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Isolation and Characterization of Antibiotic and Heavy Metal-Resistant *Pseudomonas aeruginosa* from Different Polluted Waters in Sohag District, Egypt

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Abstract Different polluted water samples were collected from a wastewater treatment plant, agricultural drainage canals, the River Nile, and irrigation canals. The samples were examined for the enumeration of *Pseudomonas aeruginosa* in the Sohag area, Egypt over a period of one year. A total of 240 isolates were collected and tested for their resistance to 12 common antibiotics and 6 heavy metals. The isolates were found to be less resistant to norfloxacin (1.7%), ofloxacin (4.6%), amikacin (9.6%), tobramycin (10.4), carbenicillin (15.4), and gentamycin (41.3%), yet more sensitive to rifampicin (75%), kanamycin (89.6%), ampicillin (90.8%), chloramphenicol (91.7%), streptomycin (92.9%), and tetracyclin (96.3%). In contrast, 7.1%, 12.9%, 25.4%, and 53.7% of the isolates were resistant to lead, cadmium, mercury, and zinc, respectively. None of the isolates had developed a resistance to silver or molybdenum. The high frequency of metal-antibiotic double resistance existed between lead and amikacin (56.5%), cadmium and ofloxacin (72.7%), zinc and norfloxacin (100%), and mercury and carbenicillin (94.6%). The high occurrence of antibiotic-resistant bacteria in natural water could be related to the widespread use of antibiotics, with possible public health hazard.

Key words: *Pseudomonas aeruginosa*, heavy metals, antibiotics

The detection of *Pseudomonas aeruginosa* in an aquatic natural environment is generally related to pollution coming from sewage, therefore, it has been considered as a water quality indicator microorganism [19, 10, 16, 22]. The importance of studying *P. aeruginosa* in natural polluted waters is based on its potentiality in originating various kinds of severe infections [38, 28, 41].

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An important characteristic of *P. aeruginosa* is its high resistance to antibiotics, heavy metals, and other environmental factors [21, 32, 35, 47, 50], which is related to its survival capability in aquatic environments [8, 13, 25]. Most strains of this species show a significant degree of intrinsic resistance to a wide variety of antimicrobiol agents, including β-lactams, aminoglycosides, and fluoroquinolones [14, 27, 41].

Microorganisms that are resistant to both antibiotics and heavy metals have been frequently isolated from different aquatic environments and clinical samples [44]. The presence of a large number of resistant bacteria in natural habitats can pose a public health risk [36]. The continuous exposure of microbial populations to antibiotics, metals or other agents can result in the selection of strains that are resistant to these agents. Such selection has already been observed in water treatment systems [5] and chemotherapy [31, 43].

The present investigation was, therefore, undertaken to determine the level of antibiotic and heavy metal resistance among *P. aeruginosa* strains collected from different natural sources of water in the Sohag district.

MATERIALS AND METHODS

Sampling

Different polluted water samples were collected from different sites in the Sohag area. These samples represented different water sources; namely, untreated and treated samples of the Sohag wastewater treatment plant, the River Nile, and irrigation and agricultural drainage canals.

For determining the microbiological pollution, represented by faecal coliforms and faecal streptococci, an investigation of *P. aeruginosa* and analysis of some physicochemical properties was conducted on samples collected monthly over a period of one year. The collected samples were immediately transferred to the laboratory for investigation.

Bacteriological Examination

Appropriate dilutions were prepared from each water sample, which were then analyzed for faecal colifoms (FC) using the MPN method [4]. The faecal streptococci (FS) investigation was performed using the membrane filtration technique [4]. Samples considered as highly polluted contained more than 10^4 FC or FS/100 ml for freshwater [3, 46].

The densities of *P. aeruginosa* were determined using the membrane filtration technique on an MPA agar medium [17]. The plates were incubated at 41°C for 72 h. All colonies of 1- 2 mm in diameter with flat outer rims and slightly raised brown to dark green or black centers were counted and isolated. The isolates were primarily identified on the basis of their colonial morphology, pigment production, and an oxidase test. Confirmation of identity was based on the standard biochemical methods [26].

Antimicrobiol Susceptibility

The susceptibility of *P. aeruginosa* isolates to various antibiotics was determined by the standard disc-agar diffusion method [18]. The following antibiotic discs (supplied by Biomerieux) were used: ampicillin (AM), 10 µg; streptomycin (S), 10 µg; gentamycin (GM), 10 µg; kanamycin (K), 30 µg; amikacin (AK), 30 µg; tobramycin (TOB), 10 µg; tetracycline (TE), 30 µg; chloramphenicol (C), 30 µg; norfloxacin (NOR), 10 µg; ofloxacin (OFX), 10 µg; carbenicillin (CB), 10 µg; and rifampicin (RA), 30 µg.

Examination of Heavy Metal Resistance

The resistance to heavy metals was evaluated by the agar diffusion method [48]. The strains of *P. aeruginosa* were assayed against the following concentrations of different metal salts: Zinc sulfate (1,600 µg/ml); lead acetate (3,200 µg/ml); cadmium chloride (1,600 µg/ml); mercuric chloride (10 µg/ml); silver nitrate (128 µg/ml), and sodium molbydenate (102,400 µg/ml). Appropriate stock solutions of different metallic salts were prepared in distilled water, sterilized by membrane filtration (0.22 µm), and kept at 40°C for less than one week. The concentrations used in this study were similar to those used in previous studies [32, 16]. Physicochemical analyses were conducted according to the American Public Health Association's standard methods [4].

RESULTS AND DISCUSSIONS

Measurements of the water quality parameters for different sources of water in the Sohag area were estimated monthly. The one-year averages of physicochemical characteristics and faecal pollution indicators (FC and FS) of the tested water samples are given in Table 1. The data showed that all the examined samples, especially from the River Nile, drainage and irrigation canals, were heavily faecally polluted. The River Nile is highly polluted because it receives discharges from industrial sources and sewage outfalls as well as

Table 1. Mean value of some physicochemical characteristics and faecal pollution indicators of Sohag water sources during the investigation period of one year.

Parameters (mg/l)	Wastewater						Surface water			
	Untreated wastewater		Treated wastewater		Agricultural drainage water		River Nile water		Irrigation water	
	Min. -	Max.	Min. -	Max.	Min. -	Max.	Min. -	Max.	Min. -	Max.
PH	6-7	8.2	6.8	7.5	7	8.3	6.9	7.7	7.1	7.9
TDS	950	1750	500	700	600	900	200	250	400	800
TSS	300	520	120	250	220	600	150	480	180	560
BOD	250	390	80	145	8	28	1.5	6.3	3.5	22
TN	90	220	58	110	12	50	2.6	7.2	11.2	45
NH_3-N	50	90	18	46	5	36	0.9	2.4	2.5	22
NO_3-N	0.3	0.5	1	3.5	0.2	0.9	0.04	0.45	0.1	0.73
P	4	15	1.5	8	0.16	0.83	0.05	0.5	0.1	0.57
Na	70	140	55	100	17	66	6	45	8	55
K	14	30	9	23	0.65	10.8	0.15	8	0.43	9.5
Fe	0.01	0.3	0.0	0.2	0.01	0.9	0.016	0.09	0.0	0.09
Mn	0.02	0.2	0.01	0.2	0.05	0.2	0.0	0.07	0.0	0.04
Cu	0.0	0.2	0.0	0.1	0.01	0.03	0.01	0.03	0.0	0.04
Pb	0.08	0.8	0.05	0.5	0.016	0.4	0.006	0.05	0.0	0.07
FC/100 ml	1.0×10^8	3×10^{10}	2×10^4	4×10^6	6.8×10^3	1×10^6	8×10^2	4.4×10^5	2.8×10^3	1.6×10^6
FS/100 ml	3.0×10^8	7×10^{10}	9×10^4	6×10^6	5.0×10^3	3×10^6	2×10^3	7×10^5	9×10^3	8.5×10^6

Min. and Max., Minimum and maximum values of each parameter detected during one year; FC, Faecal coliform; FS, Faecal streptococci.

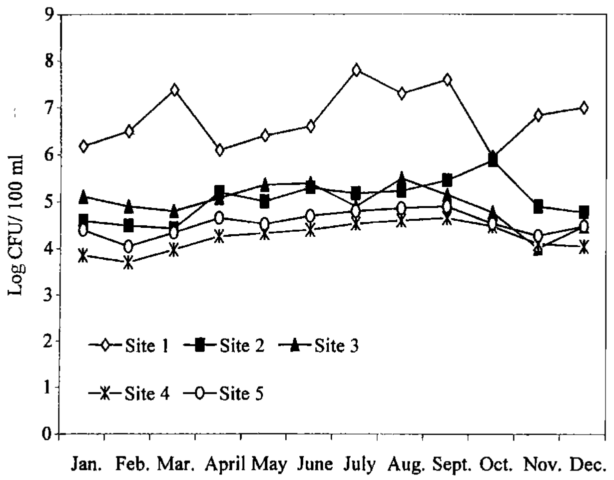

Fig. 1. Distribution of *Pseudomonas aeruginosa* in samples collected from different sources in Sohag area.
Site 1, raw wastewater; Site 2, treated waste water; Site 3, agricultural drainage water; Site 4, Nile river water; Site 5, irrigation water.

seepage from inactive hazardous waste sites. Therefore, the conditions in the River Nile seem appropriate for the selection of antibiotic or metal-resistant *Pseudomonas aeruginosa.* This would have significance on public health since the river is heavily used fore fishing and other recreational purposes.

Distribution of *P. aeruginosa*

For studying the distribution of *P. aeruginosa* resistant strains, the samples were classified, independently of the collection sites, on the basis of the faecal indicator content. The study of a nonpolluted area was not possible because *P. aeruginosa* was only isolated from faecally polluted areas (Fig. 1).

Over the course of the one-year study, the total number of *P. aeruginosa* ranged between 1.5×10^5 and 3.6×10^6 cfu/100 ml in the samples collected from untreated wastewater and between 2.7×10^3 and 7.5×10^4 in the treated wastewater samples (Fig. 1). In the agricultural drainage water, the total number of *P. aeruginosa* was within a range of 1.0×10^3 to 3.2×10^4 cfu/100 ml. The highest densities (4.5×10^3 and 8.1×10^3 cfu/100 ml) were scored during September in the River Nile and irrigation samples, whereas the lowest number (5×10^2 and 1.3×10^3 cfu/100 ml), respectively, were recorded in February. These results coincide with those previously reported [2, 37, 49].

Particular attention was paid to investigating the degree of pollution of *P. aeruginosa* in the tested water samples. The data presented in Table 2 shows that this bacterium was recorded in a relatively high frequency in Sohag area water. The widespread occurrence of this organism reflects its role as a contaminant, an indicator of pollution, and as an autochthonous member of the bacterial community in a highly polluted area [15].

Resistance of P. aeruginosa to Antibiotics

The 240 identified isolates (60 isolates for each of the four different bodies of water) were tested with various antibiotics and heavy metals. The resistance patterns of the strains

Table 2. Relative resistance of *P. aeruginosa* strains from different sites of water to various antibiotics and heavy metals.

Antibiotics and heavy metals	Untreated and treated wastewater		Agri. drainage water		River Nile water		Irrigation water		Overall	
	R	%	R	%	R	%	R	%	R	%
Ampicillin	58	96.7	56	93.3	50	83.3	54	90	218	(90.8)
Streptomycin	59	98.3	55	91.7	53	88.3	56	93.3	223	(92.9)
Gentamycin	36	60	21	35	18	30	24	40	99	(41.3)
Tobramycin	10	16.7	5	8.3	3	5	7	11.7	25	(10.4)
Norfloxacin	3	5	0	0	0	0	1	1.6	4	(1.7)
Ofloxacin	9	15	0	0	0	0	2	3.3	11	(4.6)
Carbenicillin	21	35	5	8.3	3	5	8	13.3	37	(15.4)
Kanamycin	55	91.7	54	90	51	85	55	91.7	215	(89.6)
Amikacin	11	18.3	4	6.7	2	3.3	6	10	23	(9.6)
Tetracyclin	60	100	57	95	55	91.7	59	98.3	231	(96.3)
Chloramphenicol	60	100	50	83.3	52	86.7	58	96.7	220	(91.7)
Rifampicin	52	86.7	40	66.7	45	75	47	78.3	180	(75)
Lead	9	15	6	10	0	0	2	3.3	17	(7.1)
Zinc	38	63.3	43	71.7	21	35	27	45	129	(53.7)
Cadmium	15	25	11	18.3	0	0	5	8.3	31	(12.9)
Mercury	18	30	22	36.7	8	13.3	13	21.7	61	(25.4)
Silver	0	0	0	0	0	0	0	0	0	0
Molybdenum	0	0	0	0	0	0	0	0	0	0
Total isolates	60		60		60		60		240	

R, Total number of isolates resistant to a particular antibiotic or metal; % , Percent of resistant isolates.

from different sources of water are shown in Table 2. The untreated and treated wastewater isolates exhibited sharp peaks of resistance to tetracycline (100%), chloramphenicol (100%), streptomycin (98.3%), ampicillin (96.7), kanamycin (91.7%), rifampicin (86.7%), and gentamycin (60%), whereas the resistance to the other remaining antibiotics ranged between 5% and 35%. The strains from the agricultural drainage water showed fewer variations in their resistance to antibiotics (Table 2). Most isolates (> 90%) were highly resistant to ampicillin, streptomycin, kanamycin, and tetracyclin. In addition, a high degree of sensitivity (100%) was detected in the case of ofloxacin and norfloxacin. *P. aeruginosa* is known to be highly resistant to various antimicrobial agents [11, 12, 25]. However, the resistance of 90% of the 200 *P. aeruginosa* isolates from the untreated sewage investigated by Al-Jebouri, in 1985 [1], was almost similar to our present results.

The strains isolated from the River Nile and irrigation water exhibited the highest rate of resistance (> 75%) to ampicillin, streptomycin, kanamycin, tetracyclin, chloramphenicol, and rifampicin, whereas only weak or no resistance to the other tested antibiotics was observed. These results were similar to those previously reported [16, 32, 37, 41, 43].

Based on the present results it can be stated that the ratios of antibiotic-resistant *P. aeruginosa* in the River Nile water were much lower than those in the untreated wastewater, drainage, and irrigation canals. The observation that the untreated wastewater contained multiple resistant isolates with a variable frequency compared to those from contaminated river and irrigation water (Table 2, Fig. 1) was confirmed by other studies on antibiotic resistance [1, 24, 31, 43]. These researchers concluded that the raw wastewater isolates were mostly human-derivated bacteria, whereas the isolates from contaminated river water were mainly derived from sewage and plants or soil.

Antibiotic-resistant strains of *P. aeruginosa* causing diseases in Egypt, such as otitis, meningitis, urinary tract, ear, and eye infections, and skin rashes have been previously isolated from clinical materials by Shohayeb *et al.* [42], who found that the percentages of isolates resistant to carbenicilin, amikacin, gentamycin, tobramycin, and ofloxacin were 29.2, 1.4, 40.3, 11.1, and 13.8%, respectively. They also noticed that none of the isolates were resistant to norfoxacin. Meanwhile a lower figure was recorded by Tarek and Shohayeb [45], who reported that the percentages of strains resistant to gentamycin, tobramycin, and carbenicillin were 80, 55, and 60%, respectively. Bastawrows, in 1997 [9], indicated that a high degree of (100%) isolates were detected to be resistant against 5 antibiotics (ampicillin, gentamycin, chloramphenicol, penicillin-G, and erythromycin) out of 8 antibiotic tested.

Part of the explanation for the relatively higher level of resistance to various antibiotics by *P. aeruginosa* observed in the present work may be due to widespread use of antibiotics in the area under study. The aforementioned results, described either in the current study or in the literatures, all showed marked variations in the behavior of *P. aeruginosa*. These variations are most probably due to biological alterations resulting from the misuse of different antibiotics in both medical and veterinary practices [6].

Resistance to Heavy Metals

P. aeruginosa strains resistant to various concentrations of the six heavy metal ions were investigated and the percentage is summarized in Table 2. Variable resistance levels were only observed against Cd, Zn, Pb, and Hg. The high resistance frequencies for the *P. aeruginosa* strains

Table 3. Correlation between antibiotics and heavy metals resistance in 240 isolates of *P. aeruginosa* from different polluted waters at the Sohag area.

Antibiotic		Lead			Zinc			Cadmium			Mercury		
	TN	N	%	T%	N	%	T%	N	%	T%	N	%	T%
Ampicillin	218	17	7.8	7.1	100	45.9	41.7	30	13.7	12.5	60	27.5	25.0
Streptomycin	223	14	6.3	5.8	109	48.9	45.4	30	13.5	12.5	61	27.4	25.4
Gentamycin	99	9	9.1	3.8	65	65.7	27.1	26	26.3	10.8	50	50.5	20.8
Kanamycin	215	15	7.1	6.3	100	46.5	41.7	31	14.4	12.9	61	28.4	25.4
Amikacin	23	13	56.5	6.5	19	82.6	7.9	15	65.2	6.3	20	86.9	8.3
Tobramycin	25	8	34.8	3.3	23	92	9.6	13	52	5.4	20	80.0	8.3
Tetracyclin	231	17	7.8	7.4	125	54.1	52.1	31	14.6	12.9	61	26.4	25.4
Chloramphenicol	220	17	7.7	7.1	125	56.8	52.1	31	14.1	12.9	61	27.7	25.4
Norfloxacin	4	2	50	0.8	4	100	1.7	3	75	1.3	3	75.0	1.7
Ofloxacin	11	6	54.5	2.5	10	91	4.2	8	72.7	3.3	10	90.9	4.2
Carbenicillin	37	10	27.0	4.2	30	81	12.5	17	46	7.1	35	94.6	14.6
Rifampicin	180	12	6.7	5.0	80	44.4	33.3	24	13.3	10	57	31.7	23.8
Total isolates	240	17			129			31			61		

TN, Total number of isolates resistant to the particular antibiotic; N, Number of isolates resistant to metal and antibiotics; %, Percent of total number of isolates resistant to the particular antibiotic; T%, Percent of total number of isolates (240).

isolated from untreated and treated wastewater, drainage, River Nile, and irrigation water to Zn were 63.3, 71.7, 35, and 45%, respectively, while the resistances to Hg were 30, 36.2, 13.3, and 21.7%, respectively. As for Pb and Cd, 15 and 25%, 10 and 18.3%, and 3.3 and 8.3% of the isolates from the wastewater, drainage, and irrigation water, respectively, were more tolerant toward these heavy metals. The isolates from the River Nile water were not resistant to Cd and Pb, and all the isolates were not resistant to Ag and Mo. These findings are partly in support of those previously reported [16, 21, 32].

The metal considered as the environmental marker is Hg. The frequencies of Hg resistance obtained in this study were almost similar to those reported for *P. aeruginosa* isolated from surface waters by Joly *et al.* [23] and De Vicente [16], although they were lower than the frequencies observed for strains of *P. aeruginosa* isolated from different sources [8, 32, 33].

The percentage of metal and antibiotic double-resistant strains (Table 3) was calculated by dividing the total number of isolates resistant to a specific antibiotic and also to a certain metal ion by the total number of isolates resistant to this particular antibiotic X100 [39]. The highest incidence of metal antibiotic double resistance existed between lead and amikacin (56.5%), cadmium and ofloxacin (72.7%), zinc and tobramycin (92%), and mercury and carbenicillin (94.6%). The resistance to a particular heavy metal has already been reported to correlate with antibiotics and other heavy metals resistance in certain organisms [7, 29, 39], and the role of plasmids in conferring resistance to antibiotics and metals has also previously been demonstrated [20, 30, 34, 40].

The results obtained in this study indicate that the polluted water may play an important role as a reservoir of antibiotic and heavy metal resistant *Pseudomonas aeruginosa*, resulting in increasing the potential public health hazard.

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