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Prevalence of Multidrug Resistant *Pseudomonas* species in water sample of Patna Region

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Abstract: *The global problem of antimicrobial resistance is pressing in developing countries like India. The overuse and misuse of antimicrobials has led to appearance of organisms that can evade them, the so called antibiotic resistant bacteria. Since water can be a reservoir of these resistant bacteria, there is a strong need to identify the sources of antibiotic resistant bacteria in aquatic environment. Multidrug-resistant Pseudomonas which is a significant threat to antibiotic resistance can be deadly for patients in critical care. An estimated 51,000 healthcare-associated P. aeruginosa infections occur in the United States each year. More than 6,000 (13%) of these are multidrug-resistant, with roughly 400 deaths per year attributed to these infections. Thus, the aim of the study is to see the prevalence of Multidrug Resistant Pseudomonas species in water sample of Patna Region. It was found that the prevalence rate of Pseudomonas species in Ganga water was maximum (47%) as compared to the other water samples studied. Out of 15 isolates three Pseudomonas isolates were screened on Pseudomonas Agar Base and named as P1, P2 and P3. All the three isolates were obtained from Ganga water. By studying the antibiotic susceptibility profile of the isolates P1, P2 and P3, it was observed that the strains P1 and P3 showed multidrug resistance. Isolate P1 was resistant to 13 out of 15 antibiotics studied thus, showing resistance for 86.66% of antibiotics tested. Out of the panel of 15 antibiotics tested, two commonly used antibiotics Streptomycin and Tetracycline (for which many other gram-negative bacilli are resistant) showed susceptibility towards strain P1. Therefore, these two antibiotics were further used to study the Minimum Inhibitory Concentration. The minimum inhibitory concentration of Streptomycin and Tetracycline were recorded as 7.5mg/ml and 25mg/ml, respectively, which points to their progression towards resistance. Thus, it is recommended that in order to protect public health, a series of control strategies should be developed and implemented at all stages of water disinfection system to eliminate contamination with this important human pathogen.*

Keywords: *Pseudomonas sp, antibiotic resistance, Ganga water.*

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Introduction:

Antibiotics are an important group of therapeutic agents used for the treatment of bacterial infections. The discovery and use of antibiotics led to a revolution in medical biology and an increase in agricultural productivity. However, this wide and intensive use of antibiotics has led to their continuous release in the environment. A sufficient amount of the antibiotics also keeps accumulating in the environment in the un-metabolized form. This leads to large residues of antibiotics in the recipient waters. (Nain V.K., 2015). Paradoxically, this use of antibiotics led to appearance of organisms that can evade them, the so called antibiotic resistant bacteria (ARB) which commonly emerge in areas where antibiotics are commonly used. Antibiotic resistance is a natural phenomenon and represents an evolutionary response to the strong selective pressure resulting from exposure to these compounds. Overtime bacterial resistance to all classes of antibiotics has emerged. Many studies have reported the presence of bacteria which are multi drug resistant strains or MDR. The emergence of antibiotic resistant bacteria especially MDR, thus, poses serious health problems to humans and has become a global problem. (Khurana G.S., 2015). As a result, when water bodies are contaminated with fecal matter containing antibiotic resistant bacteria, they could serve as a source and/or reservoir of antibiotic resistant genetic elements that could possibly be transferred to other bacterial species and make the quality of water very hazardous for health (Biyela and Bezuidenhout, 2004) and also create a favourable condition for the spread of antibiotic resistant genetic element from place to place (APUA, 1999). Therefore, together with water quality assessments, studies that give valuable information about antibiotic resistant microbes from environmental sources like water, food, soil and other niches should be encouraged to avoid the above mentioned complications (APUA,1999). Some bacterial spp. ***Pseudomonas*** is an opportunistic human pathogen. It is opportunistic because it seldom infects healthy individuals. Instead, it often colonizes immune compromised patients, like those with cystic fibrosis, cancer, or AIDS (Botzenhardt and Doring, 1993). It is such a potent pathogen that firstly, it attacks up to two third of the critically-ill hospitalized patients, and this usually portends more invasive diseases. Secondly, ***P. aeruginosa*** is a leading Gram-negative opportunistic pathogen at most medical centres, carrying a 40-60% mortality rate. Thirdly, it complicates 90% of cystic fibrosis deaths; and lastly, it is always listed as one of the top three most frequent Gram negative pathogens and is linked to the worst visual diseases (Fick, 1993). It also exhibits intrinsic resistance to a lot of different types of chemotherapeutic agents and antibiotics, making it a very hard pathogen to eliminate (Lederberg, 2000). This global problem of antibiotic resistance is particularly prevailing in developing countries like India where the bacterial load is high due to poor hygiene. The easy availability of antibiotics sometimes without a medical prescription has caused their widespread misuse.

Hence, today there is a strong and serious need to identify the sources of antibiotic resistant bacteria in aquatic environment which is the emphasis of the present study. Since many infections are also water borne, local antibiotic resistance surveillance was also done. Isolation of antibiotic resistant bacteria and the extent of their antibiotic resistance have been evaluated in this study in water samples from different water bodies of Patna Region.

Materials and Methods:

Collection of samples: The water samples used in this study were collected from the river Ganga at Gai Ghat, swimming pool of St. Michael's School, Digha, and the pond at Patna Women's College, Patna. The samples were brought under aseptic condition and processed within 24 hours of procurement.

Isolation and screening of bacterial strains: The water samples were serially diluted in normal saline and plated on nutrient agar under aseptic conditions. The plates were incubated at 37° C for 24 hours; the strains showing different cultural characteristics were selected and streaked on Nutrient agar slants. Further, each of these selected strains was streaked on ***Pseudomonas*** Agar Base [selective media] for the screening of ***Pseudomonas*** species. The isolates thus obtained were maintained on nutrient agar slants for further studies. **Cultural and morphological characterization of selected isolates:** Selected isolates were identified on the basis of their colony morphology, colour, texture and elevation. The morphological character of isolates was studied by performing the Gram's staining and observing the slides at 100X magnification of light microscope.

Biochemical characterization: For biochemical characterization the selected isolates catalase test, motility test, Indole production test, citrate utilization test, urease test, gelatin liquefaction, fermentation of carbohydrates, MR-VP test and nitrate reduction test were performed.

Antibiotic Susceptibility Test: To identify the resistant ***Pseudomonas*** species the antibiotic disc diffusion method (Bauer et al, 1966) was followed for a panel of antibiotics. Small inoculum of each isolate was inoculated in nutrient broth and incubated at 37°C for 24 hours. After incubation pre-prepared sterile cotton swabs were dipped into the nutrient broth containing the cultures and used to evenly inoculate pre-prepared Muller-Hinton agar plates. Thereafter, antibiotic discs of Tetracycline, Streptomycin, Erythromycin, Gentamycin, Ampicillin, Amoxicillin, Sparfloxacin, Ciprofloxacin, Tobramycin, Azithromycin, Chloramphenicol, Cefuroxime, Methicillin and Ceftrtamycin were placed on the plates. After 24 hours of incubation the diameter of the zone of inhibition (in mm) were observed. Clinical interpretation [resistant (R) and susceptible (S)] was drawn based on the zone observed and the CLSI standards for the particular antibiotic.

Identification of the Multidrug Resistant *Pseudomonas* strain: The Antibiotic susceptibility pattern of the ***Pseudomonas*** isolates were studied, the isolates showing resistance to three or more than three antibiotics were considered as multidrug resistant ***Pseudomonas***.

Minimum Inhibitory Concentration: The strain showing maximum multidrug resistance was then selected and MIC (broth dilution method) was performed for selected antibiotics (Tankeshwar Acharya, 2013). Two sets of 10 sterile test tubes were taken for testing the minimum inhibitory concentration for each antibiotic. Tubes were labeled from 1 to 10. Using sterile pipette, 2 ml nutrient broth was added to the tubes labeled 2 to 10 (Fig. 2). The first tube of both sets does not contain the broth. 2ml of the antibiotic to be tested was added to the tube 1 and 2 of both the sets. After that, using sterile pipette 2ml solution from tube 2 was transferred to tube 3. It was mixed well and then 2ml solution from tube 3 was transferred to tube 4. This process was continued through tube 9 and at last 2ml from tube 9 was taken and was discarded. Thus tube 10 receives no antibiotic and serves as a positive control. Again using a sterile pipette 2ml of multi drug resistant ***Pseudomonas*** was inoculated in each tube. Then both sets of tubes were incubated for 12-18 hours at 37°C.

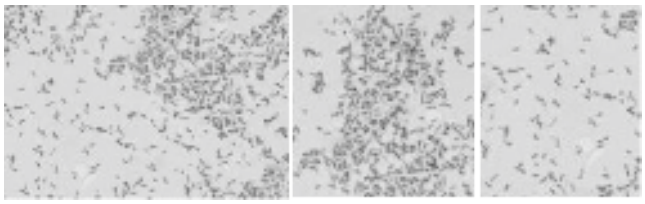
Results:

Isolation and screening: Out of 32 colonies isolated on nutrient agar 15 colonies were selected on the basis of difference in cultural characteristics and were streaked on ***Pseudomonas*** agar base. Further out of 15 isolates streaked on ***Pseudomonas*** agar base, three strains were screened as ***Pseudomonas*** species and were named as P1, P2 and P3. It was found that the prevalence rate of ***Pseudomonas*** species in Ganga water was maximum (47%) as compared to the other water samples studied.

Cultural and morphological characterization of isolates: The cultural characteristics like colour, texture, opacity and margin of isolates P1, P2 and P3 has been shown in table 1. The isolates were further subjected to grams staining. The microscopic view of isolates P1, P2 and P3 at 100X magnification of a light microscope have been shown in fig 1.

Table 1. Cultural and morphological characterization of Pseudomonas isolates P1, P2 and P3

Strains	Colony Characteristic				Gram's reaction	Shape
	Colour	Texture	Opacity	Margin		
P1	*White*	*Slimy*	*Opaque*	*Irregular*	*Negative*	*Bacillus*
P2	*White*	*Slimy*	*Opaque*	*Irregular*	*Negative*	*Bacillus*
P3	*White*	*Slimy*	*Opaque*	*Irregular*	*Negative*	*Bacillus*



a) Isolate P1 **b) Isolate P2** **c) Isolate P3**

Fig.1. Microscopic view of isolates P1, P2 and P3 at 100X magnification of a light microscope

Biochemical characterization: The isolated strains were further subjected to a series of biochemical tests. The results of the test have been tabulated in table 2.

Table 2. Observations of the biochemical tests for selected Pseudomonas isolates P1, P2 and P3

Biochemical tests	***P1***	***P2***	***P3***
Catalase test	Positive	Positive	Positive
Urease test	Negative	Positive	Negative
Gelatin liquefication test	Positive	Positive	Positive
MR-VP test	Negative	Negative	Negative
Nitrate reduction Test	Positive	Positive	Positive
Motility test	Negative	Negative	Negative
Lactose fermentation	Negative	Negative	Negative
Sucrose fermentation	Negative	Negative	Negative
Dextrose fermentation	Negative	Negative	Negative
Casein hydrolysis test	Negative	Negative	Negative

Thus on the basis of cultural, morphological, and biochemical characterizations the isolates P1, P2 & P3 have been confirmed as ***Pseudomonas*** sp***.*** However, for the identification at species level molecular characterization by 16srRNA sequencing should be performed.

Antibiotic Susceptibility Test: Observation of the antibiotic susceptibility test performed to detect the susceptibility pattern of ***Pseudomonas*** species along with the CLSI (Clinical and Laboratory Standard Institute) standards has been tabulated below (table 3)

Table.3. Diameter of the zone of inhibition (in mm) along with the standards given by CLSI

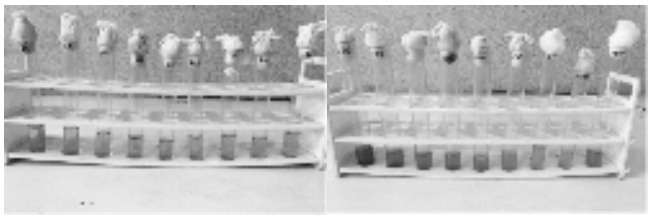
Sl. No.	Antibiotics	Diameter of zone of inhibition						
		Disc Conc. (mg)	Resis-tant (in mm)	Inter-mediate (in mm)	Sensi-tive (in mm)	P1 (in mm)	P2 (in mm)	P3 (in mm)
1.	Amoxicillin	20	£ 13	14-17	³ 18	7 (R)	15(S)	7 (R)
2.	Ampicillin	10	£ 13	14-16	³ 17	8 (R)	14(S)	9 (R)
3.	Azithromycin	15	£ 13	14-17	³ 18	10 (R)	14(S)	10(R)
4.	Ceftriaxone	30	£ 15	16-20	³ 21	13 (R)	15(S)	15(S)
5.	Cefuroxime	30	£ 14	15-17	³ 18	9 (R)	20(S)	20(S)
6.	Ciprofloxacin	5	£ 15	16-20	³ 21	10 (R)	19(S)	16(S)
7.	Chloramphenicol	30	£ 12	13-17	³ 18	9 (R)	20(S)	10(S)
8.	Erythromycin	15	£ 13	14-22	³ 23	0 (R)	13(R)	14(S)
9.	Methicillin	5	£ 17	18-20	³ 21	0 (R)	25(S)	0(R)
10.	Sparfloxacin	5	£ 15	16-18	³ 19	12 (R)	13(R)	20(S)
11.	Streptomycin	10	£ 11	12-14	³ 11	19 (S)	13(S)	23(S)
12.	Tetracycline	30	£ 11	12-14	³ 15	19 (S)	15(S)	18(S)
13.	Tobramycin	10	£ 12	13-14	³ 15	8 (R)	16(S)	15(S)
14.	Gentamycin	10	£ 12	13-14	³ 15	10(R)	18(S)	12(S)
15.	Nalidixic acid	10	£ 12	13-14	³ 15	5 (R)	10(S)	10(R)

*R = resistant, S = susceptible

From the above table it was observed that P1 was resistant towards Amoxicillin, Ampicillin, Azithromycin, Ceftriaxone, Cefuroxime, Ciprofloxacin, Chloramphenicol, Erythromycin, Methicillin, Sparfloxacin, Tobramycin, Gentamycin, Nalidixic acid. It was susceptible to Streptomycin and Tetracycline. Strain P2 was resistant to Erythromycin and Sparfloxacin. Strain P3 was resistant to Amoxicillin, Ampicillin, Azithromycin, Chloramphenicol, Methicillin andNalidixic acid. Since isolates P1 and P3 showed resistance towards more than three antibiotics they were considered as multidrug resistant bacteria. Isolate P1 was resistant to 13 out of 15 antibiotics studied thus, showing resistance to 86.7% of antibiotics tested.

Identification of Multidrug resistant isolates: From the study of the antibiotic susceptibility pattern of the isolates P1, P2 and P3, it was observed that the strains P1 and P3 showed multidrug resistance in which P1 was resistant to 13 out of 15 antibiotics studied. Thus, the rate of resistance was higher in strain P1 as it showed resistance to 86.66% of antibiotics studied.

Minimum Inhibitory Concentration: Out of the panel of 15 antibiotics tested, two commonly used antibiotics Streptomycin and Tetracycline (to which many other gram-negative bacilli are resistant) showed susceptibility towards strain P1 which was a highly resistant strain in our study. Therefore, these two antibiotics were further used to study the Minimum Inhibitory Concentration. The minimum inhibitory concentration evident by the absence of visible bacterial growth (Tankeshwar, 2013) for Streptomycin and Tetracycline were recorded as 7.5mg/ml and 25mg/ml respectively (fig 2), which points to their progression towards resistance.



(a) (b)

Fig: 2 Observation of broth dilution method for measuring MIC of (a) Streptomycin (b) Tetracycline against P1.

Discussion: The present study entitled Prevalence of Multidrug Resistant ***Pseudomonas*** species in water sample of Patna Region was conducted to determine the incidence and the antimicrobial resistance pattern of ***Pseudomonas*** sp. The resistance of ***Pseudomonas*** spp to the antibiotic in the quinolone group is variable in different centers. In a prospective study, resistance to ciprofloxacin was reported as 8-31% in ICU patients (Tassios et al., 1998) another study reveals that the resistance rate for Pseudomonas spp against ciprofloxacin was found as 54.17% while it was 32% in Indian (Sivaraj et al., 2012), 23% in Spain (Bouza et al.,1999), 31.9% in Italy (Bonfiglio et al., 1998). Our study states that the prevalence rate of ***Pseudomonas*** spp in Ganga water was maximum (47%) as compared to the other water samples. P1 shows resistance against 86.66% of drugs tested which indicates that it is highly resistant. The resistance of the isolate P1 is on the rise which may be

due to the activities of industrial effluent, hospital discharge and sewage disposal in the environment. As a result, immunization may fail to recover by constant exposure of resistance microbes. Thus, it is recommended that in order to protect public health, a series of control strategies should be developed and implemented at all stages of water disinfection system to eliminate contamination with this important human pathogen. More epidemiological studies are needed to determine the possible Ganga water as a source and/or reservoir of ***Pseudomonas*** spp. Even though of medical improvement, the antimicrobial resistance still becomes an age-old problem. So, proper implementation of antibiotic policies and guidelines must be there in every hospital. Also the development of effective vaccine against ***Pseudomonas*** spp is necessary in the modern world.

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