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RESEARCH ARTICLE OPEN ACCESS

Occurrence of Multidrug Resistant *Pseudomonas aeruginosa* Isolated from Waste Water Samples of Dhaka, Bangladesh

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Abstract

***Pseudomonas aeruginosa* is a prevalent gram-negative pathogenic bacterium ubiquitous in natural environment. Aquatic environment of wastewater serves as reservoirs of this bacteria and their wide resistance phenomenon to a number of antibiotics is frequently increasing. This study was conducted to determine the prevalence of *P. aeruginosa* in 10 industrial waste water and 10 tannery waste water samples of whole Dhaka and 65% (13/20) water samples were found positive for *P. aeruginosa* which was confirmed by both biochemical test & Biolog™ Microbial Identification System. Kirby Bauer disc diffusion method was applied for antimicrobial susceptibility testing and isolates showed resistance to most of the commercial antibiotics except neomycin, gentamycin, streptomycin, ciprofloxacin and nalidixic acid, hence confirmed the multidrug resistance (MDR) of *P. aeruginosa* in wastewater which is one of the life-threatening public health issues all over the world causing ineffectiveness of several antibiotics. So, it is recommended to make sure surface water or food samples not to be contaminated by this antibiotic resistant *P. aeruginosa* that might be transferred to animal and human. In these circumstances, not only the hygiene practice is the first and foremost prerequisite but also management practices with effective wastewater disposal system can also be a part of awareness. Additionally, appropriate and logical use of antibiotics must be applied to reduce the emergence of multidrug pathogens to environment.**

Keywords: Antibiotics, Antimicrobial Susceptibility Testing (AST), Hygiene, Multidrug Resistance (MDR), Pathogens

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INTRODUCTION

Pseudomonas species are considered as a leading organism found from soil, water, food and plants. It is an aerobic, gram-negative and motile rod bacterium belonging to the family *Pseudomonadaceae*. Widespread occurrence of this bacterium relating to water, is an increasing concern of public health now-a-days. Transmission of *Pseudomonas* sp. to human body initiates foodborne illness by taking food & water contaminated with this bacterium. Some species of *Pseudomonas* are medically significant because they are considered as opportunistic pathogens for humans and animals while others are very important in the agricultural sector.[1] Data covering 1986-2003 from the National Nosocomial Infections Surveillance system showed *P. aeruginosa* is the most common cause of pneumonia and urinary tract infection.[2]

The occurrence of *P. aeruginosa* in sewage effluent sample is very common. Although number of this bacteria is variable in fresh water bodies, its level is minimum in drinking water for good management and hygiene practices. *P. aeruginosa* can metabolize a wide variety of compounds, and proliferate in waters with low concentrations of dissolved compounds, showing its ability to adapt in environmental challenges with minimal nutritional requirements.[3] This organism's ability to form biofilms, results their protection against adverse environmental conditions and also shows tolerance and resistance to antibiotics, which are secondary metabolites produced by microorganisms and chemically synthesized/semi-synthesized molecules inhibiting proliferation of others.[4] Usage of antibiotics is increasing promptly and studies say that it will reach up to 200%.[5] Besides, it has been found that, very low percentages get completely metabolized by humans and animals whereas 20-90% get excreted through urine and feces reaching and contaminating the environment.[6] As a result, most *Pseudomonas aeruginosa* infections are difficult to treat due to high levels of antibiotic resistance. The use of antibiotic as growth promoters and feed enhancer in farming and livestock management are responsible for spreading the antibiotic resistance genes in the environment.[7] Besides, Antibiotic resistance is an accommodative genetic feature present in few bacterial subpopulations and due to this, they get survival capacity rather killing under therapeutic doses.[8] Because antibiotics at low concentration create selective pressure on bacterial colonies which emphasize their resistance profile.[9,10] Moreover, bacteria can explore multidrug resistance (MDR) creating a challenge to give treatments in hospitals.[11] Not only that, several virulence factors are spread out due to multidrug resistance (MDR) in health care centers and others community settings impacting economic and social aspects, less productivity and high poverty rate.[12]

Considering the concerning fact relating to multidrug resistance of *P. aeruginosa*, the present study was designed to isolate and identify environmental *P. aeruginosa* from 2 types of waste water samples and show their antibiotic resistance pattern. Besides, pH, temperature, DO (Dissolve Oxygen), TDS (Total Dissolve Solids), TVC (Total Viable Count), TC (Total Coliform) of the collected water samples were also documented as well.

MATERIALS AND METHODS

Sample collection

A total of 20 water samples (first 10 from different industry effluent sites and next 10 from different tannery sites at Savar in Hemayetpur, Dhaka) were aseptically collected for detecting microbiological and physicochemical properties. Prior to collection, bottles were washed, rinsed thoroughly several times with distilled water and then autoclaved. For the accuracy of the results, the physicochemical parameters were measured immediately from the collected samples and the samples were labeled and transported to the laboratory for microbiological analysis.

Physico-chemical analysis

Temperature is the most responsible physico-chemical factor to determine the quality of water and is measured by thermometer.[13] pH measures acidity and alkalinity of a solution. pH has a major influence on bacterial population growth, whereas pH value of 7 is the neutral one.[13] The DO, TDS and pH of the samples were estimated at the point of collection using portable DO meter, TDS meter and pH meter respectively.

Microbiological analysis

Collected water samples were analyzed according to the standard method of APHA 1995 (American Public Health Association). TVC was performed by serial dilution method, followed by pour plating in plate count agar (PCA) media. The plates were incubated at 37°C for 24 hours. All the experiments were performed at 37°C for 24 hours in duplicate manner. To monitor water quality, the enumeration of total coliforms (TC) was done by following Most Probable Number (MPN). Presence of *P. aeruginosa* was detected by inoculating 10 ml of sample into 100 ml Tryptic soy broth (TSB).[14] After proper mixing, broth was subjected to incubation for 48 hours at 35-37°C. After that, a loopful growth was streaked on cetrimide agar (CEA) plate from TSB and again incubated for 48 hours at 35-37°C. 30 distinct colonies exhibited fluorescent green pigment were primarily selected as *P. aeruginosa*.

Biochemical confirmation of selected *P. aeruginosa*

Selected *P. aeruginosa* colonies were purified for 3 months continuously in Plate Count Agar (PCA) medium and 16 biochemical tests were performed for their possible identification according to Bergey's Manual for Systematic Bacteriology, Vol. 2.[15] All tests were done in duplicate and the incubation period was 37°C for 24 hours.

Biolog confirmation of *P. aeruginosa*

Preliminary selected *P. aeruginosa* were also confirmed by Biolog™ Microbial Identification System (Biolog Inc., USA) using GEN III Micro Plate™. 24 hours bacterial cultures were swabbed by inoculators and taken in inoculation fluid supplied by the company. The solution was poured into reservoir and 100 µl was transferred into each GEN III MicroPlate™ plate well by multichannel pipette tips followed by incubation of the plates at 37°C for 24 hours. After this period, plates were placed in Biolog™ Microbial Identification System machine and read by the system software OmniLog® which gave a specific bacterial name.

Antibiotic susceptibility pattern of *P. aeruginosa*

The antibiotic susceptibility testing was performed on Muller Hinton agar using Kirby Bauer disc diffusion method[16] against the following commercial antibiotics- ampicillin (AMP),

Table 1. Physico-chemical parameters and microbial load of collected waste water samples

No.	Temp (°C)	pH	DO (ppm)	TDS (ppm)	TVC (cfu/ml)	TC (MPN/100 ml)	*Pseudomonas aeruginosa*
1.	29.9	8.3	4.43	262	2.2×10^4	>2400	Present
2.	30.2	8.2	4.23	78	Absent	Absent	Absent
3.	29.3	8.5	4.85	94	Absent	Absent	Absent
4.	29.9	7.6	4.42	68	4.6×10^4	>2400	Present
5.	29.8	7.7	4.00	82	1×10^3	>2400	Present
6.	29.8	7.7	3.90	80	Absent	Absent	Absent
7.	29.8	7.3	3.62	81	3.4×10^4	>2400	Present
8.	29.8	7.2	3.18	75	1.3×10^4	>2400	Present
9.	30	8.0	4.07	33	8×10^3	>2400	Present
10.	29.6	8.0	4.22	68	Absent	Absent	Absent
11.	30.3	7.5	4.44	56	1.02×10^5	>2400	Present
12.	29.7	7.7	4.23	78	1.45×10^4	>2400	Present
13.	30.1	7.6	4.11	73	3.5×10^6	>2400	Present
14.	29.8	8.5	3.93	64	Absent	Absent	Absent
15.	31.0	8.3	3.78	81	4.5×10^3	>2400	Present
16.	28.9	8.1	3.17	49	2.4×10^4	>2400	Present
17.	29.9	7.8	3.69	86	Absent	Absent	Absent
18.	30.3	7.6	4.32	88	2.7×10^3	>2400	Present
19.	30.2	7.1	4.43	91	5.2×10^4	>2400	Present
20.	29.7	8.2	3.56	79	Absent	Absent	Absent

methicillin (MET), ceftriaxone (CRO), ceftazidime (CAZ), cefixime (CFM), rifampicin (RIF), penicillin G (PEN G), neomycin (NEO), nitrofurantoin (NIT), fusidic acid (FA), amoxicillin (AMX), cefaclor (CEC), vancomycin (VAN), gentamycin (GEN), streptomycin (STR), ciprofloxacin (CIP), erythromycin (ERY), chloramphenicol (CHL) and nalidixic acid (NAL).

RESULTS AND DISCUSSION

The initiative to create a resistance profile of waste water is a new undertaking in Bangladesh. The spread pattern of Multiple drug resistance (MDR) explores a threatening risk for human boosting up morbidity, mortality and cost.[12] Untreated waste water may facilitate more spread of multi drug resistance (MDR). In the present study, the physico-chemical parameters of collected samples were measured and the results of the physico-chemical parameters of collected samples are shown in Table 1.

The pH level of samples ranged between 7.1-8.5 and the temperature was more or less 29°C. DO differed among the sampling areas, with a maximum of 4.85 ppm and a minimum of 3.17 ppm. In most of the cases, maximum dissolved oxygen concentrates vary with temperature. But for living organism, 4 mg/L (4ppm) of minimum DO should be in water otherwise living organism cannot survive.[17] So, it can be said that our collected wastewater sites were not a good example for aquatic growth.

Figure 1 represents the total viable count (TVC) of waste water in Plate count agar media. Highest number of total bacterial count (5.2×10^4) was found in sample no 19 from tannery site. The total coliform no was also beyond the limit (>2400) that confirmed unsatisfactory water quality. *P. aeruginosa* was detected in 13 water samples showing fluorescent green pigment on CEA plate and among them, 30 colonies were subjected to biochemical test responses in different media. The overall same result for all selected colonies was presented in Table 2.

All strains gave positive result in citrate utilization, oxidase and catalase test. Besides, all the strains were motile and pigmented. On the other hand, these suspected colonies were found to be negative in indole test, methyl red test and VP test. Among the glucose, mannitol, sucrose and lactose, all the selected isolates were able to ferment glucose only. Finally, all the suspected colonies were biochemically confirmed as *P. aeruginosa* by comparing phenotypical data of pathogen with the published data of Bergey's Manual for Systematic Bacteriology, Vol. 2.[15] Moreover, the Biolog™ Microbial Identification System machine also clearly indicated the colonies as *P. aeruginosa* (Figure 2).

Being intrinsically resistant to several classes of antibiotics, *P. aeruginosa* limit the treatment choices. Unluckily now, problems with antimicrobial treatment are becoming complicated

Table 2. Overall biochemical test results of selected isolates

No.	Name of the test	Result
1.	Colony morphology	Rod
2.	Gram Stain	Negative
3.	Indole production	Negative
4.	Methyl red test	Negative
5.	Voges- Proskauer test	Negative
6.	Citrate	Positive
7.	Oxidase	Positive
8.	Catalase	Positive
9.	Pigment	Positive
10.	Motility	Positive
11.	Urease	Negative
12.	H_2S production	Negative
13.	Glucose	Positive
14.	Sucrose	Negative
15.	Mannitol	Negative
16.	Lactose	Negative

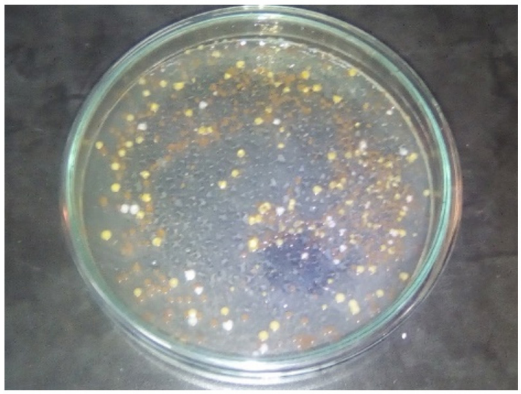
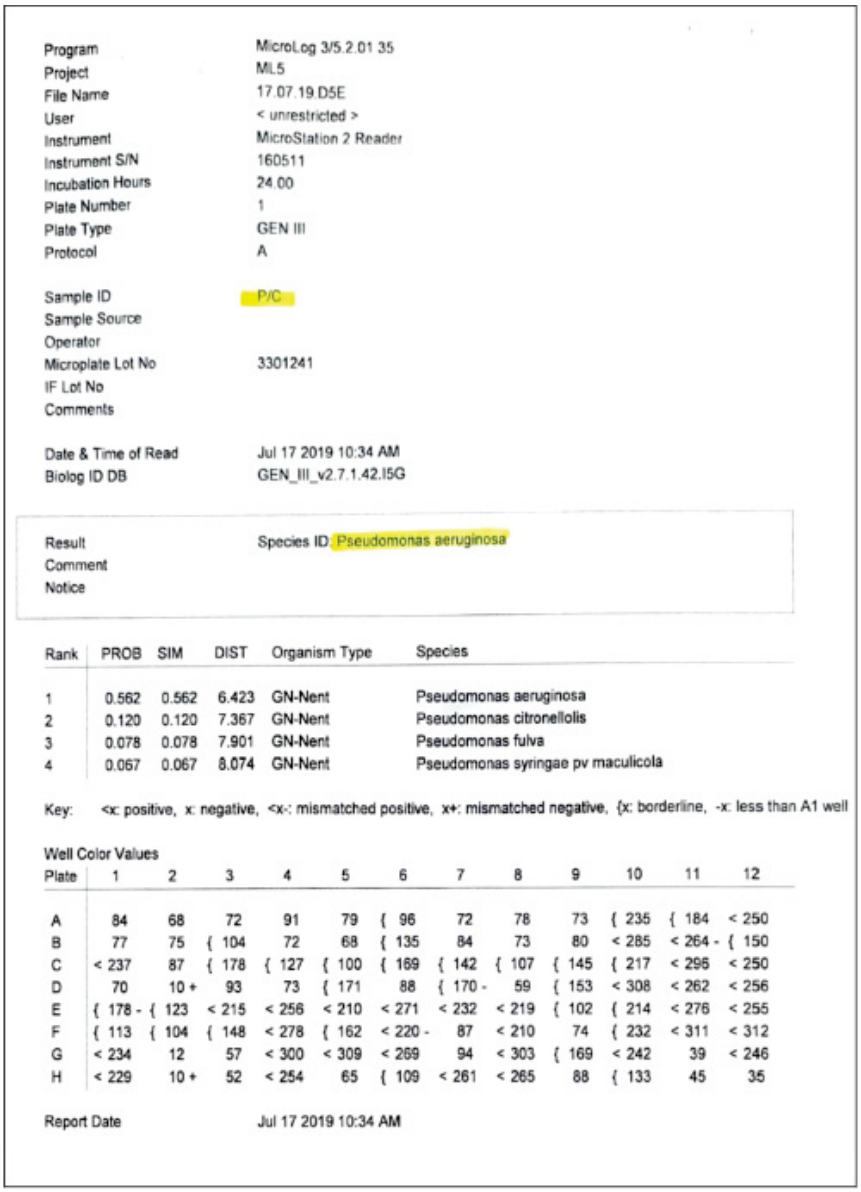


Figure 1. Total bacterial count (cfu/ml) in PCA medium



Program	MicroLog 3/5.2.01 35
Project	ML5
File Name	17.07.19 D5E
User	< unrestricted >
Instrument	MicroStation 2 Reader
Instrument S/N	160511
Incubation Hours	24.00
Plate Number	1
Plate Type	GEN III
Protocol	A
Sample ID	P/C
Sample Source	
Operator	
Microplate Lot No	3301241
IF Lot No	
Comments	
Date & Time of Read	Jul 17 2019 10:34 AM
Biolog ID DB	GEN_III_v2.7.1.42.I5G
Result	Species ID: Pseudomonas aeruginosa
Comment	
Notice	

Rank	PROB	SIM	DIST	Organism Type	Species
1	0.562	0.562	6.423	GN-Nent	Pseudomonas aeruginosa
2	0.120	0.120	7.367	GN-Nent	Pseudomonas citronellolis
3	0.078	0.078	7.901	GN-Nent	Pseudomonas fulva
4	0.067	0.067	8.074	GN-Nent	Pseudomonas syringae pv maculicola

Key: <x: positive, x: negative, <x-: mismatched positive, x+: mismatched negative, {x: borderline, -x: less than A1 well

Well Color Values

Plate	1	2	3	4	5	6	7	8	9	10	11	12
A	84	68	72	91	79	{ 96	72	78	73	{ 235	{ 184	< 250
B	77	75	{ 104	72	68	{ 135	84	73	80	< 285	< 264 -	{ 150
C	< 237	87	{ 178	{ 127	{ 100	{ 169	{ 142	{ 107	{ 145	{ 217	< 296	< 250
D	70	10 +	93	73	{ 171	88	{ 170 -	59	{ 153	< 308	< 262	< 256
E	{ 178 -	{ 123	< 215	< 256	< 210	< 271	< 232	< 219	{ 102	{ 214	< 276	< 255
F	{ 113	{ 104	{ 148	< 278	{ 162	< 220 -	87	< 210	74	{ 232	< 311	< 312
G	< 234	12	57	< 300	< 309	< 269	94	< 303	{ 169	< 242	39	< 246
H	< 229	10 +	52	< 254	65	{ 109	< 261	< 265	88	{ 133	45	35

Report Date Jul 17 2019 10:34 AM

Figure 2. Biolog confirmation of *Pseudomonas aeruginosa*

for their rising acquired or mutational resistance.[18] Multiple reports of multidrug-resistant (MDR) *P. aeruginosa* have been found worldwide and this resistance is thought to be driven by several mechanisms including efflux mechanism, enzymatic deactivation, loss of outer membrane protein (porin) and target mutations.[19] Similarly, in our study, antimicrobial susceptibility testing of *P. aeruginosa* showed resistance to most of the commercial antibiotics except neomycin, gentamycin, streptomycin, ciprofloxacin and nalidixic acid (Table 3).

In a study of Frederick Bert 1997, 13.3% and 16.1% of *P. aeruginosa* were resistant to amikacin and ceftazidime respectively.[20] Similarly, 66.7% resistance to ceftazidime was found in a study done by Shakir *et al.*[21] This study was in agreement with Sulaiman and Abdulhasan who

Table 3. Antibiotic name, class, target and overall susceptibility test result of selected *Pseudomonas aeruginosa*

No.	Name of Antibiotics	Class /Sub class	Target	Potency	Overall Susceptibility result of *P. aeruginosa*
1.	Rifampicin	Rifamycin	RNA synthesis	10 µg	R
2.	Penicillin G	Penicillin	Cell wall	10 µg	R
3.	Neomycin	Aminoglycosides	Protein synthesis, 30S	10 µg	S
4.	Nitrofurantoin	Nitrofurane	Multiple	300 µg	R
5.	Fusidic acid	Fusidane	Protein synthesis	10 µg	R
6.	Amoxicillin	Aminopenicillins	Cell wall	25 µg	R
7.	Cefaclor	2nd generation Cephalosporins	Cell wall	10 µg	R
8.	Vancomycin	Glycopeptides	Cell wall	30 µg	R
9.	Gentamicin	Aminoglycosides	Protein synthesis, 30S	10 µg	S
10.	Streptomycin	Aminoglycosides	Protein synthesis, 30S	10 µg	S
11.	Ciprofloxacin	2nd generation fluroquinolones	DNA synthesis, DNA gyrase	5 µg	S/R (variable)
12.	Erythromycin	Macrolide	Protein synthesis, 50S	35 µg	R
13.	Chloramphenicol	Chloramphenicol derivatives	Protein synthesis, 50S	10 µg	R
14.	Nalidixic acid	1st generation fluroquinolones	DNA synthesis, DNA gyrase	10 µg	S
15.	Ampicillin	Aminopenicillin	Cell wall	25 µg	R
16.	Methicillin	Penicillinase-resistant-penicillins	Cell wall	5 µg	R
17.	Ceftriaxone	3rd generation Cephalosporins	Cell wall	10 µg	R
18.	Ceftazidime	3rd generation Cephalosporins	Cell wall	30 µg	R
19.	Cefixime	3rd generation Cephalosporins	Cell wall	30 µg	R

R=Resistant; S= Sensitive

pointed on 66% resistance to ceftazidime.[22] On the other hand, lower resistance against ceftazidime was found as 17.5% in Iraq.[23] Another study of MM Loureiro *et al.*, revealed high antimicrobial resistance percentage of this bacteria to ß-lactams, chloramphenicol, trimethoprim-sulfamethoxazole and tetracycline.[24] Almost 91% sensitivity of *P. aeruginosa* against ciprofloxacin was reported by Dinesh Shubedi in 2017 whereas we found both resistant and susceptible phenotype among the isolates.[25] A study done by Souli *et al.* shows the data from 23 countries on the European Antimicrobial Resistance Surveillance System (EARSS) on resistant rates of aminoglycosides, carbapenems, quinolones and ceftazidime antibiotics which were 0–51.9%; 9–50.5%; 7.2–51.9% and 4–48.5%, respectively. In this study, around 18% of the isolates were documented as multi drug resistant (MDR).[26]

There are different kinds of wastewater systems, like municipal sewage systems, hospital wastewater systems which have higher risks of spreading MDR.[27] In a study of Ghana, researchers found the presence of multidrug-resistant bacteria in hospital wastewater making up *Escherichia coli* (30.6%), *Klebsiella pneumoniae* (11.2%) and *Pseudomonas mendocina* 5.4%.[28] In Saudi Arabia, Wang *et al.* conducted a study on a specific COVID-19 hospital wastewater and they assured antimicrobial resistance (AMR) due to the usage of antimicrobial agents during the pandemic.[29] In China, various antibiotics were determined in frightening concentrations in hospital wastewater, containing high incidence of antibiotic resistance genes such as blaGES-1, qnrA, blaOXA-1, blaOXA-10 and blaTEM-1.[30]

CONCLUSION

Wastewater creates great burning issues when the untreated or inadequately treated water mixes with our natural environment. As a result, number of diseases and health problems have been occurring every year. Wastewater is a potential source for irrigation purposes, but it can also be an issue of concern when a good number of *P. aeruginosa* are found in the water. If human or animals get exposed to *P. aeruginosa* from wastewater, severe infections can happen. Moreover, the chemical component discharged from industries can affect the oxygen demand, which ultimately affects the aquatic ecosystem also. Antibiotic resistant pathogen also creates emergence of antibiotic resistance which can spread through aquatic system to human body. So, it can be concluded that presence of multidrug resistant *P. aeruginosa* and their risk of transmission to human is an acute health issue. The most challenging part to prevent this bacterial disease outbreak is that, these bacteria are continuously changing their ways to fight against the antibiotics used to treat the infection. Our study provided information on environmental reservoirs with *P. aeruginosa*, recommending the importance of hygiene practice. Moreover, this work might be a good foundation for further research on multidrug resistance mechanism of *P. aeruginosa* and their pattern of transmission as well.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHORS' CONTRIBUTION

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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DATA AVAILABILITY

All datasets generated or analyzed during this study are included in the manuscript.

ETHICS STATEMENT

Not applicable.

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