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High Prevalence of Multi-drug Resistant Pseudomonas aeruginosa Recovered from Infected Burn Wounds in Children

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Abstract

Background: *Pseudomonas aeruginosa* is a very common cause of health care acquired infection and represents a major threat to critically ill patients particularly burn patients. The emergence of multidrug resistant strains is up surging leading to problematic control. Thus we aimed in this study to investigate the resistance profiles of *P. aeruginosa*, the frequency of ESBL and the presence of integron class 1.

Methods and Findings: Pus samples from 250 children were collected and screened by culture on suitable media for isolation of *P. aeruginosa* that were identified by culture characteristics, Gram stain, and biochemical reactions. The susceptibility of the isolates to commonly used antibiotics in pediatric cases was performed using the disc diffusion method. ESBL detection was performed phenotypically by double disc method in addition to detection of bla_{TEM} and bla_{SHV} genes by PCR. Class 1 integron was amplified by real time PCR. A total of 50 (20%) of the isolates were identified as *P. aeruginosa*. The mean age was 9.2±3.5, with higher rate of isolation among females (70%) and in children below 10 years (62%). High resistance to ceftazidime (86%), and cefotaxime (72%) was observed. No resistance was found to imipenem. Twenty-eight isolates (56%) were multi-drug resistant and 71.4% of them had integron class 1. ESBL was detected in 51% of the ceftazidime resistant strains of which only 12.5% harbored bla_{TEM} gene and none had bla_{SHV} gene.

Conclusion: High frequency of integron class 1 is a possible reason for dissemination of antibiotic resistance in the pediatric burn unit in Minia governorate.

Limitations: CTX-M was not tested for due to very limited resources as well as integron class 1 sequencing.

Keywords: *P. aeruginosa*; Burn; ESBL; Integron

Introduction

Despite of the variable degrees of burns, infection remains the most common cause of mortality amongst burn patients [1]. Burn wound infections can be caused by bacteria, fungi, or viruses. The emergence of multi-drug-resistant strains of bacteria and fungi have increased the incidence of burn wound infections, sepsis, and associated death [2]. As recognized by Infectious Disease Society of America, the commonly recovered organisms from infected patients in the burn ICU are members of the ESKAPE (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa*, and Enterobacter species) group of pathogens [3]. *P. aeruginosa* is responsible for about 10% -20% of nosocomial

infections in the form of septicaemia in intensive-care units (ICUs), cystic fibrosis, burn and wound infections, etc. [4].

The immune suppression in burn patients in addition to the fact that *P. aeruginosa* has a predilection for moist and warm wound environments made it a major challenge for burn patients [2,5]. *P. aeruginosa* demonstrates increased resistance to various antimicrobials. A multidrug resistant (MDR) *P. aeruginosa* phenotype is defined as a bacterium which is resistant to three or more anti-Pseudomonal anti-microbial classes; carbapenems, fluoroquinolones, penicillins /cephalosporins and aminoglycosides [6]. The mechanism of resistance in MDR *P. aeruginosa* is possibly through the production of several enzymes that inactivate beta-lactams and carbapenems such as extended spectrum beta lactamases (ESBLs) and metallo-β-lactamases (MBLs) [7]. Integron is one of the mobile elements known to be the natural gene capture systems in bacteria. The presence of resistance gene cassettes in class 1 integrons is associated with multi- drug resistance among Gram negative bacteria including *P. aeruginosa* [8]. The prevalence of *P. aeruginosa* infection amongst pediatrics burn patients in Minia governorate has not been studied before.

Therefore, the aim of this study is to determine the prevalence of *P. aeruginosa* infection amongst pediatrics burn patients. In addition, the *in-vitro* activity of 11 commonly used antimicrobial agents to treat *P. aeruginosa* infections in pediatrics burn patients are investigated as well as screening for ESBL production. The prevalence of class I integron is also investigated in this study as the presence of integron substantially indicate a great possibility of transferring antibiotic resistance to sensitive strains.

Methods

Isolation and identification of *P. aeruginosa*

This cross-sectional study was conducted in the Microbiology and Immunology Department, Faculty of Medicine, Minia University, Egypt. Wound swabs were taken from 250 pediatric patients suffering from moderate to severe burn wounds with clinical symptoms and signs of burn wound infection attending the Burn unit at El Minia General Hospital between September 2012 and April 2014. Samples were collected after the consent of children parents was taken.

Swab specimens were inoculated within two hours onto Tryptic Soy Broth (TSB) and the primary isolation was performed on blood agar and McConkey agar. *P. aeruginosa*, isolated from burn wounds, was identified by standard bacteriological methods which included: colony morphology, Gram staining, pyocyanin pigment production, growth at 44°C, catalase, oxidase and Triple Sugar Iron (TSI) fermentation tests.

Antimicrobial susceptibility

The drug susceptibility test was carried out for all the isolates on Mueller-Hinton plates and Kirby-Bauer agar disk diffusion method was used to measure zones of inhibition in accordance with the recommendations of clinical and laboratory standards institute (CLSI) [9]. The antimicrobial agents used in this test were chosen from the commonly prescribed antibiotics for children. The test antibiotics were: Ofloxacin (10 µg), Ampicillin (10 µg), Chloramphenicol (30 µg), Streptomycin (10 µg), Ceftazidime (30 µg), Cefotaxime (30 µg), Imipenem (10 µg), Cefepime (30 µg), Ampicillin\Sulbectam (20 µg), Piperacillin (100 µg), Gentamycin (10 µg) (Bioanalyse, Ankara, Turkey). The results were interpreted according to CLSI guidelines [9]. Multi-drug resistant isolates were defined as those resistant against at least three of the four following groups: (1) imipenem; (2) cefepime or ceftazidime; (3) piperacillin (4) aminoglycosides and (5) ofloxacine.

ESBL detection

ESBL production was primarily screened by the disk diffusion method of cefotaxime and ceftazidime as mentioned earlier. The isolates, which showed an inhibition zone of 27 mm for cefotaxime, 22 mm for ceftazidime were investigated for producing ESBL enzyme. The suspected isolates were further determined by using double disc method [10].

DNA extraction and amplification of bla_{TEM} , bla_{SHV} and class 1 integron

DNA was extracted from the study samples using a DNA extraction kit according to the manufacturer's instructions (Intron Biotechnology, Gyeonggi-do, South Korea). DNA was used immediately or stored at –20°C.

For detection of bla_{TEM} and bla_{SHV} genes, the following primers were used: TEM-F (5′- CTGGGAAACGGAACTGAATG -3′) and TEM-R (5′- GGGGTATCCCGCAGATAAAT-3′) for the TEM-1 gene (858bp product) and SHV-1-F (5′- ATGAGTATTCAACATTTCCG -3′) for a and SHV-1-R (5′-CCAATGCTTATTCAGTGAGG -3′) for the SHV gene (308bp product) [11]. The PCR mixture was done in 50 µl reactions and each consisted of 10 pmol of each primers, 1 µl DNA sample (2.5 µg/µl), and 25 µl of PCR master mix (Thermoscientific). The PCR conditions for bla_{TEM} were: 5 min at 95°C, followed by 30 cycles of 94°C for 30 s, 55°C for 1 min and 72°C for 1 min, with extension at 72°C for 10 min and for bla_{SHV}: initial denaturation step for 5 min at 95°C, followed by 32 cycles of 94°C for 1 min, 57°C for 1min and 70°C for 1min, with a final extension step at 72°C for 10 min. he amplification products were analyzed by gel electrophoresis on a 1.5% agarose gel and visualized by ethedium bromide staining. 100 bp DNA ladder was used as DNA molecular weight marker.

Class 1 integron specific primers were as follows: forward primer: 5`- GGCATCCAAGCAGCAAG-3`, and reverse primer: 5` -AAGCAGACTTGACCTGA-3`[12]. PCR products were obtained using 20 µl reactions prepared as follow: (10 µl of PCR master mix (Thermo Scintific), 0.6 µl of forward primer, 0.6µl of reverse primer, 1µL of template DNA and 7.8 µL distilled water). The amplification reactions were carried out in a real time PCR machine under the following conditions: pretreatment 50°C for 2 minute by 1 cycle, Initial denaturation at 95°C for 10 min by 1 cycle, and by 40 cycle denaturation at 95°C for 15 seconds, annealing at 60°C for 30 seconds, with a final extension step at 72°C for 30 sec.

Statistical analysis

The results were statistically analyzed by Chi squire tests and p-values of less than 0.05 were considered as significant.

Results

The patients were within the age range of one to 15 years but the mean age of the patients was 9.2 ± 3.5, while the rate of isolation was significantly higher amongst female children (70%) and those below 10 years of age (62%) **(Table 1)**. Out of 250 children burn patients, 50 (20%) *P. aeruginosa* isolates were recovered from the swabbed burn wounds of patients with moderate to severe burned wound in different body sites.

The highest antibiotic resistance rates were recorded for ceftazidime 86% and cefotaxime 72%, while the lowest was recorded for imipenem (0%), piperacillin (2%) and gentamicin (20%) **(Table 2)**. Various antibiotypes were detected and the commonest antibiotype was for isolates against ceftazidime alone (6 isolates) **(Table 3)**. None, of the isolates showed resistance against all the used antibiotics, however, 56% of the isolates were multi-drug resistant.

After initial screening, 86% and 72% of the *P. aeruginosa* isolates were resistant to ceftazidime and cefotaxime respectively. Of those resistant strains, ESBL production was detected by double disc method in 51% of these resistant strains. The bla_{TEM} gene was found in only 12.5% of ESBL producer strains, while bla_{SHV} was not detected. Integron class 1 gene was detected by real time PCR, where 40% (20 isolates) of the isolates carried class 1 integron. Integron 1 was found in 71.4% of the MDR isolates. The presence of integron was significantly associated with most of the used antibiotics except for ofloxacin **(Table 4)**.

Discussion

P. aeruginosa is commonly implicated as a cause of health care acquired infections with high mortality rates [13]. One of the reasons for the high pathogenicity of *P. aeruginosa* is the intrinsic high resistance to several antibiotics, as well as the development of multidrug resistance in the hospital environment [14]. This study was the first on *P. aeruginosa* prevalence and profiles of burns in children in Minia governorate. *P. aeruginosa* was isolated from 20% of the swabbed burn wounds from patients with moderate to severe burned wound in different sites on the body in children with ages from 1 to 15 years. The frequency of *P. aeruginosa* in the current study is similar to other studies carried out on burn wounds in Iraq (27%) [15], Tunisia (27%) [16] and South Africa (14.5%) [17], as well as a previous study in the same governorate, in which (19.5%) *P. aeruginosa* prevalence rate was reported. But in that study, *P. aeruginosa* was isolated from various sources in addition to burn wounds [18]. However, higher frequency rates were recorded in other similar studies on burn patients in Pakistan and Egypt [19,20].

Table 1 Demographic characteristics of the studied-burn patients.

Data	Cases N=50		P
	N	%	
Age groups **Less than 10** **More than 10**	31 19	62% 38%	**0.04***
Sex **MALE FEMALE**	15 35	30% 70%	**0.004***
Residence **Urban** **Rural**	**23** **27**	**46%** **54%**	**0.2**

Table 2 Antibiotic Susceptibility patterns of *P. aeruginosa* isolates associated with clinically significant burn wound infection.

Antibiotics	Resistant		Intermediate		Sensitive	
	N	%	N	%	N	%
Cefotaxime (ctx)	36	72	9	18	5	10
Imipenem (ipm)	-	-	------	---	50	100
Chloramphenicol (c)	23	46	6	12	21	42
Ampicillin (am)	21	42	15	30	14	28
Streptomycin (s)	7	14	2	4	41	82
Ampicillin\sulbactam (sam)	16	32	3	6	31	62
Gentamicin (cn)	9	18	1	2	40	80
Cefepime (fep)	21	42	13	26	16	32
Piperacillin (prl)	--	--	1	2	49	98
Ceftazidime (CAZ)	43	86	3	6	4	8
Ofloxacin (OFX)	17	34	-	--	33	66

The results of susceptibility pattern of *P. aeruginosa* isolates in our locality to antimicrobial agents showed that the 50 *P. aeruginosa* strains tested against 12 antimicrobial agents; were highly resistant to Ceftazidime (CAZ) 43 isolates (86%). Higher resistance was, also, recorded for Cefotaxime (CTX): 36 isolates (72%) and Cefepime (FEP): 21 isolates (42%), Ampicillin (AM): 21 isolates (42%), Ofloxacin (OFX): 17 isolates (34%). On the other hand, there was relatively low resistance to Chloramphenicol (C) 23 isolates; (46%), Ampicillin\Sulbactam (SAM) 16 isolates; (32%), Gentamycin (CN) 9 isolates; (18%), Streptomycin (S) 7 isolates; (14%). There was no resistance to impenem (IPM); and piperacillin (PRL). Only ampicillin, ampicillin-sulbactam, cefepime, gentamicin, chloramphenicol and ofloxacin were studied in the previous report done in Minia [18], while all the sensitivity of other anti pseudomonals was tested for the first time in our study.

The Ceftazidime resistance in our study was relatively high (86%), which is in agreement with that done in Egypt by Mahmoud et al. (91%) [20], and by Zafer et al. (60%) [21]. Moreover, many other studies showed high resistance rates of ceftazidime as well as other third generation cephalosporins as in Benin (67.5%) [22]. High sensitivity to ceftazidime is noticed in studies carried in developed countries as in Brazil (28%) [23] and USA (4.5%) [24], probably due to less empirical use of antibiotics. All our *P. aeruginosa* isolates were sensitive to imipenem, which is consistent with other studies carried in Egypt as Badr et al. who found sensitivity rate of 95.7% [25]. On the other hand, two other studies carried by Zafer et al. in Cairo, and by Diab et al. found a higher frequency of imipenem resistance; 39.34% and 72% respectively [21,26].

Table 3 Groups of P. aeruginosa Isolates with similar antibiotypes.

Profile number	No. of isolates	Antibiotic resistance pattern	Total number of resistant isolates (%)
1(7)	3	AM, CRO, CTX, C, SAM, CAZ, OFX	6%
2(4)	3	SAM, AM, CTX, CRO	6%
3(4)	2	AM, CTX, CAZ, FEP	4%
4(5)	2	AM, OFX, CAZ, CTX, FEP	4%
5(5)	3	AM, SAM, S, CRO, CTX	4%
4(4)	3	AM, CAZ, CTX, C,	6%
5(3)	5	CTX, CAZ, FEP	10%
6(2)	5	CAZ, CTX	10%
7(2)	2	CAZ, FEP	4%
8(1)	6	CAZ	12%
9(8)	1	AM,CRO, CTX, SAM, CAZ, OFX, CN, FEP	2%
10(8)	1	AM, CRO, CTX, C, SAM, CAZ, OFX, FEP	2%
11(5)	2	CRO, CTX, CN,CAZ, AM	2%
12(7)	1	CRO, CTX, C, SAM, AM, FEP, CN	2%
13(5)	1	CAZ, CTX, SAM, FEP, OFX	2%
14(4)	1	CAZ, CTX, FEP, CN	2%
15(5)	1	CAZ, CTX, C, FEP, OFX	2%
16(4)	1	CRO, CTX, AM, FEP	2%
18(5)	1	CAZ, CTX, OFX, FEP	4%
19(5)	1	SAM, CAZ, S, OFX,C	4%
20(5)	1	CTX, CAZ, CN, OFX,C	2%
21(4)	1	FEP, CAZ, S, C,	2%
22(5)	1	SAM, CTX, OFX, FEP, AM	2%
23(3)	1	CAZ, CTX, OFX	2%
24(4)	1	AM, CAZ, CTX,CN	2%
25(3)	1	AM, CAZ, S,	2%
26(2)	1	CAZ, CTX,	2%
27(2)	1	AM, CAZ	2%
28(2)	1	OFX, CTX,	2%
29(5)	1	CAZ, CTX,CRO, FEP, OFX	2%
30(2)	1	OFX, CAZ,	2%

Ceftazidime resistant strains were phenotypically investigated for ESBL production by the double disc method and 51% of those strains were found ESBL producers. Our results are in agreement with a study carried by Al Agamy et al. in Saudi Arabia, who found a higher frequency of ESBL (51%) in their ceftazidime resistant strains [27]. Our rate of ESBL production is relatively high compared a previous study done in India [28] and Brazil [23]. Our data suggest the widespread of ESBL enzymes as a causative of ceftazidime resistance compared to other mechanisms as metallo-β-lactamase and AmpC- β-lactamases [23]. However, molecular detection of two of the ESBL genes bla_{TEM} and bla_{SHV} revealed that only 12.5 of the ESBL producers carried the bla_{TEM}

Table 4 Characteristics of multi-drug resistance (MDR) isolates and the presence of class 1 integrons among *P. aeruginosa* strains.

Characteristic	No. of isolates	Integron +ve	Integron -ve	*P* value
MDR	28	20(71.4%)	8(28.5%)	0.006
Resistant antibiotics				
Cephalosporins	28	20(71.4%)	8(28.5%)	0.006
Ampicillin	21	16(76.2%)	5(23.8%)	0.003
Ofloxacin	15	8(53.3%)	7(46.7%)	0.3
Gentamicin	5	4(80%)	1(20%)	0.02
Chloramphenicol	13	9(69.2%)	4(30.6%)	0.03
Streptomycin	2	2(100%)	0(0%)	0.02

and none carried the bla_{SHV}. This is in agreement with the results of Al-Marjani et al. [29] who found none of the above genes in *P. aeruginosa* isolates. ESBL genes other than TEM and SHV could be responsible for ESBL production as the emerging CTX-M type, which should be investigated in further work. The exact definition of what an MDR organism is variable and in our study an MDR is an organism with resistance to any representatives of three or more classes of antimicrobial agents [30]. MDR strains were detected in our study with a frequency of 51%. Our results suggest an increase in MDR *P. aeruginosa* compared to the previous work done in Minia by Gad et al., who found a frequency of 36% [18].

MDR *P. aeruginosa* out-breaks in clinical settings are probably due to both intrinsic and acquired resistance mechanisms [31]. Antibiotic resistance is on the rise due to gene dissemination via horizontal transfer. Horizontal gene transfer via integrons, is responsible for multi-drug resistance in many gram-negative bacteria particularly pseudomonas [8]. No previous reports are there for the integron class 1 in *P. aeruginosa* in Egypt. We hereby, investigated the presence of integron 1, and found that 20% of our isolates have integron 1 gene. Higher frequency of integron 1 was reported in South Nigeria and Brazil where 57% and 41.5% of *P. aeruginosa* isolates contained integron class 1, respectively [32,33]. A very low frequency of integron one was recorded in a multicenter study carried in Turkey with a frequency of 4.5% of class 1 integron [34].

In this study there was a significant correlation between MDR strains and the presence of integron class 1 as 71.4% of the MDR strains carried the integron 1. Our results are consistent with a study carried in China [12]. Moreover, a significant correlation was found between the presence of integron and the resistance to particular antibiotics as the penicillins, cephalosporins, gentamicin and chloramphenicol. Therefore, proper infection control measures are required to prevent the increase in the frequency of drug resistant *P. aeruginosa*. The presence of relatively high frequency of MDR *P. aeruginosa* amongst burn patients requires proper optimization of antimicrobial. The presence of MDR strains could be possibly attributed to the presence of integron 1 cassettes, which require further sequencing to demonstrate the possible carried resistance genes. Imipenem is still the antibiotic of choice for treatment of pseudomonal burn wound infections.

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