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Co-Selection of Multiple Heavy Metals Resistance and Multiple Antibiotics Resistance in Coliform Bacteria Present in Hasdeo River in Korba and Champa Region

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Abstracts

Prevalence of multiple antibiotics resistance in microorganisms was well known but also represents a serious threat to public health. Pollution of Hasdeo river due to heavy metals and Coliform bacteria increases with industrialization, urbanization and continuous discharge of domestic waste from livestock facilities. Pollution of river water was one of the greatest concerns for the water consumers with respect to the quality of river water of Hasdeo in Korba and Champa region. The present study deals with isolation identification and enumeration of Coliforms bacteria isolated from Hasdeo river in Korba and Champa region, and examine pattern of their multiple heavy metals resistance capacity and multiple antibiotic resistance together.

Keywords: Coliform Bacteria; Microorganisms; Antibiotics.

Introduction

Ecosystem of any region deal with the quantitative interaction of species population and their communities in complex system and resources with various biotic and abiotic factors affecting directly or indirectly. Water plays crucial role in the entire ecosystem as it was most important necessity of life which has been exploited most as compared to any other resource for the sustenance of his life; hence the quality of Water contaminated by various pathogenic microorganisms, Coliforms and various industrial effluents was considered to be one of the greatest concerns. Heavy metals contamination of river water pose severe threat to the biodiversity by entering into the food chain thus effecting health of human as they persists for long time in food chain and causes irreversible damage to health. With the increasing industrialization and urbanization and various anthropogenic activities such as industrial discharge, human activities, agricultural waste disposal and sewer drained in to river water causing heavy metals pollution and microbial pollution of water which are mostly enteric origin (Bonetta et al., 2011; Bayoumi Hamuda and Patko, 2012) Hasdeo river which was tributary to Mahanadi and most important river of Chhattisgarh was found to be most polluted at Korba region, due to industrial drainage of various power plants and small scale industries located in Korba, (Vaishnav and Hait, 2013; Rajshree and Shweta, 2015). Microbial contaminationof water due to pathogenic Coliforms was one of the most important problems for the water quality management and important factor for water pollution for Hasdeo river water. Like wise microbial pollution, pollution of the Hasdeo River water due to toxic heavy metals was also the most persistent problem and was increases continuously with industrial progress although levels of pollution and its impact on environment differ from place to place.

The abilities of bacteria species to adapted and become metal tolerant to toxic concentrations of heavy

metals through various resistance mechanisms (Avezzu et al., 1995; Hemme et al., 2010; Davis et al., 2003; Lee et al., 2006) can be used as a tool in bioremediation for the treatment of effluents contaminated. Studies suggest that *Staphylococcus* was found to resistant against Cd, Cu, Pb, Zn, As and Fe. *Acinetobacter radioresistens* was resistant towards As and Ni (Raja et al., 2009; Bisht et al.,2012), ability of *Bacillus pumilus* and *Staphylococcus sp.* to reduce Cr was reported by Farah et al., 2010. Which was also supported by Nanda and Abraham, 2011).

The discharge of domestics waste such as pharmaceutical waste and antibiotics were also discharged in river water causing Development of multiple antibiotic resistances in bacterial species present in water resources was serious threat for safety of environment and human health as it carries a negative impact for public health (Fick et al., 2009), exposure on microorganisms to the multiple drug residues and their constant exposure may lead to mutations and development of new strains for better survival (Calomiris et al., 1994) which pose a great threat to human health management. Identification of antibiotic resistance bacteria also reveals about the source of pollution (Middleton and Salierno, 2013; Shah et al., 2012) and had added a new dimension to the risk posed by the presence of antibiotics and drug residues.

Materials and Methods

Description of Sampling Sites

Water samples were collected from different locations of Korba and Champa in four replicates aseptically in sterile 500mL Duran Schott glass bottles, labeled properly and transported on ice bucket to the laboratory for analysis. Aliquots of the samples were used for heavy metals and selective isolation of different bacterial species based on standard microbiological procedures.

Analysis of Heavy Metal Concentration

Contents of the heavy metals in water samples were analyzed by AAS (Thermo Scientific, UK) following the method of American Public Health Association (APHA). Concentration of all heavy metals in water expressed as mg/L and all the samples were tested in four replicates.

Isolation of Heavy Metal Resistant Bacteria from Hasdeo River Water

Bacterial species resistant to Cd, Cu, Fe, As and Pb were isolated from water samples by diluting them in sterile phosphate buffer saline solution and 200µl from each dilutionswere plated on Nutrient Agar supplemented in the form of metal salts as $CdCl_2$, $CuCl_2$, $PbCl_2$, $Fe_3SO_4$and $AsCl_2$ at the concentration of 50ppm. The plates were incubated at 37° C for 24-48 hours. Bacterial colonies were isolated and were sub-cultured to obtain pure culture on Nutrient agar. The isolates were partially identified according to Bergey's Manual of Systematic Bacteriology (Bergey et al., 1994).

Estimation of Total Coliforms

Most Probable Number (MPN) test was performed to assess the domestic pollution level in the selected area for total Coliform count. The technique involves three successive steps namely, presumptive test, confirmatory test and completed test. This method has direct application in quantification studies for media and for alternate microbiological methods. The number of broth tubes producing gas used to determine the statistical range of coliform. Confirmed and positive test are used to calculate the MPN.

Cultural Characteristic of Bacteria

Bacterial isolates were identified based on their morphology, staining reaction and molecular marker. Microorganisms show diverse culture characters and the diversity depending upon the type of medium used for culture. Cultural characteristic such as size, colour, texture margin, elevation, consistency etc. were observed for the identification of the bacterial isolates. Thesecolony characters, morphology and staining reaction helps in preliminary identification.

Biochemical Characterization of the Isolates

The biochemical tests performed include Gelatin hydrolysis test, Starch hydrolysis test, Casein hydrolysis test, Catalase activity test, Glucose Fermentation test, Citrate utilization test, Nitrate reduction test, Oxidase Test, VP -Voges-Proskauer Test ("Vi"), Urease Test. These biochemical tests were performed as per standard Microbiological methods (Cappuccino and Sherman, 2007).

Antibiotic Sensitivity

Antibiotic resistance of the metal tolerant species was determined by Kirby-Bauer disc diffusion method (Barry and Thornsberry, 1981) with using 6 mm diameter discs antibiotics discs: tigecyline, terramycin, lactoclave, Amoxycillin, Penicillin,

Ecoflox, Ofloxacin and tetracyclin (Himedia, India). Inhibition zones formed around antibiotic discs were evaluated according to the Clinical and Laboratory Standards Institute (CLSI) criteria (CLSI, 2011). Whereas antibiotics sensitivity of Ticarcillin. Piperacillin, Clavulanic acid, Ceftazidine, Cefoperazone/sulbactam, cefepime, aztreonam, doripenem, imipenam, meropenam, amikacin, gentamicin, ciprofloxacin, levofloxacin, minocyclin, tigercyclin, colistin, trimethoprim/sulpamethoxazole, kanamycin, erythromycin, streptomycin, oxytetracyclin and neomycin were tested in VITEK-2C (Biomerieux, France) as per manufacturers instruction. The bacterial isolates were scored assusceptible, intermediate or resistant to a given antibiotic bythe inhibition zone diameter around the antibiotic disc.

Multiple antibiotics resistance (MAR) index were calculated as A/B, where 'A' represents the number of antibiotics to which the isolate was resistant and 'B' represents the total number of antibiotics tested (Kruperman, 1983).

Results and Discussions

In the present study water samples were collected from Hasdeo river water at different locations of Korba and Champa, and bacterial species resistance to heavy metals were isolated, identification and characterization based on staining techniques, morphological examination, colony characteristics, biochemical tests reveals that the bacterial species were *E.coli, Klebsiella pneumonia, Salmonella typhi, Pseudomonasa aeruginosa, Shigellaflexneri, Yersinia entrocolitica, Staphylococcus aureus, Bacillus pumilus, Bacillus altitudinis, Bacillus subtilis , Pseudomonas stutzeri.* 200 colonies were screened from initial level of heavy metal supplemented LB medium. The bacterial isolates showed optimum growth at 30°C and pH 7.0. The morphological, colony characteristics and biochemical characteristics of sewage bacteria were shown in table 1, 2, 3.

Table 1: Table represents the morphological Characteristics of bacteria species isolates from river water samples of Hasdeo at different locations of Korba and Champa

S. No.	Names of bacteria	Gram staining	Shapes	Arrangement	Sizes in (μm)
1	*E.coli*	-ve	Rods	Single	3μm
2	*Enterobacterfaecalis*	-ve	Rods	Single	4 μm
3	*Staphylococcus aureus*	+ve	Cocci	Grapes like cluster	1 μm
4	*Pseudomonas sp.*	-ve	Cocci	Single/pair/short chain	1 μm
5	*Yersinia sp.*	-ve	Rod	Single/chain/cluster	-.
6	*Shigella sp.*	-ve	Rod	Single/pair/short chain	-
7	*Klebsiella pneumonia*	-ve	Rod	Single/pair/short chain	2 μm
8	*Salmonella typhi*	-ve	Rod	Single	2 μm
9	*Bacillus pumilus*	+ve	Rod	Single/cluster	1.0-5.0 μm
10	*Bacillus altitudinis*	+ve	Rod	Pair/Cluster	2.0-3.0 μm
11	*Bacillus subtilis*	+ve	Rod	Cluster	2 μm

Table 2: Data represent the cultural characteristics of bacteria species isolates from hasdeo river water samples at different locations ofKorba and Champa

S. No.	Names of Isolated organisms	Form	Color	Margin	elevation	Odour
1.	*E. Coli*	Circular	White/metallic sheen	Entire	Raised	Fecal odour
2.	*Enterobacterfaecalis*	Irregular, large	Lack of sheen	Entire	convex	Fecal odour
3.	*Staphylococcus aureus*	Circular	Golden yellow	Entire	convex	Unpleasant
4.	*Pseudomonas aeruginosa*	Circular	Flour yellow	Undulate	Raised	Fruity
5.	*Salmonella typhi*	Circular	Colourless	Entire	Raised	Unpleasant
6	*Yersinia sp.*	Circular	White	Entire	Raised	Fruity pineapple
7	*Klebsiella pneumonia*	Irregular	Blue colour	Entire	convex	Yeasty odour
8	*Shigellasp*	Irregular	Yellow colour	Lobate	convex	Fruity odour
9	*Bacillus pumilus*	Irregular	White	Regular	convex	Unpleasant
10	*Bacillus Subtillus*	Circular	Golden brown	Entire/Undulate	Convex	Foots odour
11	*Bacillus altitudinis*	Circular	creamy	Entire	Convex	Ammonia like

Table 3: Data showing the biochemical characterization of bacterial isolates isolated from the different location of river water Hasdeo in Korba and Champa

	Gelatin hydrolysis	Starch hydrolysis	Casein hydrolysis	Catalase activity	Glucose Fermentation tubes	VP -Voges-Proskauer Test	Citrate utilization test	Nitrate reduction test	Oxidase Test:
E. Coli	**-ve**	**-ve**	**-ve**	**-ve**	**-ve**	MR+VP-	**-ve**	**+ve**	**-ve**
Klebsiella pneumonia	**-ve**	**+ve**	**-ve**	**+ve**	**+ve**	MR-VP+	**+ve**	**-ve**	**-ve**
Pseudomonas sp.	**+ve**	**-ve**	**-ve**	**+ve**	**-ve**	MR-VP-	**+ve**	**-ve**	
Staphylococcus aureus	**+ve**	**-ve**	**+ve**	**+ve**	**+ve**	MR-VP-	**D**	**+ve**	**-ve**
Baciluspumilus	**-ve**	**+ve**	**-ve**	**+ve**	**+ve**	MR-VP	**-ve**	**+ve**	**+ve**
Bacillus altitudinis	**-ve**	**+ve**	**-ve**	**+ve**	**+ve**	MR-VP-	**-ve**	**-ve**	**+ve**
Enterococcus faecalis	**-ve**	**+ve**	**-ve**	**-ve**	**+ve**	MR-VP-	**+ve**	**-ve**	
Bacillus subtilis	**-ve**	**+ve**	**+ve**	**+ve**	**+ve**	MR-VP-	**-ve**	**-ve**	**+ve**

Isolation and Identification of Metal resistant bacteria and evaluation metal tolerance

Table represents sample analysis from the river water of Hasdeo, Data reflects the presence of *E.coli, Pseudomonasa sp., Bacillus sp.*, and *Enterococcus faecalis* in the river water of Hasdeo and these are showing resistant to the heavy metal pollution.

Table 4: Table shows the average density (Percent) of microorganisms having heavy metal remediation capacity isolated from Hasdeo river water from different location of Korba and Champa

S. No	Name of organisms	Average density (%) of organism
1.	*Bacillus subtillus*	14%
2.	*Pseudomonas aeruginosa*	13%
3.	*Pseudomonas stutzeri*	12%
4.	*Enterococcus faecalis*	20%
5.	*Bacillus pumilus*	11%
6.	*E. Coli*	20%
7.	*Bacillus altitudinis*	10%

Maximum metal remediation capacity was observed in *Pseudomonas sp.* towards multiple heavy metals (Cd, Cu, Pb, Zn, As and Fe) with varying degree of removal capacity in optimal growth conditions. The results were also supported by various other researchers. Lin and Harichund, (2011) reported heavy metal resistance of *Pseudomonas areuginosa* towards Cd, Co, Cu, Pb and As. Metal tolerance capacity of *Pseudomonas fluorescens* towards Pb, Ni, Cu, Cr and Cd was also reported by Wasi et al., (2011) and Selvi et al., (2012). *Bacillus sp*. was also showing great degree of metal resistance towards various metals.

Antibiotic Resistance

An antibiotic is a kind of ubiquitous contaminant in the aquatic environment with industrial effluents and sewage discharge. All the isolates were showed significant resistant against all the tested antibiotics. E. coli showed highest degree of resistance against all the antibiotics than other isolated bacteria.

It was also reported that the metal resistance capacities of the microbes are mainly associated with antibiotic resistance. Antibiotics resistance was tested against *Pseudomonas sp.,* and *Bacillus sp.,* All the bacterial strains showed different degrees of sensitivity against Ticarcillin. Clavulanic acid, Ceftazidine, Cefoperazone/sulbactam, cefepime, aztreonam, doripenem, imipenam, meropenam, amikacin, gentamicin, ciprofloxacin, levofloxacin, minocyclin, tigercyclin, colostin, trimethoprim/ sulpamethoxazole, kanamycin, erythromycin, streptomycin, oxytetracyclin, lactoclave, amoxycillin, penicillin, ofloxacin and neomycin but all bacterial strains showed resistance against Ecoflox, terramycin, tetracycline, and bacitracin. *Pseudomonas sp., Bacillus sp.* were shown to sensitive against almost all the antibiotics used but maximum sensitivity was reported with doripenem, imipenam, meropenam (MIC<=0.25). All the species show were showing least sensitivity to trimethoprim/Sulfamethoxazole (MIC=20). It was previously reported that the metal resistance has been reported to hold an association with antibiotic resistance (Verma et al., 2001). It was assume that under metal stress, metal and antibiotic resistance in microorganisms possibly helps them to adopt faster by the spread of resistant factors rather than by mutation and/or natural selection (Silver and Misra, 1988).

Table 5: MAR Index of Sample Collection site (Korba, Champa) + sign indicates sensitive against antibiotics, whereas – sign represents resistance towards antibiotics

	Antibiotics	Korba	Champa
1	ticardin	+	+
2	piperacillin	+	+
3	ceflazidime	+	+
4	cefepime	+	+
5	Aztreonam	+	+
6	doripenem	+	+
7	doripenem	-	+
8	Imipenem	-	+
9	Amikacin	+	+
10	Gentamicin	+	+
11	Ciproflaxacin	+	+
12	minocycline	+	+
13	Colistin	+	+
14	trimethoprim	+	+
15	Sulphamethoxazole	+	+
16	tigecyline	+	+
17	terramycn	-	-
18	lactoclave	+	+
19	Amoxycillin	+	+
20	Penicillin	+	+
21	Ecoflox	-	-
22	Ofloxacin	-	-
23	Tetracyclin	+	+
25			
MAR Index		0.208	0.130

MAR index of Water samples collected from Champa region were recorded 0.16 whereas it was recorded 0.208; MAR index more than 0.2 indicated that the isolates were from high-risk contamination sources such as human wastes, commercial animal farms where antibiotics used often, MAR index less than 0.2 indicated that the strain was from animals in which antibiotics were seldom or never used (Matyar et al., 2008; Kruperman, 1983; Vivekanandhan et al., 2002). Based on MAR index and heavy metal analysis, River water at Korba was found to be most polluted among all the collection sites.

Conclusion

This study revealed the prevalence of multiple antibiotics resistant and multiple heavy metal resistance in bacterial species present in Hasdeoriver in Korba and Champa region. Presence of multiple antibiotic resistances in bacteria; species showing multiple heavy metal resistance was most likely due to the spread and evolution of antibiotic resistance may be triggered by anthropogenic pollutant such as heavy metals (Baker-Austin et al., 2006) and co-selection of both antibiotics and heavy metal resistance in bacterial species.

It was very clear that maximum discharge of domestic waste and settled effluent were deposited at river in Korba region, which was considered to be the reason for high level of pollution of river water at that region. The feacal coliform bacteria are numerous in this riverine water might be due to the various anthropogenic activity particularly sewage. These microorganisms may be repeated from the total coliform group by their capability to grow and proliferate at elevated temperature. This study was thus pertinent to the present situation of environmental pollution and climate change. Due to these plenty of reasons, importance has been put on the antibiotic resistance status of such bacterial isolates and causing various bacterial species resistance to multiple antibiotics resistance.

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