**PSEUDOMONAS AERUGINOSA CLONAL DISSEMINATION IN ADULT INTENSIVE CARE UNIT IN BENHA UNIVERSITY HOSPITAL**

By

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**ABSTRACT**

To investigate the sources and spread of *Pseudomonas* in the Adult Intensive Care Unit (ICU), Benha University Hospital, 60 *Pseudomonas aeruginosa* strains were isolated from patients, staff and environmental samples and were typed using the randomly amplified polymorphic DNA (RAPD) and the enterobacterial repetitive intergenic consensus (ERIC) polymerase chain reaction (PCR) methods. Testing for extended spectrum beta lactamses and metallo-β-lactamase (MBL) production was also performed. 50% of patient samples were positive for *Pseudomonas aeruginosa*. 33% of the environmental samples were positive for *Pseudomonas aeruginosa*. Highest frequencies of *Pseudomonas* isolation were from Ambu bags (100%), stethoscope (100%), suction apparatus tubing (100%), water tap/sink (80%) and floor (75%). 13% of staff hand samples were positive for *Pseudomonas aeruginosa*. MBL production was highest in patient strains (92%), less in environmental strains (19%) and was not detected in staff hand samples. The difference in MBL distribution between patient and environmental/stuff samples was statistically significant (P < 0.001). All the *Pseudomonas aeruginosa* isolates were typable by both RAPD and ERIC-PCR methods. Seven RPAD patterns (RAPDI-RAPDVII) and eight ERIC patterns were obtained. ERIC typing method gave higher discriminatory index (0.7955) than RAPD (0.7706), still the combination of both gave the highest discriminatory index (0.7977). Water-tap and suction apparatus played a central role in the spread of *Pseudomonas aeruginosa* in the ICU. Both water-tap and suction apparatus were epidemiologically linked and both had been epidemiologically linked to patients. Water-tap was molecularly linked to staff hands and artificial ventilation fluid reservoir. Suction apparatus was linked to medical trays and stethoscope. Epidemiological linkage has been also proved between patients and artificial ventilation tubing. The patient MBL-producing strains were epidemiologically linked to water tap and suction apparatus tubing.

**INTRODUCTION**

*Pseudomonas aeruginosa* is a common pathogen that causes nosocomial infections in intensive care units (ICUs) [1, 2]. In spite of significant changes in the spectrum of organisms causing nosocomial infections in ICUs, *Ps. aeruginosa* has held a nearly unchanged position as an important pathogen. Today, the organism is isolated as the second most frequent organism causing ventilator-associated pneumonia, the fourth
causing catheter-associated urinary tract infections, the fifth causing surgical site infections and the seventh causing central line–associated bloodstream infections [3].

*Ps. aeruginosa* has inherent resistance to many antibiotic classes, can acquire resistance to all relevant treatments via mutations, and can harbor integrons with multiple resistance genes, as those coding for metallo-β-lactamases (MBLs), which can cleave the most active antimicrobial agents against *Ps. aeruginosa* and Enterobacteriaceae: the carbapenems [4].

Optimal control of *Ps. aeruginosa* outbreaks may require rapid identification and strain differentiation. *Ps. aeruginosa* has traditionally been typed on the basis of its phenotypic characteristics [5]. However, strain typing by traditional phenotypic methods may lack discriminatory power and stability. Molecular techniques offer a considerable improvement, and can complement phenotypic data to obtain a better understanding of bacterial diversity [6].

Pulsed filed gel electrophoresis (PFGE) is considered the ‘gold standard’ for *Ps. aeruginosa* Deoxyribonucleic acid typing [7-9]. However, this method is limited by technical complexity, expense and prolonged turnaround times for results [6]. As an alternative to PFGE, repetitive-element-based polymerase chain reaction (rep-PCR) and randomly amplified polymorphic DNA polymerase chain reaction (RAPD-PCR) have shown considerable potential as DNA typing tools in the laboratory [6, 10].

In many bacterial species there are repetitive DNA sequences spread throughout the genome. Polymerase chain reaction (PCR) primers can be designed for these elements to amplify the genomic DNA between the repetitive elements when two of the elements are in relatively close proximity. The regions located between the repeated elements often vary in size due to difference among separate strains, and thus fragments of different sizes will be amplified, creating unique profiles following gel electrophoresis. These different banding patterns are compared to one another to genotype the organisms [11].
In RAPD, DNA synthesis is primed at low stringency with single oligonucleotides of arbitrarily chosen sequence from sites in genomic DNA to which the oligonucleotide is fortuitously matched or almost matched, and strain-specific arrays of amplified DNA fragments are obtained from pairs of closely spaced sites [10].

**SUBJECTS AND METHODS**

To investigate the possible sources and the spread of *Pseudomonas* in the adult ICU of Benha University Hospital, samples were taken from three different source categories; patients, environment and staff. Fifty two patient samples were examined; 27 urine samples, 21 sputum samples, 2 blood samples and 2 wound swab samples.

Hand impressions of sixteen nursing staff working in the ICU were taken on *Pseudomonas* isolation agar. Sampling was performed at midday, by which time staff members had been in contact with patients for several hours.

Ninety six environmental samples were taken throughout the ICU, concentrating on damp surfaces and areas with maximum potential for cross-infection. The sampled sites can be seen in fig. 1. Surfaces were swabbed with sterile cotton swab sticks, which were used to inoculate nutrient broth tubes. Fluid samples such as antiseptic solutions were pipetted using sterile disposable plastic pipette and inoculated into nutrient broth. Fluid medium was selected as primary culture medium to dilute disinfectants and encourage growth of low organism numbers.

After 24 h incubation subcultures were made to the selective medium *Pseudomonas cetrimide* agar (Oxoid).

*Pseudomonas cetrimide* agar plates were incubated at 37°C for 24 hours. Bacteria growing on the *Pseudomonas* selective cetrimide agar were further identified using bacterial morphology by Gram stained films, exopigment production, oxidase test, growth on subculture at 42 °C, examination of motility, gelatin liquefaction and sugar utilization tests [12].
Extended spectrum β-lactamase production was evaluated by the double-disk synergy test [13]. Screening for MBLs was performed by meropenem-EDTA double-disk synergy test [14] and E-Test MBL (AB Biodisk®).

All strains were freshly cultured on nutrient agar before DNA extraction. DNA extraction was performed by using DNA extraction kit K512 (Feremntas®) according to manufacturer instructions.

**RAPD PCR**

RAPD analysis was performed as described previously, with slight modifications [15] using arbitrary sequence (5’ AGC GGG CCA A 3’). RAPD reaction was carried out in a final volume of 100 µl containing 1x DreamTaq Master Mix, 1.5 µmol/L oligonucleotide primer, 2.5 µl of chromosomal DNA and a total 4.5 mmol/L MgCl₂. Each reaction mixture was overlaid with 25 µl of mineral oil and amplified with a Biometra® DNA Thermal Cycler model 050-400 The reaction conditions were as follows: 94 °C for 2 min, followed by 35 cycles with each cycle consisting of 94 °C for 30s, 35 °C for 30s and 72 °C for 2 min, and a final elongation step at 72 °C for 10 min. [15]:

**ERIC PCR**

PCR with enterobacterial repetitive intergenic consensus (ERIC) primers (ERIC-PCR) was performed as reported previously [16] using ERIC1R (5’ ATG TAA GCT CCT GGG GAT TCA C 3’) and ERIC2 (5’ AAG TAA GTG ACT GGG GTG AGC G 3’) primers.

Polymerase chain reactions were performed in a final volume of 50 µl containing 1x DreamTaq™ Master Mix, 0.5 µmol/L of ERIC1R and ERIC2 primers, and 4 µl of chromosomal DNA.

Each reaction mixture was overlaid with 25 µl of mineral oil and amplified with a Biometra® DNA Thermal Cycler model 050-400.
Polymerase chain reaction conditions were as follows: 94°C for 1 minute, followed by 35 cycles at 94°C for 45 seconds, 52°C for 45 seconds, 72°C for 2 minutes, and a final extension at 72°C for 10 minutes [16].

Amplification products were visualized by electrophoresis on 2% agarose agar and staining by ethidium bromide (0.5 ug/ml in the running buffer). Photographs were then taken for data analysis.

**RAPD and ERIC-PCR data analysis**

Photographs were used to score the data for RAPD and ERIC analysis. All fragments generated by RAPD-PCR and ERIC-PCR were considered and analyzed for determining the RAPD-types and ERIC-types respectively.

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), and the relationships between the pattern profiles were displayed as dendrograms. 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 web address. [17].

The numerical discriminatory index (D) which is a measure of the discriminatory ability of the typing methods was calculated according to Hunter [18]. The index (D) is based on the probability that two unrelated strain samples from the test population will be placed into different typing groups.
RESULTS

50% of patient samples were positive for *Pseudomonas aeruginosa*. Highest rate of *Pseudomonas* isolation was from sputum samples (62%) followed by urine samples (48%). Blood samples and wound swaps yielded no *Pseudomonas aeruginosa* isolates.

33% of the environmental samples were positive for *Pseudomonas aeruginosa*. *Pseudomonas aeruginosa* strains were isolated from air conditioner (50%), Ambu page (100%), artificial ventilation fluid reservoir (20%), artificial ventilation tubes (50%), floor (75%), kidney trays (33%), patient side drawers (50%), stethoscope (100%), suction apparatus tubing (100%) and water tap/sink (80%) (fig 1). Only 13% of staff hand samples were positive for *Pseudomonas aeruginosa*.

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![Fig. (1): Frequency of isolation of *Pseudomonas aeruginosa* from environmental samples.](image-url)
Both Meropenem-EDTA double-disk synergy test and E-Test MBL gave equivalent results. MBL production was highest in patient strains (92%), less in environmental strains (19%) and was not detected in staff hand strains.

All the *Pseudomonas aeruginosa* isolates were typable by RAPD method. Seven RPAD patterns (RAPD1-RAPD7II) were obtained consisting of 2 to 7 bands ranging from 200 bp to 2000 bp (fig. 2).

All the 60 isolates were typable by ERIC-PCR and eight ERIC patterns were obtained (ERIC1-ERICVIII). The obtained patterns consist of 1 to 5 amplification bands ranged in size from 75 bp to 7000 bp (Fig. 3).

The dendrograms showing relationships among various RAPD and ERIC genotypes is shown in fig. 4.

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.

![Fig. (2): Different RAPD patterns for isolated *Pseudomonas aeruginosa* strains](image-url)
DISCUSSION

Resistant \textit{Ps. aeruginosa} is an emerging threat to patients [19-21].

With the rising spread of antibiotic-resistant organisms, 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 [22-24]. Evidence-based prevention strategies targeting specific pathogens should be based on a thorough knowledge of their epidemiology, reservoirs in the ICU, and modes of transmission. [25].

In the present study, we try to elucidate the sources and spread of *Pseudomonas aeruginosa* in the adult ICU. *Pseudomonas* isolates (n = 60) from environmental, staff and clinical sources were sub-typed using RAPD, ERIC-PCR profiling to investigate possible relationships. Testing for extended spectrum beta lactamases and metallo-β-lactamase production was also performed.

In the present study, *Pseudomonas aeruginosa* strains were isolated from 50% of clinical samples. This ratio was high as compared with other studies [26-28]. This high isolation ratio implies that the studied unit was facing an outbreak of *Pseudomonas aeruginosa* infections. The highest rate of isolation was from respiratory samples (62%) which is very high when compared with other studies [27]. This high isolation rate may be attributed to the raised overall isolation rate.

One third (33%) of environmental samples was positive for *Pseudomonas aeruginosa*. This figure reflects the fact that *Pseudomonas* is ubiquitous in the hospital environment. This ratio was in accordance with other studies [26, 27].

Isolating *Pseudomonas aeruginosa* per se is not enough to conclude the importance of the site of isolation. In the present study *Pseudomonas aeruginosa* was isolated from ten different sites; however, only three sites have been proved to have direct epidemiological relationships with patients (fig. 5). These condemned sites were water tap/sink, suction apparatus tubing and artificial ventilation machine tubes. Therefore, strain typing can increase the efficiency of infection control procedures by finding out actual sources of patient infections to be stressed upon and finding out sources that contain environmental pseudomonads that may receive less stringent treatment.
In the past, horizontal transmissions were regarded as the most relevant route of *Ps. aeruginosa* strain acquisition in the ICU. However, during the last 10 years, a significant proportion of *Ps. aeruginosa* isolates were demonstrated to stem from ICU water sites [29]. The present study showed that water taps were important reservoir of *Pseudomonas* in the ICU. 80% of water tap samples were positive for *Ps. aeruginosa*. 50% of water tap *Pseudomonas* isolates has been proved to be related to patient isolates by sharing ERICVIII and RAPDVII genotypes (fig. 5). This important role of water taps is in accordance with many previous studies [30, 31].

*Pseudomonas* was isolated in 13% of samples taken from hands of health care workers. Hands of health care workers have been previously identified as an important reservoir of *Ps. aeruginosa* [32]. Although *Pseudomonas* was isolated from hands of nursing staff, molecular typing methods failed showed direct link with stains isolated from patients (fig. 5). Other studies revealed that staff hands play important role in the spread of *Pseudomonas aeruginosa* in the adult ICU [32] and neonatal ICU [33]. This failure may be attributed to small staff sample size.

In recent years, the metallo-ß-lactamases (MBLs) have emerged as one of the most feared resistance mechanisms because of their ability to hydrolyze virtually all ß-lactam agents, including the carbapenems, and because their genes are carried on highly mobile elements [34]. This study showed that 92% of *Pseudomonas* strains isolated from patients were MBL producers. The discovery of MBL in *Pseudomonas* isolates in any location is alarming. MBL producing *Pseudomonas* strains have not been previously reported in Egypt in internationally published journals, however, MBL producing *Pseudomonas aeruginosa* was reported in an MD thesis in EL-Mansoura University [35].

This study showed striking difference between MBL distribution between patient isolates and environmental/staff isolates. While 92% of patient strains were positive for MBL production, only 18% of environmental/staff isolates were positive in MBL production tests (P value < 005). The higher MBL production rate in patient isolates can
be attributed to selection of MBL producing strains in patients by antibiotic administration pressure.

In the present study the circulation of *Pseudomonas* strains in the ICU was analyzed, this may has important results that help controlling *Pseudomonas aeruginosa* in the ICU (fig. 5):

- In the present study, patient strains with RAPDVI/ERICVII genotypes were only isolated from patient samples. No environmental source that has these genotypes was detected. We can conclude that these strains were transmitted by horizontal transmission from patient-to-patient probably by hands of health care workers. Patient-to-patient transmission is considered to occur when a patient acquires a strain that has a genotype matching another patient strain genotype and that is not present in any likely environmental source [36]. Instances of apparent *Pseudomonas aeruginosa* cross-transmission have been reported previously [37, 38].

- Several environmental sources were discovered in this study. Water-tap and suction apparatus had played a central role in the spread of *Pseudomonas aeruginosa* in the ICU. Both were linked epidemiologically by harboring strains that share ERICVIII and RAPDVII genotypes. And both had been linked to patient by harboring stains that share the ERICVIII and RAPDVII genotypes. Water-tap had epidemiological relations with staff hands and artificial ventilation fluid reservoir. Suction apparatus was linked to medical trays, stethoscope, and floor. Epidemiological linkage has been also proved between patient and artificial ventilation tubing by harboring strains belonging to the ERICVI genotype.

- In the present study, unique patient genotypes (i.e. genotypes present only in single patient), which are possibly of endogenous source [39] couldn’t be found. All genotypes were shared by at least two patients and/or shared by some environmental isolates.
In the present study, MBL producing strains were found to have two genotypes RAPDVI/ERICVII and RAPDVI/ERICVIII (fig. 5).

Isolates with RAPDVI/ERICVIII genotype were found in patient samples as well as in suction apparatus tubing and Water tap/sink environmental samples. These strains may be attributable to environmental source.

Isolates with RAPDVI/ERICVII genotypes were only isolated from patient samples and were not found in any environmental samples. These strains may be attributable to patient-to-patient transmission.

The finding that metallo-β-lactamase producing strains belong to several genotypes can be explained by the fact that metallo-β-lactamase genes are harbored in mobile genetic elements and integrons. Integrons carrying gene encoding metallo-β-lactamase have been characterized in Pseudomonads strains [40]. The same explanation can be used to explain that some non MBL producing strains shared the same genotype as MBL producing strains. The acquisition of metallo-β-lactamase genes is a fact that no longer questioned according to Rossolini [41].

In conclusion, MBL producing Pseudomonas aeruginosa is an emerging threat facing health care facilities in Egypt. The application of strict infection control procedures is absolutely recommended. Infection control procedures should include the eradication of possible environmental reservoirs especially water tap/sink and suction devices. Patient-to-patient transmission was proved and screening and isolation procedures are highly recommended. The antibiotic pressure in the ICU should be reduced, to prevent the development, selection and spread of antibiotic resistant strains.
Fig. (5): Graphical analysis of epidemiologic relationships proved by molecular typing methods among different sites of *Pseudomonas* isolation.
REFERENCES


