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RESEARCH ARTICLE

Resistant Pseudomonas Aeruginosa is an emerging threat to patients in the Intensive Care Unit

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Abstract

Background: Pseudomonas aeruginosa infections in the ICU are a constant problem and its prevention is extremely important. P. aeruginosa colonization has been reported to originate from exogenous sources such as tap water, fomites and/or patient-to-patient transmission, or as an endogenous etiology related to antibiotic use. The aim of this study is rapid diagnosis of Pseudomonas aeruginosa and proper identification of the source of transmission using new molecular techniques with determining the presence of MBL (metallo-B-lactamase) producing Ps. aeruginosa and avoiding the existence of resistant strains in the ICU.

Material and Methods: samples are taken from adult patients (50), health staff (18) and environment (100). After isolation and identification of pseudomonas, Antimicrobial susceptibility testing was performed for ten different therapeutically relevant antibiotics by the VITEK2 automated machine. Screening for metallo-B-lactamase (MBL) production was done. All the pseudomonas aeruginosa isolates were typed by RAPD-PCR (Randomly amplified polymorphic DNA PCR) typing method. The 124 to 127-bp enterobacterial repetitive intergenic consensus (ERIC) primers were performed. All fragments generated were analyzed for determining the RAPD-types and ERIC-types.

Results: 32% of patient samples were positive for Pseudomonas and only 11% (n=2) of staff hand samples were positive. The highest frequency of Pseudomonas isolation from environmental samples was from suction apparatus bags (86%), water tap/sink (78%). The (MBL) producing Pseudomonas is much more resistant to all antibiotics tested except Colistin to which nearly all Pseudomonas strains are sensitive. ERIC typing method gave higher discriminatory index (0.7955) than RAPD (0.7706) and that the combination of both (0.7977) was superior to either alone. Antibiogram gave the lowest discriminatory index (0.7232).

Conclusion: Hand hygiene, environmental cleaning and disinfection of patient objects could reduce environmental reservoirs of Ps. aeruginosa. It is better to limit the usage of antibiotics in the ICU as carbapenems to be used only when absolutely needed. RAPD and ERIC molecular typing methods were superior to antibiotic typing and should be used in tracing the source of infection.

INTRODUCTION

The last 2 decades have witnessed significant changes in the spectrum of microorganisms causing nosocomial infections. Gram-negative enterobacteria, which in the 1970s and 1980s accounted for 30% to 50% of all disease associated isolates in ICUs, **(Allen et al., 1981 & Weber et al., 1992)** have been to a large extent replaced by gram-positive microorganisms such as staphylococci, enterococci and corynebacteria **(Spencer, 1996 & Pfaller et al., 1999).** In spite of these significant changes, Pseudomonas aeruginosa has held nearly unchanged positions in the rank order of pathogens causing ICU-related infectons for more than four decades. In the US National Nosocomial Infections Surveillance (NNIS) system report, Pseudomonas aeruginosa continues to represent the third most frequent organism associated with wound or pulmonary infections, the fourth in causing UTI, the fifth most frequent organism isolated from blood cultures in cases of septicemia **(Richards et al., 2000; NNSI, 2004)**. Recent data was published in 2015 showing that pseudomonas spp. represent 61% of the total infections in the ICU **(Maechler et al., 2015).**

Pseudomonas aeruginosa is a Gram-negative rod of variable length, motile by mean of single polar flagellum. It is a ubiquitous organism, able to persist in a large number of environments. This is due to its ability to utilize different organic compounds as energy sources and to survive for lengthy periods as long as sufficient moisture is available through the action of multidrug efflux pumps **(Talon and Bertrand, 2003).** Furthermore, the ability to form biofilm on a range of inanimate surfaces also contributes to disinfectant resistance as well as impending physical removal **(Kerr and Snelling, 2009)**.

Ps. aeruginosa has inherent resistance to many drug classes, can acquire resistance to all relevant treatments via mutations and can harbor integrons with multiple resistance genes, as those coding for metallo-B-lactamases (MBLs), which can cleave the most active antimicrobial agents as carbapenems **(Livermore, 2002)**.

Avoidance of antibiotics may be the best prevention strategy. The overwhelming majority of Ps. aeruginosa pneumonias occur in patients already exposed to antibiotics **(Rello et al., 1993).** Despite its significant virulence and ubiquitous presence, pseudomonas appears to require suppression of normal host bacterial flora by antibiotics to cause infection. The eradication of normal human bacterial flora, particularly anaerobic flora, facilitates the overgrowth of Ps. aeruginosa. Avoidance of unnecessary antibiotic therapy may not prevent the development of pneumonia, but less antibiotic pressure Ps. aeruginosa from being the causative agent **(Wunderink and Mendoza, 2007).**

The aim of this study is the rapid diagnosis of Pseudomonas aeruginosa, proper identification of the source of transmission using new molecular techniques. Determining the presence of MBL and ESBL producing Ps. aeruginosa. Controlling the existence of resistant strains in the ICU.

Patient & Methods

This study was carried out in the Clinical Pathology Departement and the Adult Intensive Care Unit, Faculty of Medicine, Zagazig university in the period between January 2015 and July 2015. A total of 50 clinical samples were examined. Different samples were taken sputum (20), urine (16), Blood (8), wound swabs (6). Samples (18 samples) were taken from health staff members working in the ICU on pseudomonas isolation agar. Sampling was performed at midday, by which time staff members had been in contact with patients for several hours.

One hundred environmental samples were taken throughout the ICU, concentrating on damp surfaces and areas with maximum potential for cross infection. Surfaces were swabbed with sterile cotton swab sticks, which were used to inoculate nutrient broth tubes. Fluid medium was selected as primary culture medium to dilute disinfectants and encourage growth of low organism numbers. After 24h incubation subcultures were made in Pseudomonas cetrimide agar.

Fluid samples such as antiseptic solutions, air-conditioning drainage water and artificial ventilation fluid reservoirs were pipetted using sterile disposable plastic pipette and inoculated into nutrient broth as well.

A-Isolation and Identification of Pseudomonas:

Clinical samples were cultured on the selective medium Pseudomonas cetrimide agar (Oxoid), which were incubated at 37^{o}C. Bacteria growing on this media were further identified by:

1. Characteristic colony morphology and exopigments production.
2. Microscopic examination of gram stained film.
3. Oxidase test was performed using Oxidase Detection Strips (Oxoid).
4. Growth on subculture at 42 ^{o}C **(Cheesbrough, 2006)**.

5. Gelatin liquefaction test **(Collee et al., 1996):**

A stab culture was made in the nutrient gelatin test tube, incubated at 37 ^{0}C for 24 hours, then removed and held at 4^{o}C for 30 min, positive results is indicated by liquefaction of the gelatin.

B-Antibiotic Susceptibility:

Antimicrobial susceptibility testing was performed for ten different therapeutically relevant antibiotics by the VITEK2 automated machine

The VITEK2 ESBL test is based on the simultaneous assessment of the antibacterial activity of cefepime, cefotaxime and ceftazidime measured either alone or in the presence of clavulanate on card wells, which are introduced into the VITEK2 machine. For each antibiotic tested, turbidity is measured at regular intervals. The proportional reduction of growth in wells containing a cephalosporin combined with clavulanate is then compared with that achieved by the cephalosporin alone and is interpreted as ESBL-positive or negative through a computerized expert system (Advanced Expert System**) (Drieux et al., 2008).**

C-Screening for metallo-B-lactamase(MBL) production:

- Two meropenem discs (10μ) were initially placed on the Muller Hinton plates inoculated with the 0.5 McFarland bacterial suspensions.
- 930 μg EDTA (5 μl of 0.5 M EDTA solution) was added to one of them.
- The inhibition zones of the meropenem and meropenem-EDTA discs were compared after 16-18 h of incubation in air at 35 ^{0}c.
- An increase of $\geq$ 7mm in zone diameter in the presence of 930 μg EDTA compared to those with MEM tested alone was considered to be a positive test for the presence of a MBL **(Pitout et al., 2005).**

D-DNA Extraction for PCR:

All strains were freshly cultured on nutrient agar before DNA extctraction, which was performed by using DNA extraction kit k512 (Feremntas R) according to manufacturer instructions. RAPD-PCR typing (Randomly amplified polymorphic DNA PCR, which is also known as arbitrarily primed PCR (AP-PCR). It is a rapid PCR-based genomic fingerprinting method **(Mcpherson and Moller, 2000)**. Strains can be differentiated by determining the characteristic number and size of the amplified fragments as reflected in band patterns on gel electrophoresis **(Wu and Della-Latta, 2002).**

The use of defined primers for PCR amplification of interspersed repetitive DNA elements present at distinct locations in prokaryotic genomes is referred to as repetitive element sequence based-polymerase chain reaction **(Hiett and Seal, 2009)**. It has become a widely used technique for strain typing because of its speed and relative low cost **(Wu and Della-Latta, 2002).** The 124 to 127-bp enterobacterial repetitive intergenic consensus (ERIC) primers were performed **(Hulton et al., 1991).**

RAPD and ERIC-PCR DATA ANALYSIS

Dendrograms construction

All fragments generated by RAPD-PCR and ERIC-PCR were considered and analyzed for determining the RAPD-types and ERIC-types.

Photographs were used to score the data for RAPD analysis, and DNA fragment sizes were estimated by comparisons with DNA size markers run on the same gel. The presence (1) or absence (0) of a particular band was recorded to generate a binary table.

Dice′s similarity coefficients were then calculated and strains were grouped by using the unweighted pair group method with arithmetic averages (UPGMA). The relationships between the pattern profiles are displayed as dendograms. These calculations were made with a dendrogram construction utility which is available in the internet address [http⁄⁄genomes.urv.cat ⁄UPGMA ⁄index.php]. The generated binary table was entered in the form that is available at the mentioned address. **(Garcia-Vallvē, 2009).**

The numerical index (D) which is a measure of the discriminatory ability of the typing methods was calculated according according to Hunter **(Hunter,1990).** It is based on the probability that two unrelated strain samples from the test population will be placed into different typing groups. This index (D) is given by the following equation:

$$D=1-\frac{1}{N(N-1)}\sum_{j=1}^{s} nj(nj-1)$$

Where N is the total number of strains in the sample population, s is the total number of types described and nj is the number of strains belonging to the jth type.

Simpson′s index of diversity ranges from 0.0 to 1.0, where (1.0) indicates that a typing method is able to distinguish each member of a strain population from all other members of that population and conversely (0.0) indicates that all members of a strain population are of an identical type. An index of 0.5 means that if one strain

was chosen at random from a strain population, then there would be a 50% probability that the next strain chosen at random was indistinguishable from the first **(Hunter,1990).**

Results:

Table (1): Isolated Pseudomonas aeruginosa strains from patients samples:

Sample types	Positive		Negative	
	N	%	N	%
Sputum samples (n=20)	8	40	12	60
Urine samples (n=16)	6	37.5	10	62.5
Blood samples (n=8)	0	0	8	100
Wound swaps (n=6)	2	33.3	4	66.7
Total (n=50)	16	32	34	68

This table shows that 32% of patient samples were positive for Pseudomonas aeruginosa. Highest rate of pseudomonas isolation was from sputum samples (40%).

Table (2): Staff hand samples that were positive for Pseudomonas aeruginosa:

Samples	**n (%)**
Positive	2(11)
Negative	16(89)
Total	18(100)

This table shows that only 11% (n=2) of staff hand samples were positive for Pseudomonas aeruginosa.

Table (3): Comparison between patient, environment and stuff samples for metallo-B-lactamase

Positive samples for Ps.aeruginosa	MBL positive n (%)	MBL negative n (%)
Patient Samples (n=16)	10 (62.5)	6 (37.5)
Enviromental Samples (n=37)	6 (16.2)	31 (83.8)
Stuff Samples (n=2)	0 (0)	2 (100)
Total (n=55)	16 (29)	39 (71)

This table shows that the frequency of MBL production was significantly higher in strains isolated from patient samples when compared to strains isolated from environmental samples and stuff samples.

The highest frequency of Pseudomonas isolation from environmental samples was from suction apparatus bags (86%), water tap/sink (78%), Ambu bags (63%), Stethoscope (48%), and floor (30%).

Table (4): Antibiotic resistance pattern for isolated Ps. aeruginosa strains by doing Antibiogram Analysis

Pattern	AK	CN	CIP	PRL	CT	TOB	FEB	CAZ	ATM	MEM	IPM
A1	S	S	S	S	S	S	S	S	S	S	S
A2	S	S	R	S	R	R	S	S	S	S	S
A3	R	R	S	S	S	R	S	S	R	S	S
A4	S	R	R	S	S	R	S	S	R	S	S
A5	S	R	R	R	S	R	R	R	R	S	S
A6	R	S	R	R	S	R	R	R	S	R	R
A7	R	R	R	R	S	R	R	R	R	R	R

The above table shows that the isolated pseudomonas aeruginosa strains showed 7 antibiotic sensitivity patterns that were ranged from A1-A7, where pattern A1 is sensitive to all tested antibiotics and pattern A7 is resistant to all tested antibiotics except colistin.

The MBL producing Pseudomonas is much more resistant to all antibiotics tested except (CT) colistin to which nearly all Pseudomonas strains (both MBL and non-MBL producing strains) are sensitive.

RAPD ANALYSIS

All the pseudomonas aeruginosa isolates were typed by RAPD method. The patterns consisted of 2 to 7 bands ranging from 200 bp to 2000 bp. The typing method showed seven RAPD patterns (RAPDI-RAPDVII).

Table (5): Observed RAPD patterns for isolated Ps. aeruginosa strains

RAPD	Observed bands

pattern	200	350	400	450	500	600	700	850	1000	1250	1500	1750	2000
I	0	0	1	0	1	1	0	0	0	0	0	0	0
II	0	0	0	0	0	1	1	0	1	0	0	0	0
III	0	1	0	0	1	0	1	0	0	0	0	0	1
IV	0	0	1	0	1	0	0	0	0	0	0	0	0
V	1	0	1	1	0	1	0	0	0	0	0	0	0
VI	0	0	1	0	0	1	0	1	1	1	1	1	0
VII	0	0	1	0	0	1	0	1	1	0	0	0	0

1=present; 0=absent

RAPD patterns were analyzed visually and the presence (1) or absence (0) of bands was recorded to generate the above binary table.

The Similarity Matrix computed with Dice coefficient for different observed RAPD patterns showed that the similarity varied from 0 to 0.8 the larger the number the more the correlation. Highest correlation was between RAPDIV and RAPDI (0.8).

ERIC ANALYSIS

ERIC-PCR yielded 1 to 5 amplification bands. The size of amplified DNA bands ranged from 75 bp to 7000 bp. All the 55 isolates were typed by this method. Eight ERIC patterns were obtained (ERICI-ERICVIII).

Table (6): Observed ERIC patterns for isolated Ps. aeruginosa strains

	Observed bands (kb)										
	4oo	450	500	600	700	850	1000	1250	1500	1850	2000
I	1	0	0	0	0	0	1	1	0	0	0
II	1	0	0	0	1	1	0	0	0	0	0
III	0	0	0	0	0	0	0	1	0	0	0
IV	1	0	0	0	0	1	1	0	1	0	0
V	1	0	0	0	0	0	1	0	0	0	1
VI	0	0	1	1	0	1	0	0	1	0	0
VII	0	1	0	0	0	0	0	0	0	1	0
VIII	0	1	0	0	1	0	1	0	1	0	0

1=present; 0=absent

ERIC patterns were analyzed visually and the presence (1) or absence (0) of bands was recorded to generate the above binary table.

The Similarity Matrix computed with Dice coefficient for different observed ERIC patterns showed that the similarity varied from 0 to 0.667 the larger the number the more the correlation. Highest correlation was between ERICI and ERICV (0.667).

Table (7): Numerical discriminatory index of ERIC, RAPD and Antibiogram

	No. of different strains	No. of strains belonging to the most numerous type	Numerical discriminatory index
ERIC	8	16	0.7955
RAPD	7	16	0.7706
Combined ERIC/RAPD	9	16	0.7977
Antibiogram	7	16	0.7232

The above table showed that ERIC typing method gave higher discriminatory index (0.7955) than RAPD (0.7706) and that the combination of both (0.7977) was superior to either alone. Antibiogram gave the lowest discriminatory index (0.7232).

After analyzing the data taken we found that the patient isolates belong to RAPD genotypes VI and VII. Environmental isolates belong to RAPD genotypes I, II, III, IV, V, VII. Genotype VII contains both environmental and patient isolates.

The same was done to ERIC, where the patient isolates belong to ERIC genotypes VI, VII, VIII. Enviromental isolates belongs to ERIC genotypes I, II, III, IV, V, VI,VIII. Genotypes VI and VIII contain both environmental and patient isolates.

Our results show that water-tap and suction apparatus has central role in the spread of Pseudomonas aeruginosa in the ICU and both of them had been linked to patient by harboring strains that share the ERIC VIII and RAPD VII genotypes especially patients harboring MBL-producing strains.

Water-tap has epidemiological relations with staff hands and artificial ventilation fluid reservoir by harboring strains that share the genotypes RAPD I and ERIC I. Suction apparatus was linked to medical trays, stethoscope and the floor by harboring strains that share ERICII and RAPDII genotypes.

ESBL testing done on the VITEK show positivity in only one Ps.aeruginosa isolate in our study, which is detected in one of the patient's samples.

Discussion

Pseudomonas aeruginosa is a common pathogen that causes nosocomial infections in intensive care units (ICUs) **(NNIS, 2004 & Wisplinghoff et al., 2004).** Resistant Ps. aeruginosa is an emerging threat to patients, so clinical laboratories must focus more and more on the epidemiology of hospital-acquired infections. Strain typing is an extremely useful tool in tracking the spread of nosocomial infections **(Paterson, 2006).**

Evidence-based prevention strategies targeting specific pathogens should be based on thorough knowledge of their epidemiology, reservoirs in the ICU and mode of transmission **(Trautmann et al., 2005)**.

Isolating Pseudomonas aeruginosa per se is not enough to conclude epidemiological importance of the site of isolation, so strain typing can increase the efficiency to control the infection by finding out actual sources of patient infections and finding out sources that contain environmental pseudomonads that may receive less stringent treatment.

In our study, we try to detect the epidemiology of Ps. aeruginosa in the adult ICU. Several patients and environmental samples have been typed using RAPD and ERIC-PCR to investigate possible relationships. Antibiogram was performed as a typing method and the antibiotic susceptibility tests for extended spectrum beta lactamses and metallo-B-lactamase production.

In the present study, Pseudomonas aeruginosa strains were isolated from 32% of the positive clinical patients samples **(Table 1).** This ratio is relatively high when compared with other studies. In Minia, Egypt, **Gad and his coworkers** isolated the organism from 18.2% of samples **(Gad et al., 2007).** Ndip and coworkers isolated it from 24.7% of samples **(Ndip et al., 2005) &** Olayinka et al. isolated it from 10.5% **(Olayinka et al., 2004).**

In the present study the environmental sampling had shown that one third (30%) of the samples were positive for Pseudomonas aeruginosa, where it is relatively high level. Our results were near to others, where **Ndip and coworkers** reported 28% of samples & **Gad and his coworkers** reported 19.5%. The water taps were important reservoir of the infection in the ICU (78%), 40% of them has been proven to be related to patient isolates by sharing ERICVIII and RAPDVII genotypes. This important role of water taps is in accordance with many previous studies as that done by **Trautmann and colleagues (2008)**, found that 97% of tap water samples in the surgical ICU were positive for Ps. aeruginosa, where the rate has been dropped to 0% after applying point of use water filteration.

In this study, ends of suction tubes were found to be an important reservoir for the organism. This is in accordance with the study done by **Yorioka and colleagues (2010).**

Although pseudomonas was isolated from hands of health staff, molecular typing methods failed to show direct link with strains isolated from patients. Other studies revealed that staff hands play important role in the spread of pseudomonas in adults ICU **(Foca et al., 2000).** This discrepancy of results is may be due to small staff sample size.

In recent years, the metallo-B-lactamases (MBLs) have emerged as one of the most feared resistance mechanisms because of their ability to hydrolyze virtually all B-lactam agents including the carpapenems as the genes are carried on highly mobile elements **(Walsh et al., 2005).**

Our study showed that 62.5% of Pseudomonas strain isolated from patients was MBL producers **(Table 3),** near results were detected also **(Szabo et al., 2008)** in Hungarian hospital.

In our study we found that MBL resistant pseudomonas isolates were also resistant to multiple other antibiotics, which make treatment options very limited **(Table 4).** The MBL producing strains in this study fall into two antibiotic sensitivity groups A6 or A7. The strains showing the A6 pattern are more benign being sensitive to gentamycin, aztreonam and colistin. The A7 pattern is the most alarming, where it is resistant to all tested antibiotics with the exception of colisitin, so they are called pan resistant isolate. Some authors suggest that we must revert back to colistin and polymyxin as systemic treatment alternative to other antibiotics **(Walsh et al., 2005).**

Another problem reported in our study is the existence of colistin resistant Pseudomonas strains **(pattern A2),** where those drugs may be the only treatment options for infection with pan-resistant strains.

Pseudomonas isolates (n=55) from environmental and clinical sources were subtyped using RAPD, ERIC-PCR profiling and were analyzed to help us in controlling Pseudomonas infection in the ICU.

In the present study, patients strains having the genotype RAPDVI and ERICVII showing no sharing with any environmental source, so we can assume that these strains were transmitted by horizontal transmission from

patient to patient by the hands of healthcare workers. In other studies cross transmission have been reported **(Cuttelod et al., 2011).**

In our study, we couldn′t find unique genotypes and all genotypes were shared by at least two patients and/or shared by some environmental isolates. Also we found that MBL producing strains were found to have two genotypes the first is RAPDVI/ERICVII and the second is RAPDVII/ERICVIII. The RAPDVII/ERICVIII genotype was found in patient samples as well as environmental samples (suction apparatus tubing and water tap/sink).

The finding that metallo-B-lactamase producing strains belong to several genotypes can be explained by the fact that metallo-B-lactamase genes are harbored in mobile genetic elements and integrons. This was also an explanation stated by **(Poirel et al., 2010).**

In the current study we found that MBL producing strains and non-MBL producing strains share the RAPDVI pattern. (**Rossolini, 2005)** gave an explanation for that finding , where the MBL producing strains may have originated from the non-MBL strains that acquired the genes through some mobile genetic elements.

In our study ERIC typing method gave higher discriminatory index (0.7955) than RAPD (0.7706), but still the combination of both gave the highest discriminatory index (0.7977). Antibiogram gave the lowest discriminatory index (0.7232). These results comes in agreement with **(Liu & Wu, 1997)**, who compared ERIC and RAPD for Acinetobacter typing. The discriminatory index was 0.75 for ERIC and 0.71 for RAPD, which are close to our results.

Lastly we recommend hand hygiene, environmental cleaning and disinfection of patient objects to reduce environmental reservoirs of Ps. aeruginosa. Reducing the usage of antibiotics in the ICU as carbapenems to be used only when absolutely needed. RAPD and ERIC molecular typing methods were superior to Antibiotic typing and should be used in tracing the source of infection.

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