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Isolation and Characterization of Multiple Antibiotic-resistant Bacteria from Diverse Polluted Water Bodies of Darbhanga, Bihar.

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Abstract.

Presence of antibiotic resistant bacteria in surface water like ponds and such alike water bodies has been seen as a major public health concern. Viewing this as one of the major concerns this study proceeds with aiming to isolate and identified different types of bacteria and to determine different antibiotic resistivity over them from two famous ponds of Darbhanga. 12 isolated bacteria from both ponds were morphologically, biochemically identified and their antibiotic resistant profiles were determined by commonly used disc diffusion method and MAR indices were calculated. MAR (Multiple Antibiotic Resistance) values of all isolates were calculated between 0.2-0.8 indicated severe antibiotic pollution in the ponds water. Maximum MAR index was calculated for MK02 and identified as *Pseudomonas aeruginosa*, which may be because of alternation in its gene. These types of variation may cause severe environmental problems if not controlled by controlling flow of sewage with antibiotics.This study would attend to coverhow, the contamination of pond waters by antibiotics or other pollutants supports the development of antibiotic resistance in bacteria.

Keywords: Pond Water, Bacterial Identification,Multiple Antibiotic Resistance (MAR), *Pseudomonasaeruginosa.*

Introduction

Discovery of penicillin by Alexander Fleming in twentieth century was one of thegreatest achievements in the field of medical sciences.Till then Antibiotics have become the best substance for recovering from diverse types of diseases in human life and theother living organisms as well. These antibiotics can be of natural or anthropogenic origin. These have been seen useful in animal farming, fish farming, poultry farming, mushroom culture, agricultural purposesand various other field of concern. So its production in pharmaceutical industries can be seen at a large scale.Though it is necessary to produce its amount as per the market demand but it cannot be ensured that the total consumption witheco-friendly approach is possible.There is an annual estimationof its production is 1,00,000tons in world around (Bbosa and Mwebaza 2013).Seeing this with a lens of hope is true but excess use, improper handling, release of improper or untreated medical and industrial waste, make the antibiotic ubiquitous pollutant for environment. No propertreatment of wastewater from agriculture, animal husbandry, pharmaceutical industries, slaughter houses, and hospitals enter into surface water and spread into the whole environment. Presence of antibiotic resistant bacteria in surface water like ponds and rivers has become a major public health concern (Ayandiran *et al*., 2014) including aquatic bodies (Baquero F., *et al. 2008.*) and it has been increasing for last few

decades (Mohanta and Goel, 2014).When any single bacterium becomes resistant to more than one antibiotic it can be said multi-drug resistant. Several authors from all over the world have been reported multi-drug resistant bacteria from sewage water (Mulamattathil., *et al*. 2014) , household water (Mukhopadhyay., *et al*. 2012) and other environment. Excess use and misuse of antibiotic, in human andveterinary medicine, agriculture and aquaculture, causes multi drug resistance (MDR) in bacteria (Mc Manus and Stockwell 2001).In present study water of two ponds of Darbhanga has, therefore, been undertaken to isolate and determined the presence of antibiotic resistant bacteria and their MAR indices as well.

Materials and Methods

For the bacteriological analysis water sample from two different ponds of Darbhanga town namely Gangasagar pond (26°08'18.0"N, 85°54'19.2"E) and Mirza Khan pond (26°07'42.5"N, 85°53'54.2"E) were collected in autoclaved glass bottles inJuly 2015 and transported to laboratory in the ice box. For decreasing the number of bacterial colonies, serial dilutions of the samples were performed and different level of diluted samples were cultured and enumerated total viable count of those plates which were containing 30- 300 bacterial colonies (Alef and Nanninpiri, 1996). Bacterial colonies grown on different types of cultivation media were isolated by streaking method. Identification of bacteria were performed by their morphology, biochemistry, gram staining, TSI (Triple Sugar Iron) and sugar fermentation test (Holt et al. 1994; Forbes *et al*., 2007)

Once all the bacterial isolates were identified their antimicrobial susceptibility testing were performed against five most frequently used antibiotics (Hi-Media, India) like Ampicillin (10 µg/disc), Tetracycline (30 µg/disc), Gentamicin (10 µg/disc), Tobramycin (10 µg/disc), Vancomycin (30 µg/disc) by disc diffusion method on Mueller-Hinton agar (Bauer *et al*., 1966). For that, Separate bacterial inoculums were prepared by suspending the bacteria in 2 ml of sterile salin and the turbidity was adjusted to 0.5 McFarland (prepared by mixing 0.5 ml of 1.175% barium chloride and 99.5 ml of 0.36N sulfuric acid) standards. Inoculation of the Mueller-Hinton Agarplate followed by placement of the antibiotic disks were performed carefully and incubated at 35°C. After incubation (18-24 h) zone of inhibition were measured in millimeter with the help of scale and compared with National Committee for Clinical Laboratory Standards (NCCLS) guidelines (2000) to categorize into three categories like susceptible (S), intermediate (I) and resistant (R).

The MAR (Multiple Antibiotic Resistance) indices for all the isolates were calculated by the following formula described by Krumperman (1983)

$$MAR\ Index = \frac{Number\ of\ antibiotics\ to\ which\ isolate\ is\ resistant}{Total\ number\ of\ antibiotics\ against\ which\ isolate\ was\ tested}$$

Result and discussion:

Initially bacterial colonies were distinguished on the basis of their colony color and morphology, and then for identification of 12 distinguished isolates, gram staining, biochemical, TSI (Triple Sugar Iron) and sugar fermentation tests were performed. Isolates like GS02, GS03, GS04, MK01, MK02, and MK03 grown on MacConky agar were detected gram negative rod shapes and rest of the isolates like GS01, GS05 GS06, MK04, and MK05 which did not grow on MacConky agar were detected Gram positive rod shape except MK05 (cocci).

During TSI test GS01, GS05, GS06 and MK04 showed K/A (alkaline red slant/acidic yellow butt) and none of them produced gases. GS02, GS04 MK03 MK05 and MK06 showed A/A (acidic yellow slant/ acidic yellow butt) among them in all tube release of gases were observed except in MK05. Rest of the isolates GS03, MK01 and MK02 showed K/K (alkaline red slant/ alkaline red butt) without gas production. None of the isolates H_2S

production was detected and in TSI test which were also confirmed by separate H_2S production test. All the results of morphological, biochemical, gram staining and TSI test are mentioned in table 1 and sugar fermentation patterns are mentioned separately in table 2. Finally on the basis of above observation all the isolates were identified as mentioned in table 3.

Tests	Bacterial Isolates Code											
	GS01	GS02	GS03	GS04	GS05	GS06	MK01	MK02	MK03	MK04	MK05	MK06
Gram	+	-	-	-	+	+	-	-	-	+	+	-
Shape	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Cocci	Rod
Growth on MA	-	+	+	+	-	-	+	+	+	-	-	+
TSI	K/A	A/A	K/K	A/A	K/A	K/A	K/K	K/K	A/A	K/A	A/A	A/A
Indole	-	-	-	+	-	-	-	-	-	-	-	+
Methyl red	-	-	-	+	-	-	-	-	-	-	+	+
Vogues Proskauer	+	+	-	-	+	+	-	-	+	+	+	-
Citrate	+	+	+	-	+	+	+	+	+	+	+	-
Nitrate red.	+	+	+	+	+	+	+	+	+	+	-	+
Catalase	+	+	+	+	+	+	+	+	+	+	+	+
Oxidase	+	-	+	-	-	+	+	+	-	+	-	-
H2S production	-	-	-	-	-	-	-	-	-	-	-	-

Key: MA = MacConkey agar, TSI = Triple Sugar Iron, + = Indicates positive results, – = Indicates negative results, A/A = Acidic yellow slant/ Acidic yellow butt K/A = Alkaline red slant/Acidic yellow butt K/K = Alkaline red slant/ Alkaline red butt

Table 1: Biochemical test results of bacterial isolates

Sugar fermentation test

Sugars	Bacterial Isolates											
	GS01	GS02	GS03	GS04	GS05	GS06	MK01	MK02	MK03	MK04	MK05	MK06
Lactose	-	+	-	+	-	-	-	-	+	-	+	+
Maltose	+	+	-	-	-	-	-	-	+	+	+	-
Mannitol	-	+	-	+	-	-	-	-	+	-	+	+
Sorbitol	-	+	-	+	-	-	-	-	+	-	ND	+
Sucrose	v	+	-	v	+	+	-	-	+	v	+	v

Key: + = Indicates positive results, – = Indicates negative results, V = Variable, ND = Not done,

Table 2: Sugar fermentation test results of Bacterial isolates

Bacterial isolates	**Identified bacteria**
GS01	*Bacillus cereus*
GS02	*Klebsiellapneumoniae*
GS03	*Pseudomonas aeruginosa*
GS04	*E. coli*
GS05	*Bacillus subtilis*
GS06	*Bacillus cereus*
MK01	*Pseudomonas aeruginosa*
MK02	*Pseudomonas aeruginosa*
MK03	*Klebsiellapneumoniae*
MK04	*Bacillus cereus*
MK05	*Staphylococcus aureus*
MK06	*E. coli*

Table 3: Identified bacterial isolates.

All the 12 isolates were exposed to 5 commonly used antibiotics for their resistant tests. Their resistivity were observed by measuring zone of inhibitions with the help of scale and documented in mm (millimeters). Among all bacterial isolates lowest resistant against single antibiotic ampicillin was recorded in GS01 with MAR (Multiple Antibiotic Resistance) Index 0.2, While the highest resistant against 4 antibiotics namely ampicillin, tetracycline, tobramycin and vancomycin were recorded in MK02 with 0.8 MAR index. Isolates like GS03, GS06, MK01 and MK04 showed resistivity against 3 antibiotics (MAR Index 0.6) and GS02, GS04, GS05, MK03, MK05 and MK06 showed resistance to two antibiotics with 0.4 MAR indices. All isolates were resistant to ampicillin and none of the isolates were recorded resistant against gentamicin. MAR index values of bacteria greater than 0.2 indicates high risk source of contamination in the environment where antibiotics are often used (Osundiya et al,. 2013). Intermediate susceptibility was also recorded for some isolates like GS05 to tetracycline, GS02 and MK03 to gentamicin, GS03 and MK01 to tobramycin and GS01 to both tetracycline and tobramycin. 50% of the total isolates were determined 0.4 MAR indicis. MAR indices ranged 0.2 – 0.8 were recorded for all isolates (Table 4.) which revealed the prevalence of multiple antibiotic resistant bacteria in both Gangasagar and Mirza Khan Ponds of Darbhanga. Detection of such high MAR indices value indicates the incorporation of antibiotics from the vicinity of the ponds because, for any given ecological niches, MAR indices are the useful tools for determining the level of antibiotic pollution. (Krumperman PH 1983). Bacterial isolates MK02 with highest MAR index 0.8 was isolated from Mirza Khan Pond and identified as *Pseudomonas aeruginosa*. The reason for the high MAR index of *Pseudomonas aeruginosa* may be to wash the hospital's clothes in the Mirza Khan pond along with release of sewage. So the high resistivity detected among bacteria against different antibiotics may be attributed to genetic modification.

Bacterial isolates		Name of Antibiotics					MAR Index
		Ampicillin	Tetracycline	Gentamicin	Tobramycin	Vancomycin	
GS01	*Bacillus cereus*	R	I	S	I	S	0.2
GS02	*Klebsiellapneumoniae*	R	S	I	S	R	0.4
GS03	*Pseudomonas aeruginosa*	R	R	S	I	R	0.6
GS04	*E. coli*	R	S	S	S	R	0.4
GS05	*Bacillus subtilis*	R	I	S	R	S	0.4
GS06	*Bacillus cereus*	R	R	S	S	R	0.6
MK01	*Pseudomonas aeruginosa*	R	R	S	I	R	0.6
MK02	*Pseudomonas aeruginosa*	R	R	S	R	R	**0.8**
MK03	*Klebsiellapneumoniae*	R	S	I	S	R	0.4
MK04	*Bacillus cereus*	R	R	S	S	R	0.6
MK05	*Staphylococcus aureus*	R	R	S	S	S	0.4
MK06	*E. coli*	R	S	S	S	R	0.4

Key: S =Susceptible, I = Intermediate, R= Resistant, MAR = Multiple Antibiotic Resistance

Table 4: Antibiotic resistance profiles with MAR (Multiple Antibiotic Resistance) indices of isolated bacteria.

Conclusion

This study reveals that, the contamination of pond waters by antibiotics or other pollutants supports the development of antibiotic resistance in bacteria. All isolated bacteria showed the varied antibiotic resistance but the isolated one i.e. identified as *Pseudomonas aeruginosa* from Mirza Khan Pond showed the highest MAR index, which should not be overlooked. Once antibiotic resistance develop in bacteria, it does not decrease quickly and if such types of bacteria cause any disease, could be a threatening because treating these are not quite easy always.

This study recommends that not to wash hospitals contaminated clothes into ponds and such other water bodies.Seeing these it is clear that there is a dire need for a propertreatment of wastewater to keep human and our environment as a healthier one.

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