitating its pathogenesis, studying these virulence factors and their role in disease formation may be a future tool in therapy. An Efflux pump is a mechanism by which *P. aeruginosa* extrudes different classes of antibiotics making it a MDR organism. Trying different substrates that act as efflux inhibitors may aid in the treatment of this organism.

Recommendations

Infection control measures should be sharply applied to protect the patient from nosocomial infection caused by this organism that has different virulence factors and variable mechanisms of resistance.

List of abbreviations

P. aeruginosa: Pseudomonas aeruginosa, MDR: Multi-drug resistant, (T3SS):The type III secretion system, RND: resistance nodulation cell division, DNA: Deoxyribonucleic acid, CLSI : Clinical and Laboratory Stanards Institute, PCR: Polymerase chain reaction, µl :Microliter, ESKAPE: *Enterococcus, Staphylococci, Klebsiella, Acinitobacter, Pseudomonas, Enterobacter*, ICU: intensive care unit.

Authors contributions

Noha Saber Shafik:Conceptualization, Methodology, Software

Nesma A. Mohamed: Data curation,methodology, Writing- Original draft preparation,Supervision.

Mohamed Hamdy Alrawy: Samples collections, Visualization, Investigation.

Mona Mohamed Abdelrahman:Writing- Reviewing and Editing

Ebtisam Mohammed Gad: Software, Validation.

All authors have approved the final article

Conflicts of interest

None of the authors has a conflict of interest to declare.

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