Antibiotic Resistance Prevalence and Pattern in Environmental Bacterial Isolates

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Abstract: The present study investigates the prevalence of antibiotic resistance among bacterial isolates from different environmental samples and determines their resistance patterns. Bacteria were isolated from the Ganges water, the intestine of *Labeo rohita*, soil samples from agricultural land, and clinical samples of urine, pus, and throat swab. The bacterial isolates were identified on the basis of standard cultural, morphological and biochemical characteristics. Antibiotic susceptibility of the isolates was tested by disc diffusion and agar dilution method. A total of 87 bacteria belonging to 13 different genera were isolated. The percentages of resistance detected were, Ax: amoxycillin (82.75%), Te: tetracycline (49.42%), Tr: trimethoprim (41.37%), Ch: chloramphenicol (39.08%), Nx: nalidixic acid (22.98%), Ci: ciprofloxacin (24.13%), S: streptomycin (9.19%), G: gentamycin (4.59%) and Ak: amikacin (4.59%). A majority of 57 (65.51%) strains were multi-resistant; 77 (88.5%) were resistant to at least one drug. Determination of resistance pattern revealed that 3 water isolates and 1 clinical isolate belonging to *Pseudomonas aeruginosa* (n=3) and *Proteus vulgaris* (n=1) were resistant to all the 9 antibiotics tested; a *Proteus mirabilis* strain was resistant to all the drugs except G. In the seven-drug-resistant group, *Klebsiella aerogenes* showed AxChTeNxTSCi–resistance and *P. mirabilis* strain exhibited AxChTeNxTrGCi resistance pattern. The high prevalence of antibiotic-resistant bacteria harboring diverse resistance traits could represent a potential health risk. The study of antibiotic resistance helps predict future emergence and guide the development of strategies to counteract this resistance. Therefore periodic and comprehensive survey of antibiotic resistance in the environmental bacteria is required.

Keywords: Antibiotic resistance, environmental bacteria, prevalence.

INTRODUCTION

Antimicrobial resistance in bacteria associated with different ecological niches has been a global concern. The emergence of antimicrobial resistant strains of pathogenic bacteria has become a great threat to the public health [1]. The detection of emerging trends in antimicrobial resistance of bacterial strains facilitates implementation of effective control measures. The antibiotic susceptibility testing contributes directly to patient care, and have great influence on antibiotic usage and hence on the pressures that facilitate the emergence of antimicrobial drug resistance. However, in our region, the study of antibiotic resistance of bacteria from environment like soil, water or from fish is scanty. Therefore, study pertaining to antibiotic resistance of environmental isolates is imperative to explore the antibiotic pressure in the environment.

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MATERIALS AND METHODOLOGY

Samples

Bacteria were isolated from different environmental samples such as water from Gangetic riverine regions of Hooghly belt, from the intestine of *Labeo rohita*, soil samples from agricultural land at Purulia, and clinical samples (urine, pus, throat swab) from urinary tract infection cases, and cases with fever and cold, and ulcerative skin, attending the Calcutta School of Tropical Medicine, Kolkata India, for treatment.

Isolation and Identification of Bacteria

The different environmental samples were processed for the isolation of bacteria by methods described elsewhere [2]. Morphologically distinct colonies obtained from different plates were streaked on Nutrient agar (NA), MacConkey agar (MCA), XLD agar, TCBS agar, SS agar, blood agar and DCA agar (Hi-Media, Mumbai, India) to purify. The bacterial isolates were identified on the basis of standard cultural, morphological and biochemical characteristics [3].

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Antibiotics

The antibiotics (content per disc) used in the study are Ax: Amoxycillin (25 µg); Ak: Amikacin (10 µg); Ch: Chloramphenicol (30 µg); Cf: Cefotaxime (30 µg); Ci: Ciprofloxacin; Cz: Cefazolin (30 µg); Er: Erythromycin (15 µg); G: Gentamicin (10 µg); Nx: Nalidixic acid (30 µg); S: Streptomycin (10 µg); Te: Tetracycline (10 µg); Tr: Trimethoprim (5 µg); Pb: Polymixin B (300 unit). The antibiotic discs were purchased from Hi-Media, Mumbai, India.

Antibiotic Susceptibility

Antibiotic susceptibility of the isolates was tested according to the NCCLS by disc diffusion method with an inoculum of 10^8 cfu, and agar dilution method with 10^4 cfu/spot [4, 5]. The interpretive categories were defined according to the zone diameter of inhibition and equivalent MIC breakpoints [6]. *Escherichia coli* NCTC 10418 was used as the control strain. Five bacterial strains, *Bacillus licheniformis* F102, *Pseudomonas aeruginosa* W171, *Aeromonas hydrophila* O102, *Proteus mirabilis* C114 and *Bacillus pumilus* KS23, which were capable of utilizing dimethoate as a sole source of carbon [2], were selected for MIC determination.

RESULTS

Isolation and Identification of Bacteria

A total of 87 bacteria belonging to 13 different genera were isolated from different environmental sources, and identified (Figs **1** and **2**). The strains identified (Fig. **3**) belonged to *Enterobacteriaceae* group (n=69) amongst which *Escherichia coli* (n=18), *Proteus vulgaris* (n=8), *P. mirabilis* (n=3), *Klebsiella aerogenes* (n=9), *Enterobacter aerogenes* (n=5), *Serratia marcescens* (n=2), *Providencia alcalifaciens* (n=9), *Morganella morganii* (n=2), *Citrobacter freundii* (n=8), *Salmonella typhi* (n=3), *S. typhimurium* (n=2) were found. Others included *B. licheniformis* (n=1), *Bacillus pumilus* (n=1), *Bacillus subtilis* (n=6), *Ps. aeruginosa* (n=5), *Pseudomonas pyomelanin* (n=1), *A. hydrophila* (n=2), *Plesiomonas shigelloides* (n=2).

Antibiotic Susceptibility

Antibiotic susceptibility test results for the isolated bacteria (n = 87) are represented in Fig. (**3**). The highest percentages of resistance were detected for Ax (82.75%), Te (49.42%), Tr (41.37%), Ch (39.08%), Nx (22.98%), Ci (24.13%), S (9.19%). Only 4.59% of the strains presented resistance to G and Ak. A total of ten bacteria were sensitive to all the drugs tested, which belonged to *Escherichia coli* (n=2), *Enterobacter aerogenes* (n=2) and *Providencia alcalifaciens* (n=6).

Among *E. coli* most of the isolates were resistant to Ax (88%), Ch (44%), Te (44%), Nx (45%) and Tr (27%). All the isolates of *K. aerogenes* were resistant to Ax, Tr, and 88.8% were resistant to Te and Ci. *Bacillus* spp. exhibited 87.5% and 62.5% resistance to Ax and Tr, respectively. The 80% isolates of *Salmonella* spp. were Te resistant, and all were Ax resistant. *Pseudomonas* spp. showed 100% resistance to Ax, Te, Tr, and Nx but resistance to Ch and Ci was found in 83.3% and 50% isolates, respectively.

Tables **1** and **2** shows different antibiotic resistance patterns found amongst 87 isolated bacteria. Determination of resistance patterns to 9 antibiotics revealed that 77 (88.5%) were resistant to at least one drug, and the majority 57 (65.51%) of these strains was multi-resistant. Three strains namely, W171, WA01, and C364, all belonging to *Ps. aeruginosa* were resistant to combination of all nine drugs used. The *P. mirabilis* C144 strain was resistant to all the drugs except G. In the seven-drug-resistant group, *K. aerogenes* C184 showed AxChTeNxTSCi–resistance and *P. mirabilis* C124 strain exhibited AxChTeNxTrGCi resistance pattern. *Ps. pyomelanin* W011 isolated from water showed AxChTeNxTrCi pattern of resistance. The 14.94% of the isolates belonged to five-drug-resistant group. Seven various patterns of drug resistance were found in five-drug-resistant

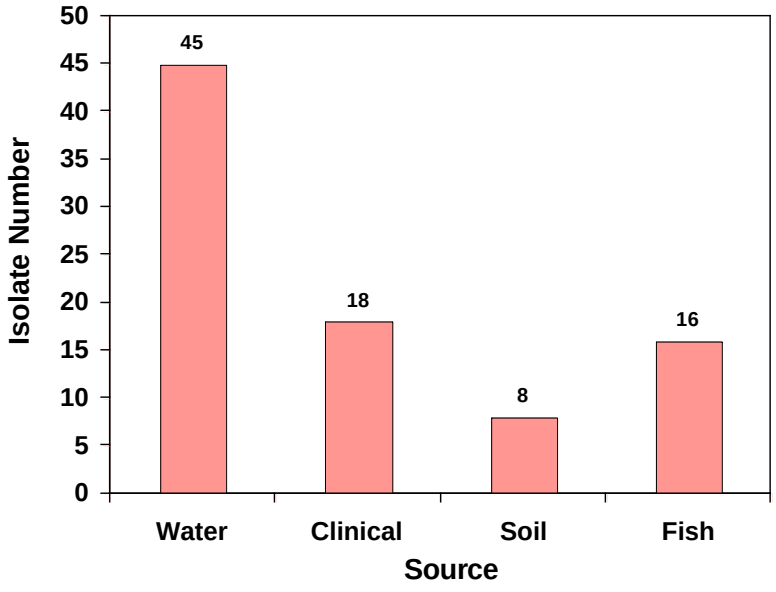

Fig. (1). Bacterial isolates from different sources.

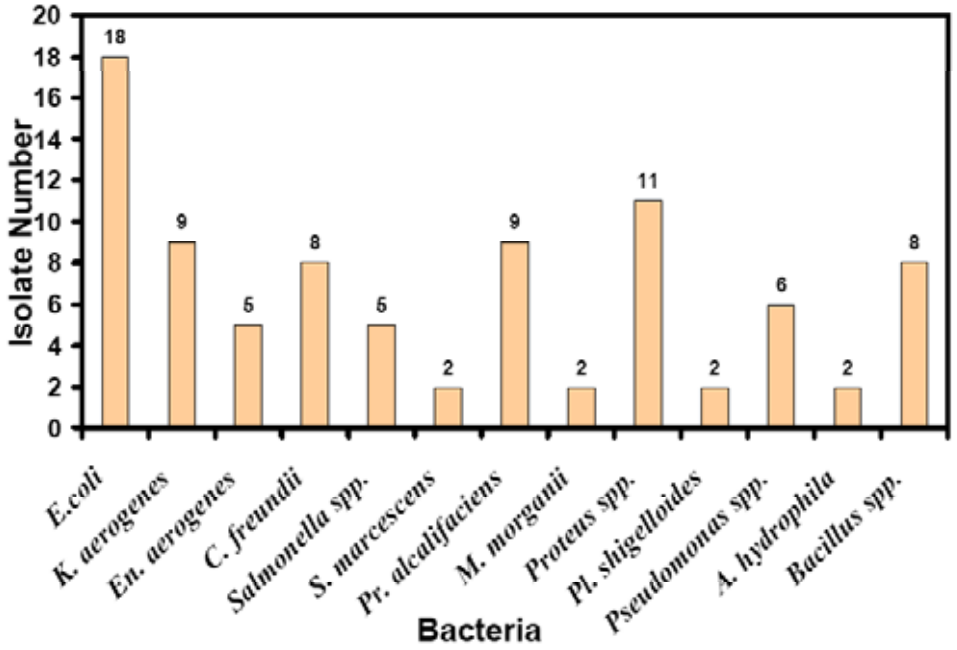

Fig. (2). Number of various isolated bacteria (n = 87).

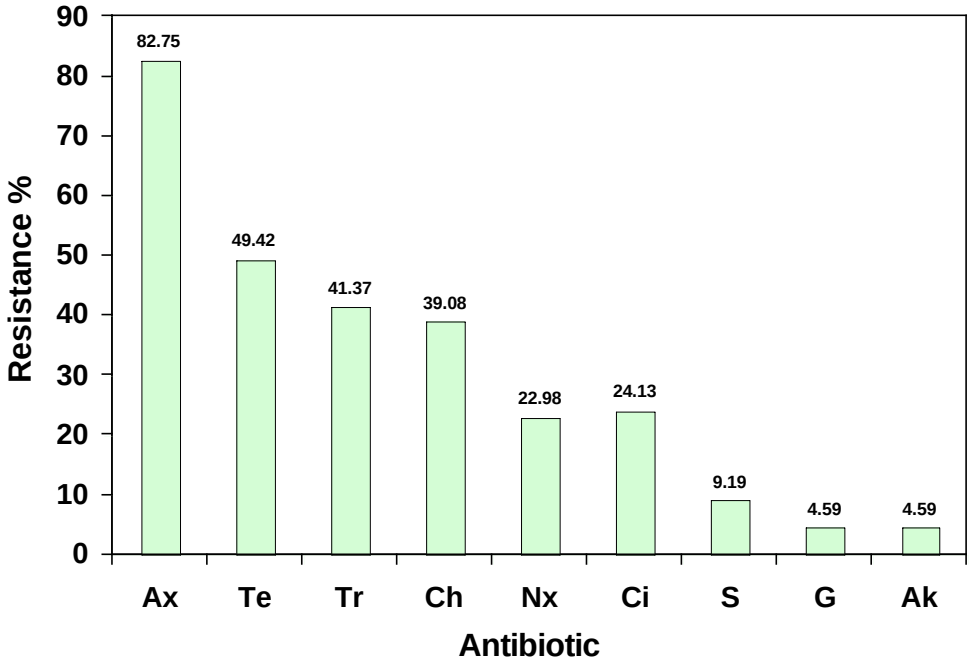

Fig. (3). Antibiotic susceptibility test results for the isolated bacteria (n = 87). Ax: Amoxycillin; Ak: Amikacin; Ch: Chloramphenicol; Cf: Cefotaxime; Ci: Ciprofloxacin; G: Gentamicin; Nx: Nalidixic acid; S: Streptomycin; Te: Tetracycline; Tr: Trimethoprim.

group. Similarly, 12 isolates showed three-drug resistance with four different patterns amongst which AxChTe (50%) was the predominant one. The 14.94% isolates belonged to the two-drug-resistant group with six different patterns. One drug resistance was found with Ax or Tr.

MIC of Antibiotic

The MICs of antimicrobial agents for five bacterial isolates *B. licheniformis* F102, *Ps. aeruginosa* W171, *A. hydrophila* O102, *P. mirabilis* C114 and *B. pumilus* KS23, are represented in Figs. (**4**-**6**). Among the isolates, MICs of Ax, Ch and Nx ranged in between 10 µg/ml and 60 µg/ml, 10 µg/ml and 100 µg/ml, 15 µg/ml and 400 µg/ml, respectively (Fig. **4**). MICs ranged from 1 µg/ml to 20 µg/ml for Te, Tr, and G (Fig. **5**), from 1 µg/ml to 10 µg/ml for Ak and S, and from 0.5 µg/ml to 3 µg/ml for Ci (Fig. **6**). The *Ps. aeruginosa* W171 strain showed highest MICs to Ax (60 µg/ml), Ch (100 µg/ml), Te (20 µg/ml), Tr (20 µg/ml), G (20 µg/ml), Ak (10 µg/ml), and S (10 µg/ml). The *A. hydrophila* O102 strain exhibited highest level of MIC to Ci (3 µg/ml),

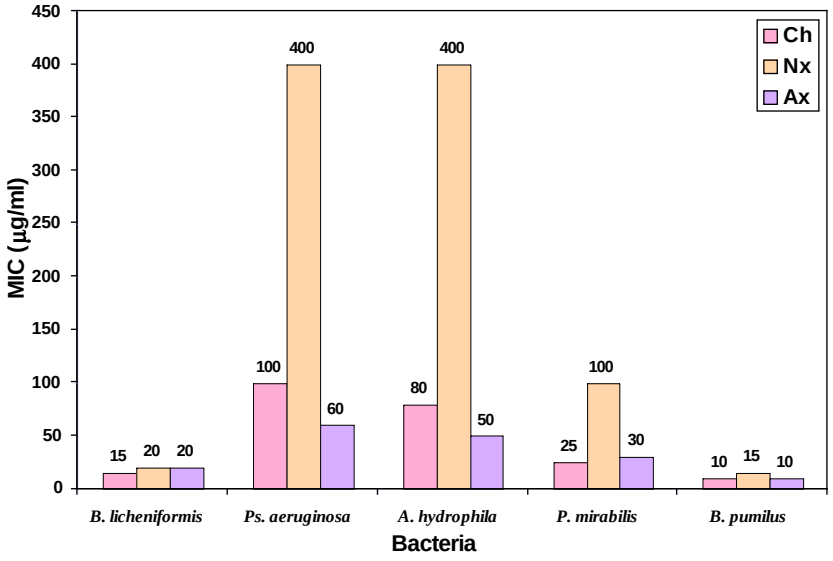

Fig. (4). Minimum inhibitory concentration (MIC) values for the five isolated bacteria to Ax (Amoxycillin), Ch (Chloramphenicol) and Nx (Nalidixic acid).

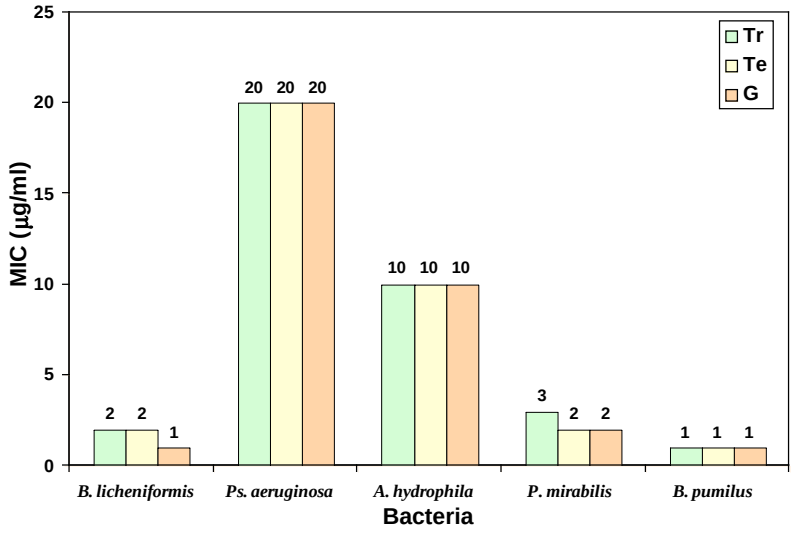

Fig. (5). Minimum inhibitory concentration (MIC) values for the five isolated bacteria to Tr (Trimethoprim), Te (Tetracycline) and G (Gentamicin).

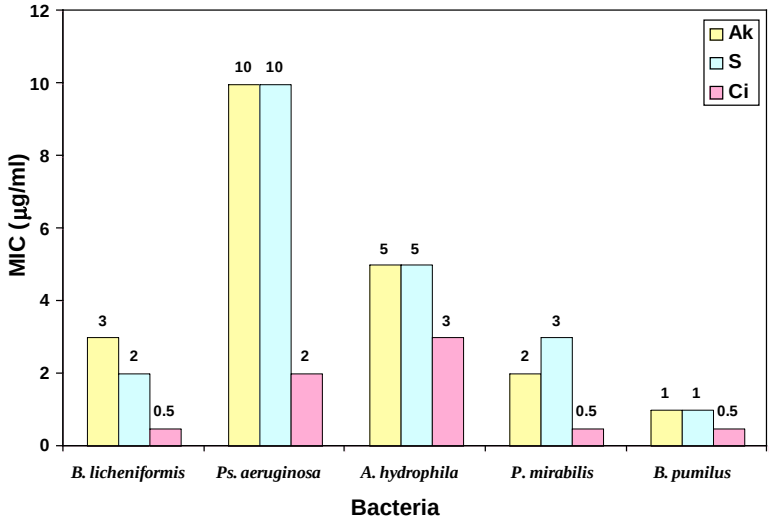

Fig. (6). Minimum inhibitory concentration (MIC) values for the five isolated bacteria to Ak (Amikacin), S (Streptomycin) and Ci (Ciprofloxacin).

Table 1. Antibiotic Resistance of Bacterial Isolates Showing 9- to 4- Drug Resistance Patterns

S. N.	Resistance Pattern	Bacteria	Strain Code	Source	Isolate Number
1	***Nine-drug***				
	AxChTeNxTrGCiAkS	*Ps. aeruginosa*	W171, WA01	Water	2
		P. vulgaris	W218	Water	1
		Ps. aeruginosa	C364	Clinical	1
2	***Eight-drug***				
	AxChTeNxTrCiAkS	*P. vulgaris*	C144	Clinical	1
3	***Seven-drug***				
	AxChTeNxTrCiG	*P. mirabilis*	C124	Clinical	1
	AxChTeNxTrCiS	*K. aerogenes*	C184	Clinical	1
	PbAxChTeCfErCz	*B. licheniformis*	F102	Fish	1
4	**Six-drug**				
	AxChTeNxTrCi	**Ps. pyomelanin**	W011	Water	1
5	***Five-drug***				
	AxTeNxTrCi	*Ps. aeruginosa*	S033	Soil	1
	AxTeNxTrCi	*K. aerogenes*	W031, W101	Water	2
	AxChTeNxTr	*Ps. aeruginosa*	W17b	Water	1
	AxChTeNxTr	*M. morganii*	S013	Soil	1
	AxCiTeSTr	*M. morganii*	C044	Clinical	1
	AxTeTrGAk	*Pr. alcalifaciens*	F182	Fish	1
	AkChTeTrG	**Pr. alcalifaciens**	F132	Fish	1
	AxChTeTrCi	*K. aerogenes*	W071, W081, W111, W211	Water	4
	AxChTeNxCi	*E. coli*	C084	Clinical	1
6	***Four-drug***				
	AxTeTrCi	*P. vulgaris*	C014	Clinical	1
	AxTeTrCi	*K. aerogenes*	C054	Clinical	1
	AxChTeNx	*P. vulgaris*	WA41, WA51	Water	2
	AxChTeNx	*E. coli*	W221	Water	1
	AxChTeNx	*P. mirabilis*	WA81	Water	1
	AxChTeNx	*A. hydrophila*	O102	Fish	1
	AxTeTrS	*S. typhimurium*	CO51, CO52	Clinical	2
	AxChTeTr	*E. coli*	W021	Water	1

Ax: Amoxycillin; Ak: Amikacin; Ch: Chloramphenicol; Cf: Cefotaxime; Ci: Ciprofloxacin; Cz: Cefazolin; Er: Erythromycin; G: Gentamicin; Nx: Nalidixic acid; S: Streptomycin; Te: Tetracycline; Tr: Trimethoprim; Pb: Polymixin B.

Table 2. Antibiotic Resistance of Bacterial Isolates Showing 3- to 1- Drug Resistance Patterns

S. N.	Resistance Pattern	Bacteria	Strain Code	Source	Isolate Number
7	***Three-drug***				
	AxTeCi	*B. subtilis*	S023	Soil	1
	AxNxTr	*B. subtilis*	C104	Clinical	1
	AxTeCi	*Se. marcescens*	W226, W263	Water	2
	AxChTe	*E. coli*	W200,WA91,WA22, W311	Water	4
	AxChTe	*S. typhi*	WA35, WA36	Clinical	2
	AxTeTr	*C. freundii*	C044	Clinical	1
	AxTeTr	*K. aerogenes*	F092	Fish	1
8	Two-drug				
	AxTr	*B. pumilus*	KS23	Soil	1
	AxTr	*B. subtilis*	S019	Soil	2
	AxTr	*C. freundii*	F142	Fish	1
	ChTe	*P. vulgaris*	WA16	Water	1
	ChTe	*P. vulgaris*	F062	Fish	1
	AxG	*P. vulgaris*	F122	Fish	1
	AxNx	*C. freundii*	F082	Fish	1
	AxCh	*En. aerogenes*	WA37, WA38	Water	2
	AxCh	*E. coli*	WA34	Water	1
	AxCh	*A. hydrophila*	W401	Water	1
	AxTe	*E. coli*	WA13	Water	1
9	One-drug				
	Ax	*B. subtilis*	S113	Soil	1
	Ax	*B. subtilis*	C024	Clinical	1
	Ax	*P. mirabilis*	C114	Clinical	1
	Ax	*Pr. alcalifaciens*	S313	Soil	1
	Ax	*S. typhi*	C304	Clinical	1
	Ax	*C. freundii*	WA21,WA31,WA14, WA18, WA26	Water	5
	Ax	*En. aerogenes*	WA11	Water	1
	Ax	*E. coli*	WA22,WA23,WA24, WA28,WA29,WA30, WA33	Water	7
	Tr	*Pl. shigelloides*	C081, C082	Clinical	2
10	All-sensitive	*Pr. alcalifaciens*	WA41,WA42,WA43,WA44,WA45, WA46	Water	6
		En. aerogenes	WA39	Water	1
		En. aerogenes	F072	Fish	1
		E. coli	WA10, WA12	Water	2

Ax: Amoxycillin; Ak: Amikacin; Ch: Chloramphenicol; Cf: Cefotaxime; Ci: Ciprofloxacin; Cz: Cefazolin; Er: Erythromycin; G: Gentamicin; Nx: Nalidixic acid; S: Streptomycin; Te: Tetracycline; Tr: Trimethoprim; Pb: Polymixin B.

while highest MIC value for Nx was 400 μg/ml as has been found in case of *Ps. aeruginosa* W171 and *A. hydrophila* O102 strains.

DISCUSSION

The widespread emergence of antibiotic resistance, particularly multidrug resistance, among bacterial pathogens has become one of the most serious challenges in clinical therapy [7, 8]. Environments containing antibiotic residues exert selection pressure and contribute to the appearance of resistant bacteria. In light of the potential health risk, many studies have focused on antibiotic-resistant bacteria from various ecosystems [9-11]. In the present study, the bacteria were isolated from different sources (water, fish intestine, clinical sample, soil) and their prevalence as well as their pattern of resistance to one or more antibiotics including Ax, Ch, Te, Nx, Nr, Tr, S, Ci and G was studied. The Ganges River has become the ultimate dumping ground of all materials including effluents from antibiotic treatment and manufacturing plants, thus posing significant threat to ecological balance as well as to public health. Hospital, municipal, agricultural sewage and aquacultural wastewater are also the sources of antibiotics and resistant bacteria in the aquatic environment [12]. In this communication, the isolated bacteria displayed resistance to Ax (82.75%), Te (49.42%), Tr (41.37%), Ch (39.08%), Nx (22.98%), Ci (24.13%), S (9.19%), G and Ak (4.59%) each. Similar findings with highest resistance to Ax were reported in coliform bacteria from waste water fed fish samples [13]. There was a stunningly high resistance in *Ps. aeruginosa* (n=2) and *P. vulgaris* (n=1) isolated from Gangetic riverine region showing resistance to all the test antibiotics. In addition, other water isolates like *Ps. pyomelanin* (n=1) had AxChTeNxTrCi resistance pattern, while 7 isolates showed AxTeNxTrCi (by *K. aerogenes*; n=2), AxChTeNxTr (by *Ps. aeruginosa*; n=1), and AxChTeTrCi (by *K. aerogenes*; n=4) resistance patterns. Herein, all the isolates showed a single plasmid co-migrated with 54 kb plasmid of *E. coli* V517 marker, and the plasmid was responsible for mediating multidrug resistance of the bacterial isolates [2]. Resistance prevalence of antibiotics against bacterial isolates such as *Ps. putida* and *Stenotrophomonas maltophilia* from wastewater effluent of the Pharmaceutical Group Corporation, Hebei Province, China, showed Tx: oxytetracycline (94.7 %), Te (95.2 %), doxycycline (83.1 %), Am: ampicillin (85.2 %), Cf (71.4 %), kanamycin (55.0 %), Ch (72.5 %), Ci (9.0 %), Er (92.1 %), rifampin (88.4 %) [14]. With an increase in the antibiotic load in aquatic environment, the resistance prevalence of a particular antibiotic increases with concomitant increase in cross-resistance in a bacterial community [14]. The high prevalence of indigenous antibiotic-resistant bacteria harboring diverse resistance traits could represent a potential health risk. Humans become infected with MDR environmental bacteria through consumption of contaminated water and vegetables. Antibiotic resistance genes might be transferred to the pathogenic bacteria infecting humans, particularly under the selection pressure of antibiotics as well as *via* the SOS response [15, 16].

Li *et al.*, demonstrated that the administration, even of a single antibiotic or long term exposure of microorganisms to high concentration of the antibiotic can select for MDR strains [14]. The current study reveals the isolation of 16 bacteria, from the intestine of *L. rohita*, of which 9 isolates presented resistance to 2 to 7 antibiotics. Amongst them, *B. licheniformis* F102 strain had high resistance to 'AxChTeCfErPbCz' pattern with MICs up to 15 μg/ml. The fish isolates *Pr. alcalifaciens* (n=2) showed AxTeTrGAk- and AkChTeTrG-resistances; one each strain of *A. hydrophila*, *K. aerogenes*, and *P. vulgaris* exhibited AxChTeNx-, AxTeTr-, and ChTe-resistances, respectively, while *C. freundii* (n=2) with AxTr- and AxNx-resistances, and *P. vulgaris* (n=2) with ChTe- and AxG-resistances. The extensive use of antibiotics and other chemotherapeutic agents in fish farms as feed additives or the direct administration thereof into fishpond water, to prevent and treat fish diseases, has resulted in an increase of drug resistant bacteria [17]. High level resistances have been recorded in aquaculture studies, where Te, Ch, and sulfonamides were either used as supplements in fish feed or poured directly into the water [18]. The fish pathogenic bacteria *A. hydrophila* and *Ps. fluorescens*, associated with epizootic ulcerative syndrome showed resistance to Ax, cloxacillin, Pn: penicillin G, and Am [19]. The antibiotics to which the fish pathogenic bacteria become resistant cannot be used in aquaculture as therapeutic agents for treating ulcers. High level of individual and multiple antimicrobial resistances to oxolinic acid, sulfadiazine-trimethoprim, Ax, Tx, and florfenicol were demonstrated by *Yersinia ruckeri*, *Flavobacterium psychrophilum*, and *A. salmonicida*, associated with enteric redmouth disease , rainbow trout fry syndrome, and furunculosis, respectively, thus indicating a substantial impact of fish farming on several groups of bacteria associated with aquacultural environments [20].

Indiscriminate use of antibiotics for the treatment of disease has caused accumulation and biomagnifications of these chemicals and emergence of resistant bacteria inside the human body. Some pathogens, such as MDR *K. pneumoniae* and *Acinetobacter baumannii*, are currently untreatable with antibiotics [21, 22]. The mechanisms by which bacteria become antibiotic resistant are either by modification of the antibiotic or the target site, or its removal from the cell [2]. Te, Pn, clindamycin resistant anaerobic strains *Bacteroides*, *Clostridium* and cocci were isolated from patients in the United States sufferring from infections involving abdominal, pelvic, and pleuropulmonary sites [23]. Pretesting is necessary to find out an effective antimicrobial agent to be used by clinicians. We found 16 clinical isolates of which 6 depicted a high level of resistance to six to nine drugs tested. The resistance prevalence of the clinical isolates was comparatively greater than the soil, fish and water bacteria probably because of direct exposure of the antibiotics during chemotherapy. The antibiotics are used in the treatment of many life-threatening diseases, and the use of new antimicrobial agent causes the pathogenic bacteria to become resistant to the relatively older antibiotics. Environmental bacteria have been shown to be reservoir and source of antibiotic resistance genes in clinical pathogens [14]. Acquisition of resistance genes through horizontal transfer facilitated by plasmids has been found to be ubiquitous in clinical pathogens [24, 25].

Various agricultural and anthropogenic activities have led to a vast number of chemicals including antibiotics

entering soil ecosystems causing a major global concern because of their toxicity and threat to human life and environment. In the current study, the soil bacteria (n=8) displayed single to five drug resistance patterns: *Ps. aeruginosa* (AxTeNxTrCi, n=1), *M. morganii* (AxChTeNxTr, n=1), *Pr. alcalifaciens* (Ax, n=1); *Bacillus spp.* with AxTeCi (n=1), AxTr (n=2), Ax (n=1). Dantas *et al.*, isolated large number of antibiotic-consuming soil bacteria with resistant to multiple antibiotics at clinically relevant concentrations, suggestive of such bacteria as the reservoir of antibiotic-resistance determinants which contribute to the increasing levels of multiple antibiotic resistance in pathogenic bacteria [26]. Most of the known antibiotics are produced by actinomycetes, commonly found in soils, compost, and other environmental sources. The soil-dwelling bacteria by evolving in an environment of antibiotic production develop diverse ways to survive or resist the toxic antimicrobial compounds produced by their neighbors.

This phenomenon of high resistance as shown in our study is an important evidence of the direct exposure of antibiotics to humans, and aquatic animals like fish; the widespread use of antibiotics and their accumulation in different ecological niches cause development of antibiotic resistance in bacteria of soil and water. The study of resistance in the environmental bacteria helps predict future emergence and guide the development of strategies to counteract this resistance.

CONFLICT OF INTEREST

None declared.

ACKNOWLEDGEMENTS

None declared.

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