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ASSESSMENT OF GROUND AND SURFACE WATER QUALITY WITH RESPECT TO ANTIBIOTICS AND ANTIBIOTIC RESISTANT BACTERIA IN RAWALPINDI, PAKISTAN

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Abstract

The study was designed to determine drinking water quality with reference to antibiotic and antibiotic resistant bacteria. Water samples were taken from Rawal dam (before and after treatment), distribution

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system and boreholes. Microbiological analysis revealed that both ground and borehole water samples were contaminated with heterotrophic bacteria, fecal streptococci, fecal coliform, total coliform and pathogenic gram-negative and gram-positive bacteria. During dry season the detection frequency of the antibiotics were high while during rainy season it was low. Most prominent antibiotics were CIP, CEF and AMO with detection frequencies at 16.7%, 7.9% and 2.4% during rainy season respectively. Out of eight only three antibiotics (AMO, CIP and CEF) were detected during rainy season. Antibiotics ranged from74 ng/L to 1 ng/L during dry season, 0 to 21 ng/L during rainy season and 0 to 43 ng/L during normal season. The pathogenic bacteria were highly resistant to KAM (91.3%) and lowest to AMC (16.67%). 191 of 193 were resistant to at least 3 antibiotics while 182 of 193 were resistant to five to eight antibiotics. Results of the study reveals that, health risk regarding the antibiotics and antibiotic resistant bacteria are high and will be even more hazardous in the future.

Keywords: Antibiotic contamination, Antibiotic resistance, Ground water, Pathogen, Potable water, Seasonal variation, Surface water

1. INTRODUCTION

Antibiotics have been in use to save human lives by controlling infections caused by bacteria from almost a century [1]. Use of antibiotics were increasing globally at a rate of 35% rate or 75% in developing countries like Brail, China, Russia, India and South African counties during 2000 to 2010. It had also been observed that about 75% of antibiotics use unnecessarily for the diseases not caused by bacteria [2]. Considerable portion of antibiotics did not breakdown into metabolites [3] and entering into aquatic environment in active form [4]. In the recent years, different concentrations of antibiotics from ng/L to µg/L have been reported in drinking water sources including surface water, ground water and rivers. Antibiotics also detected from tap and bottled water [5] [6]. Due to continuous release and presence in the environment antibiotics are known as pseudo-persistent pollutants [7]. Antibiotics and their metabolites are considered as continuous potential threat for both aquatic life and human health [8]. Because the antibiotics persist in the aquatic environment [9] concern as an essential role in producing antibiotic resistant bacteria (ARB) and antibiotic resistant genes [10]. Antibiotic resistance is a critical issue of the century [11] but it also estimated that without use of antibiotics death rate can increase many folds due to bacterial infections [12]. Municipal wastewater is a major source of antibiotics and ARB because of animal and human manure, and entering into water bodies [13]. Urgency of the problems can be assessed from the findings that both isolates of surface and ground water are resistant to different antibiotics and most of them are multi drug resistant [14] [15].

Due to rapid urbanization fresh water sources are heavily polluted with antibiotics and ARB [16]. Different gram-negative an gram-positive bacteria are grouped as heterotrophic (HPC) bacteria are inhibitor of human and animals and considered as non-pathogenic [17]. Presently occurrence of HPC bacteria used as an indicator of coliform contamination for water storage and distribution system and its concentration can be vary from 1 CFU/ml to 10000 CFU/ml. In water distribution systems permissible limit for HPC is 500 CFU/ml [18]. Increase in HPC bacteria is clear indication of poor treatment system [19] and gastrointestinal diseases increases as consequence [19]. Antibiotic resistance can transfer from pathogenic to nonpathogenic bacteria and this is

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a serious threat for public health worldwide [20] [21]. In developed countries availability of safe drinking water is not an issue [22], but in developing countries like Pakistan it is a difficult task [23]. Insufficient information is available about contamination of drinking water sources with antibiotics and ABR. Therefore, this research was designed to investigate contamination of the common drinking water sources with antibiotics and ABR at Rawalpindi city of Pakistan.

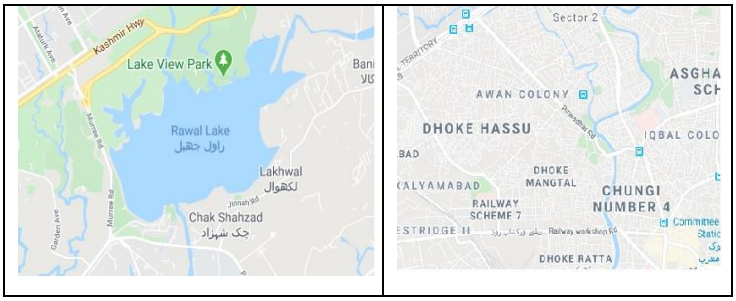

Fig. 1: Map of sampling sites

2. MATERIALS AND METHODS

In this study all the chemicals/reagents were of analytical grade and bought from local supplier, manufactured by Sigma-Aldrich (USA), BDH (UK) and Oxide (UK).

2.1 Study sites

A large area of Rawalpindi city receiving potable water from Rawal dam and ground water (borehole) is also using for drinking purpose. For representative sampling, water samples were taken from (i) Rawal dam before and after treatment and (ii) twenty randomly selected boreholes in the area. Sand filters and chlorination were only using for treatment of the water before distribution. Sampling was design to cover all type of water sources using by the local community as drinking water mainly and/or other household activities (Fig. 1). Water samples were collected four times from May to December during the year 2018, because outbreaks of gastrointestinal infections occur during these months and to cover both rainy and dry seasons.

2.2 Microbiological sampling and analysis

Water sampling for the analysis of fecal coliform, coliforms, fecal streptococci and HPC bacteria were performed according to WHO guidelines [24]. To collect the samples for bacterial analysis pre sterilized glass bottles of 1liter capacity were used. Most probable number (MPN) method with 9 test tubes were used for coliform detection with lactose as bacterial growth medium. After incubation of these tubes for 24 to 48 hours at 35 0C, inoculum from each positive tube transferred to EC and brilliant green broth media. After that, the tubes with EC media and brilliant green media were incubated for 24 hours at 44 0C and 48 hours at 35 0C respectively. In case of both media, tubes with gas (in Durham tubes) were considered positive. The 9-tube method were used for the determination of fecal streptococci, the tubes after incubation at 35 0C for 48 hours with turbidity were considered positive. Inoculum from positive tubes were transferred to

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PSE agar and inoculated at 35 0C for 24 hours. The fecal streptococci appeared in the plates with brown and black colonies and brown halos. For heterotrophic bacteria whole surface of nutrient media plates were covered with 0.1 of water sample and incubated at 35 0C for 24 hours. After 24 hours, colonies were counted and number of colonies were multiplied by 10 to calculate CFU/ml [25]. Salmonella typhi, E.coli, vibrio cholera, Streptococcus pneumonia, Bacillus cereus and Corynebacterium diphtheriae were identified and isolated by using Brilliance Salmonella agar, Brilliance E.coli/coliform selective agar, TCBS medium, KF-Streptococcus Agar, PLET Agar and Hoyle′s tellurite agar respectively [26], Bergey's manual was also used for conformation.

2.3. Antibiotic sensitivity test

Antibiotic sensitivity test for the isolation of bacteria were performed contrary to eight antibiotics by disc diffusion method according to the procedure defined by Clinical and Laboratory Slandered Institute using Mueller Hinton agar [27]. Eight antibiotics namely amoxicillin (AMO; 20 µg), ciprofloxacin (CIP; 5 µg), Cefixime (CIF; 5 µg), ampicillin (AMC; 30 µg), erythromycin (EMC; 15 µg), ceftriaxone (CEF 30 µg), streptomycin (STM; 10 µg) and kanamycin (KAM; 30 µg) were selected for human and animals pathogens and diseases (Table 1). Inoculum was prepared by direct colony suspension method. The suspension was spread on the Mueller-Hinter agar plates. The antibiotic disks were placed evenly on the inoculated Mueller-Hinter agar plates and incubated at 35 0C for 24 hours. The diameters of inhibitory zones were measured according to the method described by Clinical and Laboratory Standards Institute [28].

Table 1: List of antibiotics, bacteria, and related diseases

Antibiotics	Bacteria	Main diseases
Amoxicillin	Strephtococcus pneumonia	Pneumonia [29]
	Strephtococcus spp.	Skin infections, Pneumonia [30]
	Escherichia coli	Diarrhea, Urinary tract infection [31]
	Helicobacter pylori	Peptic ulcer [32]
Ciprofloxacin	Escherichia coli	Diarrhea, Urinary tract infection [32]
	Klebsiella pneumonia	Urinary tract infection, Pneumonia, Intro-abdominal infections [33][34]
	Salmonella typhi	Diarrhea, abdominal pain and nausea [35][36]
Cefixime	Strephtococcus aureus	Skin infections [37]
	Strephtococcus pneumonia	Ear infections, meningitis [38][39]
	Klebsiella	Blood stream infections, Wound infectons and Urinary tract infection [40][41]
	Escherichia coli	Diarrhea, Urinary tract infection [40][42]
Ampicillin	Streptococcus pneumonia	Ear infections, meningitis [43][1]
	Streptococcus pyogenes	Pharyngitis, Skin infections and wound infections [44][45]
	Enterococcus	endocarditis, intra-abdominal infection and urinary tract infection [46]
Erythromycin	Staphylococcus	Minor skin infections (pimples) to pneumonia and meningitis [47] [48]
	Haemophilus	Pneumonia and bacterial meningitis [49]
	Corynebacterium genera	Corynebacterium diphtheria [50]

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Ceftriaxone	Staphylococcus aureus	Osteomyelitis, endocarditis and furunculosis [51][52]
	Escherichia coli	Diarrhea, Urinary tract infection [53][51]
	Psudomonas aeruginosa	Infections in burned and surgical wounds and organ transplant [54][55]
Kanamycin	Pseudomonas	Pneumonia [56]
	Mycobacterium tuberculosis	Tuberculosis [57]

2.4 Antibiotic analysis

2.5 Sampling

Water samples for antibiotic analysis were collected in amber glass bottles; volume of each sample were 4 liter and moved towards the laboratory in an ice box. Sample were stored at 4 0C but not more than 12 hours (Chen et al., 2015). Total number of samples were 66.Samples were separately accumulated in dry seasons (n=22), rainy season (n=22) and normal season (n=22)

2.6 Sample preparation

For removal of suspended particles from the water samples, one liter of water sample has been passed through 0.22 µm glass filter. The samples were shaken well after mixing 0.5g N2EDTA and concentrated hydrochloric acid was added to adjust pH to 2-3. Afterwards, 150 ng 13C3-caffeine also added to the sample. The extraction of the sample was carried out by using solid phase extraction cartridge. Before passing the sample, activation of cartridges was carried out by passing 6 ml Na2EDTA (2.5 g/L) and 6 ml of ultrapure water. After the filtration of samples, 6 ml of ultrapure water passed from the cartridge and dried for half an hour under negative pressure. The targeted analytes were eluted by passing 6 ml acetonitrile and 6 ml methanol under gravity condition. The volume of collected eluent was reduced to less than 1 ml by using water bath at 40 0C. Volume of sample was reconstituted to 1 ml by using methanol. The sample was mixed with 10 µL of 13C3-caffeine (50 ppm) and transferred to injection for analysis [3] [58].

2.7 Antibiotic analysis

Prepared water samples were analyzed by using high performance liquid chromatography (HPLC) coupled with Triple quadrupole mass spectrometer. The analytical column RP-18e (250mm×4.6mm, 5µm) has been applied for the analysis of room temperature. 10 µL of sample was injected, and flow rate of mobile phase was 0.3 mL/minute. Formic acid (3%) and 0.1% ammonium format aqueous solution were utilized as an aqueous phase. Methanol and acetonitrile with 1:1 volume was used as an organic phase. Antibiotics were well separated and peaks for all the antibiotics appear on chromatograph [59] [60] [61].

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3. RESULTS AND DISCUSSION

3.1 Antibiotics in drinking water sources

All the 8 antibiotics under study were detected in drinking water sources during dry season (October, November and December) while five antibiotics (CIF, AMC, STM, EMC and KAM) out of the eight were not detected during the rainy season (July and August). This is only because of difference in dilution factor during dry and rainy seasons [62]. AMO, CIP and CEF were the only antibiotics detected during the rainy season. From the samples collected during normal season (May, June and September), two out of eight antibiotics (CIF and KAM) were not detected. Concentration of all the targeted antibiotics (Table 2) was at ng/L. In dry season, sampling of three antibiotics CIP, CEF and EMC were more dominant with detection frequency 20.2, 19.1 and 17.3 respectively. CIP was also the most prominent antibiotic detected from water samples collected (with detection frequency 16.7) during rainy season followed by CEF (detection frequency 13) AMO (detection frequency 9). Higher concentrations of antibiotic were detected from water samples collected during December (dry season). Major concentrations of the antibiotics were 74 ng/L (CIP), 55 ng/L (AMO) and 41 ng/L (CIF) respectively. Around 90% of some antibiotics taken orally excreted from body through urine or feces in active form and most of antibiotics were not easily degraded in environment and persist in water environment (Kumar et al., 2005). Therefore, antibiotics are detectable in surface and ground waters [63]. Detection frequency and amount of antibiotics was higher in dry season and less in rainy season because of dilution factor. The amount of antibiotics in drinking water samples measured in this study is less than the concentrations (from below detection limits to 11960 ng/L) reported in drinking water by Reis [62] while the measured concentration of antibiotics higher than that reported by Wang and Coworkers in tap water (0.0064 ng/L to 0.0089 ng/L) [5]. It is worth mentioning that, for drinking water there is no guideline available about the pharmaceuticals and World Health Organization still believed unwarranted to mention in guidelines for drinking-water quality standards [64].

Table 2: Summary of antibiotics detected from drinking water sources of study area (ng/L)

		AMO	CIP	CIF	AMC	EMC	CEF	STM	KAM
Dry season (n =22)	Frequency	11	20.2	8.7	9.4	17.3	19.1	6.4	4.4
	Max	55	74	41	28	29	30	29	30
	Med	27.5	46	22.5	15.5	8.5	13.5	15	12.5
	Min	7	5	3	2	1	1	2	1
Rainy season (n =22)	Frequency	2.4	16.7	0	0	0	7.9	0	0
	Max	9	21	ND	ND	ND	13	ND	ND
	Med	5.5	9.5	ND	ND	ND	4.5	ND	ND
	Min	0	0	ND	ND	ND	0	ND	ND
Normal season (n =22)	Frequency	8.6	18.3	0	3.6	5.3	12.6	2.5	0
	Max	21	43	ND	15	17	18	21	ND
	Med	10.5	20	ND	9	6.5	10.5	8.5	ND
	Min	1	2	ND	0	1	0	1	ND

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3.2. Microbiological Analysis

Results of microbiological analysis of water samples collected from Rawal dam (before and after treatment) and from distribution system given in table 3. The result reveals that catchment area of the dam is highly contaminated with sewage water; because mixing of sewage water is main source of bacterial contamination in ground water reservoirs [65] [66]. The results also exposed that current treatment system (use of sand bed and chlorination only) is reducing microbiological load but not able to convert the water source into potable water [67]. This may be due to inefficient system because amount of chlorine was varied from 1 ppm to 4 ppm in both cases. Values of microbiological study of water samples collected from the distribution system also exemplifying that microbial load increasing after entering into the distribution systems. Its indicating leakage of the system and mixing of sewage and resistance of the organisms from disinfectant or biofilm formation [68], and this is a common problem in developing countries [64].

Table 3: Biological characteristics surface water (Rawal dam) using for drinking purposes

	Heterotrophic bacteria (per ml)	**Fecal Streptococci (MPN/100ml)**	**Fecal coliform (MPN/100ml)**	**Total coliform (MPN/100ml)**
Raw water				
Maximum	1294	188	95	179
Minimum	79	16	11	37
Average	316.41	48.87	17.22	54.8
After chlorination (before distribution system)				
Maximum	38	2	0	11
Minimum	0	0	0	0
Average	9	0.13	0	2.5
Distribution system (tap water)				
Maximum	489	176	57	84
Minimum	15	0	4	5
Average	112.27	47.58	23.7	41.4

Table 4: Biological characteristics ground water (borehole) using for drinking purposes

	Heterotrophic bacteria (per ml)	**Fecal Streptococci (MPN/100ml)**	**Fecal coliform (MPN/100ml)**	**Total coliform (MPN/100ml)**
Ground water (borehole)				
Maximum	559	74	82	136
Minimum	0	0	0	0
Average	60.4	9.3	12.5	19.8

Ground water samples were also contaminated along with microorganisms (Heterotrophic bacteria, Fecal Streptococci, Fecal coliform, and Total coliform (Table 4). Source of the microorganisms to the ground water might be urban runoff, if it reaches to

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the source [69] and other sources included are septic tanks and weeping bed. Pathogen once enter into ground water can easily survive in aquifer [70]. The situation is intense that half of the wells are contaminated with pathogenic bacteria and 5.9 million illnesses reported per year in United State [71].

Percentage of coliform, fecal coliform, fecal streptococci, and heterotrophic bacteria in samples collected from Rawal dam before and after treatment and from ground water are given in table 5. The standards elucidate incidence of the isolates is more frequent in raw water samples as compared to treated and ground water samples. It's clearly indicating that only chlorination not effective for treatment of surface water [67] and ground water is also approachable to wastewater [72]. Water bodies receiving untreated wastewater usually have high microbiological load and conventional treatment plant also not effective to remove microbial pollution [73]. Environmental factors like dry season, precipitation etc. directly impact the microbial load and its usually high during dry season [74].

Table 5: Frequency of different contaminants in samples collected from different sources

Microorganisms Contamination	**Rawal dam (Before treatment)**		**Rawal dam (After treatment)**		**Ground water**	
	Percent	Number	Percent	Number	Percent	Number
Coliform	56.36	62	23.33	14	24.32	18
Fecal coliform	52.73	58	15	09	20.27	15
Fecal streptococci	31.81	35	10	06	14.86	11
Heterotrophic bacteria	62.73	69	26.25	21	31.08	23
Total	100	110	100	80	100	74

Total 436 gram-negative and gram-positive bacteria were also isolated from samples (RW: 100; TW: 47; DP1: 30; DP2:53; DP3: 26; SP1: 61; SP2: 48; SP3: 71) (Table 6). E. Coli. Were detected in 326 samples (74.7%) followed by Bacillus cereus (35.09%), Salmonella typhi (24.08%) and Streptococcus pneumonia (6.42%) while lowest detected was Corynebacterium diphtheriae (1.38%). The results also demonstrating that raw water has a greater number of bacteria in contrast to treated (chlorinated) water. This is because chlorination can reduce the number of pathogens but completely elimination is not possible.

Table 6: frequency of bacterial isolates from water samples

Group of bacterial	**Genera**	**Occurrence**	**Percentage**
Gram-negative	Salmonella typhi.	105	24.08
	E.coli	326	74.77
	Vibrio cholera	19	4.36
Gram-positive	Streptococcus pneumonia	28	6.42
	Bacillus cereus	153	35.09
	Corynebacterium diphtheriae	6	1.38
Total		436	100

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3.3. Antibiotic Resistance

Results of the antibiotics resistance for both gram negative and gram positive are given in table 7, 8, 9 and 10. All the bacteria isolates were resistant to the antibiotics to varying degree that is lowest to AMC (16.67%) and highest to KAM (91.3%). Highest resistance in gram-negative and gram-positive bacteria isolated from surface water about 91% and 77.78% against KAM respectively. Groundwater isolates at a highest resistant almost 65% (for EMC) in case of gram-negative bacterial while it was about 50% in gram-positive bacteria for CIF. It was an alarming situation that majority (191/193) were resistant to at least 3 antibiotics while 182 of 193 were resistant to five to eight antibiotics under study. It was also noted that the isolates of treated water were more resistant to the antibiotics as compared to isolates of untreated water, reason behind is that bacteria can develop resistance against different disinfectant including chlorine and the bacteria shows more antibiotic resistance against antibiotics [75]. Detection of antibiotic resistant bacteria in treated water, well water and tap water is emerging health issue worldwide [76] [77] [26]. Dramatic increase in antibiotic resistance of pathogenic bacteria is global issue and imagining as high health risk for both human and animals [78].More shocking situation is that the nonpathogenic bacteria also getting resistance against antibiotics and able to transfer the resistant genes to even different species [79] [80].

Table 7: Level of antibiotic resistance of Gram-negative bacteria isolated from raw water of Rawal dam.

Source	Number of isolates	Percentage of antibiotic resistance (%) of the isolates							
		AMO	CIP	CIF	AMC	EMC	CEF	STM	KAM
RW	58	67.24	70.69	56.90	68.97	84.48	72.41	77.59	86.21
TW	23	73.91	73.91	69.57	73.91	86.96	73.91	86.96	91.30
DP1	19	78.95	73.68	73.68	78.95	84.21	68.42	89.47	89.47
DP2	44	68.18	84.09	65.91	63.64	79.55	65.91	88.64	86.36
DP3	11	63.64	81.82	72.73	54.55	81.82	54.55	90.91	81.82

Table 8: Level of antibiotic resistance of Gram-positive bacteria isolated Rawal dam and its distribution area

Source	Number of isolates	Percentage of antibiotic resistance (%) of the isolates							
		AMO	CIP	CIF	AMC	EMC	LFX	STM	KAM
RW	42	35.71	47.62	54.76	16.67	21.43	23.81	26.19	38.10
TW	24	58.33	54.17	58.33	29.17	33.33	37.50	29.17	45.83
DP1	11	54.55	45.45	54.55	36.36	54.55	27.27	36.36	63.64
DP2	9	66.67	66.67	44.44	33.33	55.56	33.33	66.67	77.78
DP3	15	46.67	40.00	53.33	40.00	46.67	40.00	33.33	60.00

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Table 9: Level of antibiotic resistance of Gram-negative bacteria isolated from ground water

Source	Number of isolates	Percentage of antibiotic resistance (%) of the isolates							
		AMO	CIP	CIF	AMC	EMC	CEF	STM	KAM
SP1	39	43.59	56.41	53.85	48.72	58.97	23.08	41.03	61.54
SP2	32	53.13	43.75	40.63	50.00	65.63	18.75	28.13	46.88
SP3	45	37.78	42.22	46.67	51.11	44.44	20.00	22.22	40.00

Table 10: Level of antibiotic resistance of Gram-positive bacteria isolated from ground water

Source	Number of isolates	Percentage of antibiotic resistance (%) of the isolates							
		AMO	CIP	CIF	AMC	EMC	LFX	STM	KAM
SP1	22	27.27	31.82	36.36	40.91	36.36	18.18	27.27	45.45
SP2	16	37.50	25.00	50.00	25.00	31.25	31.25	31.25	25.00
SP3	26	23.08	30.77	34.62	26.92	30.77	19.23	19.23	34.62

CONCLUSION

Municipal wastewater has severally contaminated Rawal dam. Advance techniques adequate to control bacterial contamination required for the water supply system. Water distribution system is not a fool proof to prevent water from the bacterial contamination. The pollutants (antibiotics and antibiotic resistant bacteria) are approaching ground water. This may pose a serious threat to the community consuming the water for drinking purpose. A comprehensive mitigation approach should be designed to degrade the antibiotics in portable water by utilizing novel techniques. Implementation of indigenous legal polices to prevent the entry of bacterial contaminated water from point sources should be ensured to conserve the precious water resources

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