Evaluation of antibiotic resistant bacteria in underground drinking water and transfer of their resistant character to normal flora of the body

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Abstract: The untreated surface water for drinking and domestic use is an alarming situation to public health especially in prevalence of antibiotics resistant bacteria. This investigation aimed to isolate and identify the antibiotic resistance bacteria in underground water samples in district Dera Ismail Khan, Pakistan. The underground water samples were collected from four different places using hand pumps (Khyber town, riverside, Gomal University and united town). Cultured on nutrient agar media, identified by Gam staining and biochemical tests. There after antibiotic resistance assay were performed by measuring zone of inhibition of different antibiotics by disc diffusion method. Six different bacterial colonies were isolated and identified as *Enterobacteriaceae, Serriata specie, Proteues, Pseudomonas,* all these bacterial colonies were 33% resistant to chloramphenicol with and 100% resistant to amoxicillin. Some colonies were also considered as resistant, according to the criteria of National Committee for Clinical Records (NCCL) that less than 10mm zone of inhibition are considered as resistant. Subsequently, the chloramphenicol resistance bacteria were analyzed for their ability to transfer resistant gene to sensitive bacteria. In in-vitro method, an isolate M1b (resistant) was found capable to transfer resistance gene to M1a isolate (sensitive) in nutrient rich environment. It was concluded that antibiotics resistance bacteria found in underground water, moreover capable of transferring the antibiotic resistant character to suitable recipient i.e. normal flora of the body or to other pathogens by conjugation.

Keywords: Staining, chloramphenicol, antibiotic resistance, conjugation, resistant pathogens.

INTRODUCTION

A chemotherapeutic agent that kills microorganism is called antibiotic. The word antibiotic was derived from Greek word which mean against life. In the 9th century a French scientist Paul valium observed a substance called "pyocyanine" as it was inhibiting the growth of bacteria in the test tube, but it was too toxic to be used (Muhammad *et al.,* 2013). In the late 19thcentury, when the germ theory of disease acceptance of promoting research begin to antibiotic; the aim of research was to find "magic bullets" that would kill microbes (Alcoma *et al.,* 1994). In 1942 the term antibiotic was first coined by Selman Waksman and he described that any substance produce by microorganism is antagonist to other microorganisms in high dilution. Antibiotic is produced as secondary metabolic product of metabolism of microorganisms (Muhammad *et al.,* 2013). The bacteria that are not killed by the antibiotic are called antibiotic-resistant bacteria; bacteria can change its self by many ways, e.g. to use internal mechanism to change the structure so no longer antibiotics work. The resistant bacteria are not killed or their growth is inhibited by a particular antibiotic against that resistance has been developed. Therefore, newly researched antibiotic will be needed to treat. As we know that antibiotic are extremely important, but unfortunately due to excessive use of antibiotic and passing of bacteria through evolutionary changes, bacteria develop antibiotic resistance as a defense mechanism. A question has arisen in mind that how they become resistant so the answer is "survival of the fittest". The antibiotic- resistance is an outcome of evolution (Al-Lawati *et al.,* 2000).

Like other developing country, in Pakistan a general increase in antibiotic-resistance has been documented as the excessive use of antibiotic in hospital, in farm animals and poultry and sold over the counter (OTC) in drugstore without any prescription (Khursheed & Khatoo, 1984, Naqvi *et al.,* 1990, Ahmad *et al.,* 1997, Khan *et al.,* 1998). Due to insufficient data available about antibiotic-resistance, inadequate antibiotic susceptibility tests and due to lack of insufficient national surveillance plane verse conditions are expected especially in remote rural areas of the country. In Pakistan the hygienic conditions in health facilities and hospitals are also favoring the spreading of antibiotic- resistance (Muhammad *et al.,* 2013). Since the literature on underground water of Pakistan contains antibiotics resistant bacteria, therefore underground water samples for antibiotic resistance was investigated during this project.

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MATERIALS AND METHODS

Nutrient agar, Normal saline, Eosin methylene blue (EMB) agar, MacConkey agar, Lactose, Simmons Citrate Agar, Muller Hinton agar, Ampicillin, Chloramphenicol, Erythromycin, Oxytetracycline and DMSO were purchased from Sigma-Aldrich (St. Louis, MO, USA) and Merck (Germany).

Sample collection

The underground water samples were collected in sterile bottles labeled with sample identification codes, from four hand pumps located in Khyber town (M1), Indus river side (M2), Gomal University (M3) and united town (M4) in district Dera Ismail Khan, Pakistan. These samples were transported to the laboratory in an ice filled thermo cooler and stored into refrigerator (Barja *et al.,* 1989, Jones *et al.,* 1986). These samples were collected in May 2017

Isolation of pure culture

For pure culture, the sample serial dilutions were made with sterile distilled water up to 10^{-6}. Afterwards, 0.1ml of each dilution was loaded on nutrient agar media by spread plate technique and incubated at 37^{o}C for 24 hours (Ronald *et al.,* 2006). The plates were observed for bacterial growth, six different colonies (strains) were isolated, in sample M1, two morphological different colonies naming as (M1a) and (M1b), in sample M2only one type of colony observed (M2a), while sample M3 contain two types (M3a) and (M3b) and in sample M4 we found only one type of colony (M4a).The morphologically distinctive colonies were purified by streaking and maintained on nutrient agar (Li, 1929). The morphological different colonies transferred into a nutrient broth by a sterile wire loop from a nutrient agar plate of each sample and incubated at 37^{o}C for 48 hours or until turbidity of nutrient broth was achieved. The inoculated broth tubes were labeled with a sample identification code and stored at 4^{o}C in the refrigerator in order to preserve the bacteria in their native form. These stock cultures were used in further experimental through the sub culturing process.

Identification tests

Bacterial isolates were identified by performing physical and bio-chemistry test. Morphologically bacteria were identified by observing the phenotype characters like colony shape, edge shape and surface etc. (Fehmidafasin, 2003). For gram staining smear of bacterial culture were made on clean glass-slide, air dried, heat fixed and gram staining was performed. After staining the slides were examined under light microscope (As shown in table 1).

Biochemically bacteria were identified by using Eosin methylene blue (EMB) test. To perform this test, samples were streaked on the EMB agar media plates and incubated at 37C for 24hours. For MacConkey agar test bacterial strains were streaked on MacConkey agar plates. Thereafter, Simmons' Citrate Agar test was performed, Simon's citrate slants were inoculated by picking up a loop full of stock broth culture and slant test tubes were placed in incubators at 37^{o}C for 24 hours to achieve the growth (As shown in the table 2). The pouring and streaking processes were carried out in laminar air flow hood in order to decrease the microbial contamination.

Antibiotic disks preparation

The antibiotic solution concentrations expressed in ug/ml, based on the potency per disk prescribed by WHO and national committee on clinical laboratory standards (NCCL S) of the United States. It was assumed that paper disk could absorb 0.02ml solution. The following formula was used in determining the amount of antibiotic powder to be used.

$$\text{Weight of powder (mg)} = \frac{\text{volume of solvent (ml) x concentration (mg/ml)}}{\text{Potency of powder (mg/g)}}$$

Antibiotic disks were prepared by using ordinary office one-hole puncher to prepared paper disk (whatman filter paper and Neigele chromatography paper) of 6mm size and autoclaved them at 121^{o}C for 15 minutes. Antibiotic solutions were prepared by dissolving in their appropriate solvents and further diluted in distilled water. The Ampicillin with potency of 10uI, 0.00058g Ampicillin powder was dissolved per ml of distilled water with 5 % DMSO. Similarly chloramphenicol with potency of 30u I dissolved 0.0017 chloramphenicol powder. For erythromycin with 15u potency I dissolved 0.000873g powder per ml of solution in distill water with 5% DMSO. For oxytetracycllin with potency of 30u I dissolved 0.0017g powder in water with 5% DMSO. So for amoxicillin with potency 25u I dissolved 0.0014g powder in solvent and then diluted with water. Subsequently blank disks were soaked in known concentrations of antibiotic solutions and allowed to dry in an incubator at 37^{o}C for 3 hours and then kept in freezer at 4^{o}C (As shown in table 3)

Antibiotic sensitivity test

An antibiotic sensitivity test was carried out by using the disk diffusion method (Bauer *et al.,* 1966). Cells were grown overnight at 37^{o}C in nutrient broth, followed by swabbing onto Muller Hinton agar to achieve a lawn of bacterial growth. The antibiotic disks were placed on each plate and incubated at 37^{o}C for 24 hours. After incubation, the zone of inhibitions is observed, for sensitive or resistant to antibiotics.

Resistance transfer

The selected bacteria *enterobacteriaceae* (M1a) and *serratia specie* (M1b) were brought in contact for resistance transfer against chloramphenicol. The *serratia specie* (M1b) was used as a donor and *entrobacteriaceae*

(M1a) as the recipient. The donor and recipient bacteria were grown separately overnight in nutrient broth (Meheus, 1998). Thereafter, the incubated donor and recipient strains (1:10 donor/recipient) were mixed in flask containing nutrient broth and was held for 2 hours without shaking at 37^{o}C. After incubation, the donor and recipient mixture was streaked onto nutrient agar plate previously supplemented with chloramphenicol (50ug/ml) and were incubated at 37^{o}C for 24 hours. The successful trans-conjugant was detected by its ability to grow on nutrient agar plate supplemented with chloramphenicol antibiotic.

RESULTS

Isolation and identification of isolates

The isolates were obtained by culturing the sample serial dilutions on nutrient agar media, after incubation six different colonies (strains) were isolated. The morphologically distinctive colonies were purified on nutrient agar for identification.

Morphological identification

After growth on nutrient agar medium the morphologically different colonies were appeared in different shapes, colony edge shapes, suffice, elevation, opacity, and colors. On the morphological basis, they were distinguished and isolated (As shown in table 4)

Gram staining

When the smears were examined under a microscope, they were found in different colors and shapes, mostly they were appearing in pink colors under microscope and so they were gram negative (As shown in table 1).

Eosin methylene blue (EMB) test

When streaked EMB plates were examined after 24 hours incubation, the growth was found which revealed gram negative bacteria in a sample (As shown in table 5). The isolates M3a and M4 were lactose positive, as they fermented lactose in the media. The results of colonies colors appeared were matched to stander color of growth on EMB on ASM microbelibrary.org (Muray & Lupez, 1997).

MacConkey' agar test

The bacteria were identified on the basis of color appeared on the MacConkey agar media and the color were matched to a standard color searched on ASM microbelibrary.org (Muray & Lupez, 1997, Phillip, 1998, Muray *et al.*, 1990). After matching their names were given as shown in table 6. There was similar in MacConkey and EMB tests. The isolates (M1a), (M3a), and the M4 were found as lactose fermenter sand they turn media pH to acidic and caused the pH indicator (neutral red), change the color to turn pink on nutrient agar media which indicated that they were lactose positive. While the Isolates (M1b), M2 and (M3b) were not lactose fermenters and remain the media color same as before applying the isolates or slightly change to brownish yellowish. When the EMB (table 5) agar and Macon key (As shown in the table 5) results were matched, it was indicated that the (M1a) isolate belonged to specie *Enterbacteriaceae*, (M3b) belong to *Pseudomonas sppecie* and M4 belongs to *Enterobacteriaceae*. The results of colonies colors appeared were matched to stander color of growth on EMB and Macon key agar searched at ASM microbelibrary.org (Muray & Lupez, 1997, Phillip, 1998, Muray *et al.*, 1990).

Simmons' citrate agar

After 24 hours incubation period, the bacterial growth appeared and also the colors of slants medium changed from green to blue. The sample number (M1a), (M3a), (M3b) and M4 changed the media color from green to blue, which indicated their ability to utilize citrate as sole carbon source and ammonium ions as the sole nitrogen source and turned the pH indicator blue from green that indicated the samples (M1a), (M3a), (M3b) and M4 were positive to Simon's citrate agar test. While the samples (M1b) and M2 did not change the color of the media is indicated that samples (M1b) and M2 were not utilized citrate as sole carbon source and ammonium ions as the sole nitrogen source, so it was concluded that they negative to Simon's citrate agar test (As shown in the table 2).

Table 1: Gram staining of the isolated strains of the microorganisms

Isolates	Color	Gram type
M1a	Pink	Gram-
M1b	Pink	Gram-
M2	Pink	Gram-
M3a	Pink	Gram-
M3b	Pink	Gram-
M4	Pink	Gram-

Table 2: Simmons; citrate agar test

Isolate	Color	Fermentation Citrate (+,-)
M1a	Blue	+
M1b	Green	-
M2	Green	-
M3a	Blue	+
M3b	Blue	+
M4	Blue	+

Antibiotic resistance test

The six underground water samples isolates were assayed to five antibiotics resistance tests. The isolates were found as significantly ampicillin and amoxicillin resistant, while erythromycin, and oxytetracycline were considered as

Table 3: Antibiotic disk properties including size and potency of the disc

Antibiotic powder	Solvent	Diluents	Disk size	Disk potency
Ampicilline	DMSO	Water	6mm	10ug
Chloramphenical	DMSO	Water	6mm	30ug
Erythromycin	DMSO	Water	6mm	15ug
Oxytetracycline	DMSO	Water	6mm	30ug
Amoxicillin	DMSO	Water	6mm	25ug

Table 4: Morphological identification and characteristics of the identified strains

Isolates	Whole Colony Shape	Colony Edge Shape	Surface	Elevation	Opacity	Color
M1a	Circular	Smooth	Rough	Raised	Non-Transparent	Creamy White
M1b	Rhizoids	Filamentous	Waxy(Smooth)	Flat	Transparent	White
M2	Irregular		Rough Dry	Raised	Non-Transparent	White
M3a	Irregular	Smooth	Smooth	Flat	Transparent	Yellow
M3b	Rhizoids	Smooth	Smooth	Flat	=	=
M4	Irregular	Filamentous	Rough	Less Raised	Non-Transparent	White

Table 5: Eosin methylene blue (EMB) test of the pathogens

Isolates	Color	Gram stain	Fermentation (lactose)+,-	Bacteria strains type
M1a	Brownish	Gram-	-	*Enterobacteriaceae*
M1b	Brownish	Gram-	-	*Serratiaspp*
M2	Yellowish	Gram-	-	*Proteus*
M3a	Pink	Gram-	+	*Enterobacteriaceae*
M3b	Yellowish	Gram-	-	*Pseudomonas*
M4	Pink	Gram-	+	*Enterobacteriaceae*

Table 6: MacConkey's test

Isolate	Color	Gram stain	Fermentation (lactose)+,-	Bacteria strains type
M1a	Pink	Gram-	+	*Enterobacteriaceae*
M1b	Yellowish	Gram-	-	*Serratia spp*
M2	Yellowish	Gram-	-	*Proteus*
M3a	Pink	Gram-	+	*Enterobacteriaceae*
M3b	Yellowish	Gram-	-	*Pseudomonas spp*
M4	Pink	Gram-	+	*Enterobacteriaceae*

resistance according to values of the National Committee on Clinical Laboratory Standards (NCCLS) that bacteria which show zone of inhibition less than 10mm are considered as resistant. The two *serriataspp* (M1b) and *pseudomonas* (M3b) showed significant resistance to chloramphenicol, while other bacteria, *enterobacteriaceae* (M1a), *proteues* (M2), *enterobacteriaceae* (M3a) and *enterobacteriaceae* (M4), found sensitive to chloramphenicol. All strains were found resistant to amoxicillin and ampicillin (As shown in the table 7)

According to the values of NCCLS that less than 10mm zone of inhibition are resistance, 10mm are intermediate and greater then 10mm are sensitive. The frequency of sensitivity to chloramphenicol was higher than other antibiotic for all isolates except isolates (M1b) and (M3b), while chloramphenicol was more sensitive for rest all. The frequency of sensitivity to oxytetracycycline was also observed by isolate (M1a), M2 and ampicilline to isolate (M1a) and M4.The intermediate greater than 10mm were considered as sensitive (As shown in the table 8).

Resistance transfer

The resistance transfer between bacteria was defined by their ability to grow on the antibiotic supplemented nutrient agar plate. After incubation, the growth was observed on nutrient plate supplemented with chloramphenicol. This indicated that the isolate (M1a) (of rough dry colony) which was before sensitive but now it was becoming resistant (As shown in the table 9).

DISCUSSION

The 97% of global freshwater is coming from underground water and this source is a globally exploited natural resource (Mumma *et al.*, 2011). In most of areas of developing countries, drilling of boreholes for domestic use of underground water is a common. With this

Table 7: Resistant percentage of isolates towards different antibiotics, intermediate, sensitive and resistant

Antibiotics	No of Isolates	Resistance	No of Isolates	Intermediate	No of Isolates	Sensitive
Chloramphenicol	2	33%	0	0%		63%
Erythromycin	1	16%	3	50%		33%
Ampicillin	6	100%				
Amoxicillin	6	100%				
Oxytetracycline	2	33.33%	2	33.33%	2	33.33%

Table 8: Zone of inhibition of different antibiotics against isolated pathogens

Isolate No.	Isolated Bacteria	Habitat	Zone of Inhibition				
			Chloramphenicol 30ug	Erythromycin 15ug	Ampicillin 10ug	Amoxicillin 25ug	Oxytetra 30ug
M1a	*Enterobacteriaceae*	Water	13mm	11mm	7mm	3mm	14 mm
M1b	*Serriata spp*	Water	0mm	10mm	5mm	4mm	7 mm
M2	*Proteues*	Water	17mm	10mm	6mm	0mm	12 mm
M3a	*Enterobacteriaceae*	Water	16mm	12mm	4mm	0mm	13 mm
M3b	*Pseudomonas*	Water	0mm	7mm	6mm	0mm	9 mm
M4	*Enterobacteriaceae*	Water	16mm	11mm	8mm	0mm	11

Table 9: Pathogen showed transfer of resistant gene

Isolate	Bacteria	Susceptibility	Colony shape	Susceptibility after Conjugation
M1a	*Enterobacteriaceae*	Sensitive	Rough dry	Resistance
M1b	*Serriata spp*	Resistance	Smooth mucous	Resistance

background information we investigated the bacterial population resistance to antibiotics found in underground water of District Dera Ismail Khan. During underground water investigations, ampicillin resistant bacteria were observed in water samples (Herwigr *et al.*, 1997).

The similar finding has been reported in this study conducted on underground water resistance bacteria found in lake water and found ampicillin resistant bacteria from lake water samples (Jones *et al.*, 1986). The likewise, we also found that isolates were 100% resistant to amoxicillin (AML) and 33% were resistant to chloramphenicol. In earlier studies, it is also reported that the B-lactamase product is a major mechanism of resistance to ampicillin, B-lactamase enzyme breakdown the penicillin B-chain and deactivate it (Ronald *et al.,* 2006). The 93.7% *pseudomonas* isolates were found significantly resistant to both antibiotics, Tetracycline and Gentamycin (Nasreen *et al.*, 2015), while similar findings, but with elevated rates (100%) *pseudomonas* resistance to antibiotics were found in Egypt (Mahmoud *et al.*, 2013), and slightly less than this rate (99%) *pseudomonas* resistance has been reported in Pakistan (Anjum, 2010).

During current investigation, we found the *serriataspp andpseudomona* significantly resistance to all of used antibiotics specifically to chloramphenicol. In previous studies, it has been reported that increased antimicrobial resistance found in those areas where antibiotics are used indiscriminately (Harakeh *et al.,* 2006). According to other published studies, one of the major elements in antimicrobial resistance is horizontal gene transfer of mobile genetic elements (Stokes & Gilling, 2011). During an investigation, the microbial resistance against various antibiotics (Erythromycin, Ampicillin, tetracycline, Gentamycin, and Ciprofloxacin) has been evaluated at different times (Koesak, 2012).

In the present study, the multiple drug resistance pattern was observed as *Serriata specie, Proteues, Enterobacteriaceae, Pseudomonas,* were resistant to Amoxicillin and relatively less resistant to Ampicillin, while *Proteues, Enterobacteriaceae* also found sensitive to chloramphenicol and oxytetracycline. This multiple drug resistance pattern correlates with an investigation by (Mudryk, 2002), with respect to multiple drug resistances, which may be coded on several genetic elements such as plasmids, mutational events or mobile genetic materials known as transposons. This phenomena is also expected due to horizontal gene transfer between microorganisms occurring spontaneously in nature (Harakeh *et al.,* 2006).

CONCLUSION

In conclusion, the current study has described the microbial profile and antibiotic resistance pattern observed among isolates from underground water samples taken from different locations of District Dera Isamil Khan, KPK, Pakistan. Among these isolates, *serriata spp* and *pseudomonas* showed multiple drug resistance while *proteues and enterobacteriaceae*, were resistant to Amoxicillin and Ampicillin and sensitive to chloramphenicol and oxytetracycline. Moreover, it is also observed that intrinsic resistance among bacterial species

found in underground water is due to horizontal gene transfer. So it is concluded that this antimicrobial resistance pattern is associated with a major health risk.

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