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Frequency of antimicrobial resistance markers among *Pseudomonas aeruginosa* and *Escherichia coli* isolates from municipal sewage effluent water and patients in Jordan

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Abstract

The objective of this study was to compare the frequency of antimicrobial resistance markers of *P. aeruginosa* and *E.coli* isolates from effluent of sewage treatment plants and clinical specimens over the same period in Jordan. A total of 212 *Pseudomonas aeruginosa* and 54 *Escherichia coli* isolates were recovered from effluent water, in addition, 65 *P. aeruginosa* and 50 *E.coli* isolates were obtained from clinical specimens over the same period. High percentage of *P. aeruginosa* and *E.coli* isolates from both sources have similar multi-resistant to > 3 antimicrobial drugs**,** and carried common Class 1 integrons and resistance genes. The antimicrobial resistance markers; *tet*(B) gene, *tet*(A)gene, metalo-β-lactamase genes (bla_{OXA2} bla_{VIM2a}, bla_{VIM2b}) and aminoglycoside genes (*aac*A) were detected in both clinical and effluent water isolates. This study indicates that strains isolated from effluent water were highly polluted with antimicrobial resistance markers similar to those observed frequently in clinical bacterial isolates, and these results may contribute to increase accumulation of antimicrobial resistance in the natural environment and to constitute a potential health risk factor.

Keywords: *P. aeruginosa*, *E.coli*, antimicrobial resistance, patients, effluent water, Jordan.

Introduction

In recent years, high incidence of common multidrug resiance (MDR) among commensal gram-negative bacteria such as *Pseudomonas aeruginosa* and *Escherichia coli* has been demonstrated in both human intestines and aquatic environment worldwide (1-5). It has been also suggested that accumulation of antibiotic resistance genes in natural environments can cause serious adverse consequences on human health in the future (6-8).

Metallo-β-lactamases (MBLs) is rapidly emerging group of β-lactamases, which recently has been demonstrated to spread widely in many countries (9-12). MBL-producing strains in gram-negative bacteria exhibit resistance to almost all currently available antibiotics including the carbapenems, particularly MBLs-producing *P. aeruginosa* strains have caused several nosocomial outbreaks in medical care centers worldwide, demonstrating the need for proper infection control measurements (11). The MBLs genes are often found as cassettes integrated within integron structure and carried on large transferable plasmids. Class 1 integrons are capable of transferring genes responsible for resistance to tetracycline, beta-lactam, sulfonamide, aminoglycoside and quaternary ammonium antimicrobial agents in Gram-negative bacteria, particularly *P. aeruginosa* (12-15).

High prevalence of MBLs genes in commensal gram-negative bacteria either in clinical settings or water environment will eventually increase their dissemination among diverse genera of human pathogens (6, 16). There is still limited data documenting the prevalence of MBLs genes in *P. aeruginosa* and *E. coli* and their association with class 1 integrons in aquatic sources in developing countries (6-7).

This study aimed to investigating the frequency of antimicrobial resistance markers in *P. aeruginosa* and *E.coli* isolated from municipal effluent sewage water and clinical specimens over the same period in Jordan.

Materials and Methods

Sampling and culture

A total of 212 *P. aeruginosa* and 54 *E. coli* isolates were collected from effluent of the two main municipal sewage water treatment plants (Kherbet Assamra and Abo Nosair) near Amman, for the period of 4 months; September through December 2007. Abo Nosair treatment plant receives sewage water from Jordan University Hospital, whereas Kherbet Assamra receives sewage water from the most other hospitals of the capital city Amman (ca. 2 million population). Samples were collected twice each week in 100ml cleaned, sterilized bottles and transported at ambient temperature from the sampling site to the laboratory. Within 3 hrs of collection five ml of each sample was inoculated into asparagine enrichment broth for 24 to 48 hrs at 37°c, and 0.01 ml of enrichment broth was streaked onto Pseudomonas Cetremid agar (Oxoid, Cambridge, United Kingdom) and incubated at 42°c, for 24-48 hrs. The growth of *P. aeruginosa* colonies appears flat with light outer rims and bluish or greenish/brownish in color. Also, 0.01 ml of fresh effluent water samples was cultured directly on MacConkey agar plates (Oxoid, Cambridge, United Kingdom) and incubated for 24 hrs at 37°C to recover *E. coli*. In addition, over the same period, 65 *P. aeruginosa* and 50 *E.coli* isolates were randomly collected from clinical specimens (blood, urine, sputum, wounds) at the Bacteriology Laboratory, Jordan University Hospital (JUH) in Amman and were included in this investigation.

Identification of *P. aeruginosa* and E.coli isolates

All collected isolates were first identified by conventional biochemical tests: cytochrome oxidase reaction, triple sugar iron tube agar, citrate and indole and urea production. *P. aeruginosa* isolates were examined further for pigment production and growth at 42°C (5). The isolates were later confirmed by rapid biochemical Remel test (Remel Inc, KS. USA), and three colonies of *P. aeruginosa and E.coli* from each culture sample were picked up and inoculated on Brain-Heart infusion agar bottles containing 15% glycerol and kept at -20°C until additional tests were performed as described below.

Antimicrobial Susceptibility

Antimicrobial susceptibility of *P. aeruginosa* and *E.coli* isolates was determined using disc diffusion method against 9 commonly used antimicrobial drugs in Jordan and according to the guidelines of CLSI (17). Two control strains of *E.coli* (ATCC 25922) and *P.aeruginosa* (ATCC 27853) were used throughout the study.

DNA extraction

A total of 115 *P. aeruginosa* and 32 *E.coli* isolates from effluent water and clinical specimens which were resistant to > 3 antibiotics were selected for molecular investigations. Genomic DNA was extracted from overnight cultures of *P. aeruginosa* and *E.coli* isolates in 300 ul nuclease free water which was boiled for 10 minutes using the water bath. Then the mixture was centrifuged at 3000 rpm for 15 seconds to precipitate undesired substances and supernatant used for molecular analyses.

PCR detection of class 1 integrons and gene cassettes

All selected multidrug resistant isolates *P. aeruginosa* and *E.coli* were screened for class 1 integrons using PCR as previously described in detail (4). The primers used are shown in **Table 1.** The isolates were also screened for the presence of tetracycline

TABLE 1. Primer sequences used in the detection of class 1 Integrons and gene cassettes.

Gene	Primer sequences(5' to 3')	Annealing T.(°c)	Product size (bp)	References
Intl 1	GCA TCC TCG GTT TTC TGG GGTGTGGCGGGCTTC GTG	50.3 57.1	457	Shibata et al. 2003
*bla*IMP-1	ACC GCA GCA GAG TCT TTG CC ACA ACC AGT TTT GCC TTA CC	52.9 56.3	578	Poirel et al. 2000
*bla*IMP-2	GTT TTA TGT GTA TGC TTC C AGC CTG TTC CCA TGT AC	47 44.6	678	23 Shibata et al. 2003
*bla*VIM-1A	TCTACATGACCGCGTCTGTC CAA AAG TCC CGC TCC AAC GA	53.8 49.7	920	Poirel et al. 2000
*bla*VIM-2A	ATG TTC AAA CTT TTG AGT AAG CTA CTC AAC GAC TGA GCG	51.7 53.8	748	Poirel et al. 2000
*bla*VIM-2A	ATG TTC AAA CTT TTG AGT AAG CTA CTC AAC GAC TGA GCG	50.3 44.5	801	Poirel et al. 2000
*bla*VIM-2B	AGT GGT GAG TAT CCG ACA G ATG AAA GTG CGT GGA GAC	48 51	261	Tsakris et al. 2000

*bla*OXA-2	AAG AAA CGC TAC TCG CCT GC CCA CTC AAC CCA TCC TAC CC	53.8 55.8	478	Bert et al. 2002
*Aac*A	CGT ACA GGA ACA GTA CTT TTT GAA CCA TGT ACA CGG C	51.7 48.9	380	Fonseca et al. 2005
tet(A)	GTAATTCTGAGCACTGTCGC CTGCCTGGACAACATTGCTT	51.7 51.7	956	Agersø & Guardabassi 2005
tet(B)	CTCAGTATTCCAAGCCTTTG ACTCCCCTGAGCTTGAGGGG	57.9 49.7	414	3 Agersø, & Sandvag 2005
tet(39)	CTCCTTCTCTATTGTGGCTA CACTAATACCTCTGGACATCA		701	Agersø & Guardabassi 2005

genes; *tet*(A), *tet*(B) and *tet*(39) and metallo-β-lactamase genes (bla_{IMP-1}, bla_{IMP-2}, bla_{VIM-1A}, bla_{VIM-1B}, bla_{VIM-2A} and bla_{VIM-2B}) using PCR specific primers as reported by the listed authors in Table 1 (13-15,18-21). PCR reactions were performed in a final volume of 50µl containing 3µl of total DNA (50-100 ng) as a template, 3mM MgCl 250 pmol of each primer(Mid land, Texas), 250 µM each of dexynucleotiede triphosphate (dGTP, dCTP, dATP and dTTP), and 1.5U Taq polymerase. The PCR assays for the target genes were performed by using programmable thermocycler as follows: Initial denaturation for 5 min at 94 C° followed by 35 cycles of denaturation at 94C° for 30 seconds, 30 seconds primer annealing at annealing temperature for each primer as presented in Table 1, and extension at 72C° for 45seconds. Tubes were held at 4C° when the cycles ended. The PCR products were analyzed by 1.5% agarose gel electrophoresis run for 45 minutes at 80 V using horizontal apparatus. After the electrophoresis was completed, the gel was visualized under ultraviolet light. DNA molecular size marker (1kb/100bp ladder; Promega, USA) was used.

Statistical analysis

All data were analyzed using the computerized statistical program for Social Science (SPSS, Chicago, IL; version 13, 2004) to compare variables using t test. Results were considered statistically significant if the P value was <0.05.

Results

Table 2 shows the resistant profiles of all 277 *P. aeruginosa* and 104 of *E.coli* isolates to 9 examined drugs. Overall, 38% and 52% of *P. aeruginosa* and 25% and 36% of *E.coli* isolates from effluent water and clinical specimens were multi-resistant to > 3 drugs, respectively.

Tables 3 and 4 show that class 1 integrons was found slightly more common but not significant ($P > 0.05$) in isolates of clinical multidrug resistant *P. aeruginosa* (82% versus 70%) and *E.coli*

TABLE 2. Antimicrobial susceptibility patterns of *P. aeruginosa* and E.coli isolates recovered from effluent water and clinical specimens at the same period.

Antimicrobial agents	% resistant *P. aeruginosa* Water (n 212)a	Clinical (n 65)b	% resistant E.coli Water n 54) c	Clinical (n 50)d
Amikacin	Nil	18	Nil	06
Imipenem	Nil	26	Nil	04
Gentamicin	03	32	13	20
Ciprofloxacin	03	30	20	28
Pipracillin/ Tazobactam	08	26	07	22
Aztreonam	18	38	07	20
Ceftazidime	32	62	07	28
Tetracycline	60	76	56	68
Carbencillin	78	92	61	64

a) 38%, b) 52% , c) 25% and d) 36% of the isolates were resistant to > 3 drugs, respectively.

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TABLE 3. Distribution of antimicrobial resistanc genes in 115 *P. aeruginosa* multidrug- resistant (R >3 drugs) isolates from effluent water and clinical specimens at the same period.

Source	No. Isolates	N (%) positive integron	N (%) tet-A	N (%) tet-B	N (%) $bla_{VIM\ 2a}$	N (%) $bla_{VIM2\ b}$	N (%) aacA	N (%) $bla_{OXA\ -2}$
Effluent water	81*	60 (74)	0	66 (81)	0	3 (4)	24 (30)	16 (20)
Clinical specimen	34*	28 (82)	5 (15)	25 (74)	25 (74)	26 (76)	28 (82)	26 (76)

* All isolates were negative *tet*-39, $bla_{VIM1a,b}$ and $bla_{IMP1,2}$ genes.
** P < (0.05)

TABLE 4. Distributio of antimicrobial resistanc genes in 32 *E.coli* multidrug-resistant isolates (> 3 drugs) from effluent water and clinical specimens at the same period.

Source	No. Isolates	N (%) positive integron	N (%) tet-A	N (%) tet-B	N (%) $bla_{VIM\ 2a}$	N (%) $bla_{VIM2\ b}$	N (%) aacA	N (%) $bla_{OXA\ -2}$
Effluent water	12*	8 (67)	4 (33)	8 (67)	1 (8)	1 (8)	3 (25)	2 (16)
Clinical specimen	20*	14 (70)	8 (40)	14 (70)	10 (50)	10 (50)**	12 (60)**	15 (75)**

* All isolates were negative for *tet*-39, $bla_{VIM1a,b}$ and $bla_{IMP1,2}$ genes.
** P < (0.05)

(74% versus 67%) than in water isolates, respectively. Antibiotic resistance genes (bla_{VIM2a}, $bla_{VIM2\mathbf{b}}$ *aac*A, $bla_{OXA\text{-}2}$) were found more frequently and significantly (*P*< 0.05) in clinical than effluent water isolates (**Table 3 and 4**). All tested isolates of both groups were negative for *tet*(39), $bla_{VIM1(a,b)}$ and $bla_{IMP\ (1,2)}$

Discussion

Two previous studies performed in Jordan over the period 2002-2005 had shown that *P. aeruginosa* and *E.coli* isolates from drinking water and patients stool sources were featured certain common epidemiological characteristics such as serotypes, antimicrobial resistance patterns, plasmids and *tet* (A) gene. The bacterial isolates from both sources were almost equally highly resistant to older used classes of antibiotics; ampicillin, cotrimoxazole, tetracycline, carbenicillin, and exhibited relatively less resistance to currently used drugs in Jordan, such as imipenem , pipracillin-tazobactam, amikacin and ciprofloxacin (4,5).

The present study has found that multidrug resistant rates of *P. aeruginosa* and *E.coli* isolates were higher distributed but not significant (p> 0.05) in clinical than effluent water sources over the same period (**Table 2**). In contrast to our results, a recent study performed within our region in Egypt reported that environmental isolates of *P. aeruginosa* exhibited higher antibiotic resistant rates than clinical isolates, and the majority (97%) of their isolates were beta-lactamase producers (22).

This study showed that class I integrons and *tet*(B) genes were highly prevalent in all multidrug resistant *P. aeruginosa* and *E.coli* isolates from both clinical and water sources, whereas *tet*(A) gene was less common in both isolate groups. In addition, the present study demonstrated that a number of antimicrobial genes of MBLs (bla_{VIM}, bla_{OXA}) and *aacA* genes were found significantly higher (P< 0.05) in *P. aeruginosa* and *E.coli* isolates from clinical than water sources. Previous studies from different countries have observed similar results among *P. aeruginosa* (2, 10), and recently this phenomena was observed in the *Enterobacteriaceae* isolated from patients, particularly *K. pneumonia* (9,11-12).

Resistance to the carbapenems in *P. aeruginosa* is mediated by several mechanisms, often by Class 1 integron-associated MBLs such as VIM and IMP, outer membrane porin reduction or loss, and over expression of efflux pumps (15). The high rate of multidrug resistant *P. aeruginosa* producing various MBLs in clinical isolates could result in their increased occurrence in the hospital environment, waste water and may later returned back to colonize the human gut (23) . It is known that antimicrobial resistance genes may be horizontally or vertically transferred between bacterial populations in the water environment. The factors which determine the successful transmission and colonization of pathogens in environmental niches are unknown as it is a complex interplay between virulence potential and environmental spread (6-7). However, It can be excepted that contact between clinical pathogens and environmental bacteria, with the presence of selective pressure by broad-spectrum antimicrobials, is perfect for the exchange and spread of resistance genes.

In conclusion, this study shows that effluent water in Jordan is highly polluted with multidrug resistant *P. aeruginosa* and *E.coli* and this may have serious implications for public health in the future.

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