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Biochemical characterization and identification of bacterial strains isolated from drinking water sources of Kohat, Pakistan

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As the pure drinking water is inevitable for good health, it is necessary to evaluate it for bacterial contamination. This study was conducted on the different drinking water sources of Kohat, a Northern-Western District of Pakistan. Sampling was done from different drinking water sources including tap water, tube well water, home-well, bore-well and springs. Physiochemical analyses including pH, temperature, turbidity, total dissolved solids and electrical conductivity showed that all water samples were within ranges of the values prescribed by the World Health Organization. A total of 79 bacteria isolated from different samples were characterized. Eighty two percent of the strains were Gram negative and 64% of the total Gram positive bacteria were spore forming. The physiological characterization showed that 30.4% of the total bacterial strains were obligate aerobes while the rest were facultative anaerobes. Biochemical characterization and identification depicted enormous bacterial diversity where sixteen genera could be tentatively identified. Identification of 24 of the strains was further validated by using API 20E kit. Furthermore, the selected strains were analyzed for pH, temperature optimization and NaCl tolerance. *Pseudomonas* sp were the most abundant bacteria followed by *Bacillus* sp. Some of the coliform bacteria could also be identified which present a potential health hazard.

Key words: API 20E, bacterial diversity, water-borne pathogens.

INTRODUCTION

The water availability has always been of great importance for life and for every living organism. It has a life-sustaining role in welfare and growth of mankind (Kazmi, 2004). All life on the earth is dependent on water and so does many economic activities. Every human individual require about 2 litres of clean drinking water per day and this amount reaches to almost 12 millions m^3 per day for the world population (Yassi et al., 2001). About 69% of fresh water worldwide is used for domestic purpose that is for drinking, cooking sanitation, 22% in industries, 8% for irrigation.

The quality of water is important to the health, social and economic well being of people. It is important to test the suitability of the quality of water for its use as drinking

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water. Water that looks drinkable can contain bacterial contamination, which are not visible to naked eye and cannot be detected by smell, taste and sight. Variety of bacteria may be present in water even which looks clearand tasted good may not necessarily be safe to drink. Due to anthropogenic interventions the water is getting polluted and thus causing negative effects in human lives and in natural equilibrium. The problems in water sources have become one of the primary problems of human lives. Due to the water borne diseases which result from inadequate water supply, hygiene and sanitation, around 4 million people suffer from water borne diseases and 2.2 million people die from these diseases every year (UNICEF-WHO, 2008). The problem is even severe in developing countries where generally the drinking water is untreated. The infants are the most vulnerable targets of these diseases. Bacteria constitute one of the major contaminants of water (Suthar et al., 2009). They have been reported to persist even in the extreme environmental conditions and oligotrophic conditions (Sigee, 2005). Moreover, many of the bacterial species have the ability to make resistant survival structures.

A variety of techniques have been reported so far to evaluate the ecology of bacteria in drinking water. For the characterization of drinking water bacterial strains, common techniques are culture-based methods and biochemical characterization (Keinanen et al., 2004). The phenotypic techniques include morphological, cultural, and physiological analysis. In addition to this a variety of molecular tools are available. The biochemical characterization has been very useful for assessment of the bacterial ecology as well as the identification. Moreover, kits are available for reliable bacterial identification based on the biochemical analysis. Nonetheless, these tools are very effective to study the biodiversity of bacterial flora. For instance, the API-20E system is suitable for the identification of enteric bacteria and provides a convenient method to inoculate and read tests relevant to members of the family *Enterobacteriaceae* and non-fastidious, Gram-negative rods (Thaochan et al., 2010). API 20E identification system contains a strip of 20 miniaturized bio hanges, and a database. Identification is obtained by the API 20E catalogue (Thaochan et al., 2010). The kit has been used in a number of studies for identification of bacteria in water (Liguori et al., 2010; Kampfer et al., 2008).

Majority of bacteria inhabiting the drinking water belong to the classes *Alpha- proteobacteria* such as *Sphingomonas*, *Hyphmicrobium and Pedomicrobium* and *Betaproteobacteria* such as *Dechlormonas*, *Aquaspirillium*. *Gammaproteobacteria* such as *Legionella and Mycobacteria* were also found in drinking water *(*Domingo et al., 2003). Additionally, the coliform bacterial group may occur in water due to faecal contamination that is discharge of faeces by human and other animals (Kaspar et al., 1990). Coliform includes the members of *Enterobacteriaceae* example *Escherichia coli*, *Enterobacter aerogenes*, *Salmonella* and *Klebsiella* spp. These enteropathogenic bacteria in water are responsible for a variety of diseases like cholera, typhoid,dysenteries, bacillary dysentery, etc. in human and livestock (Ashbolt, 2004).

This research work was based on the ecological analysis of the indigenous bacterial strains of drinking water from different sources of Kohat District located in the North Western Province (Khyber Pakhtoonkhwa) of Pakistan using a large sampling strategy. The study focuses on the isolation and identification of bacterial strains by morphological, cultural, and biochemical methods.

MATERIALS AND METHODS

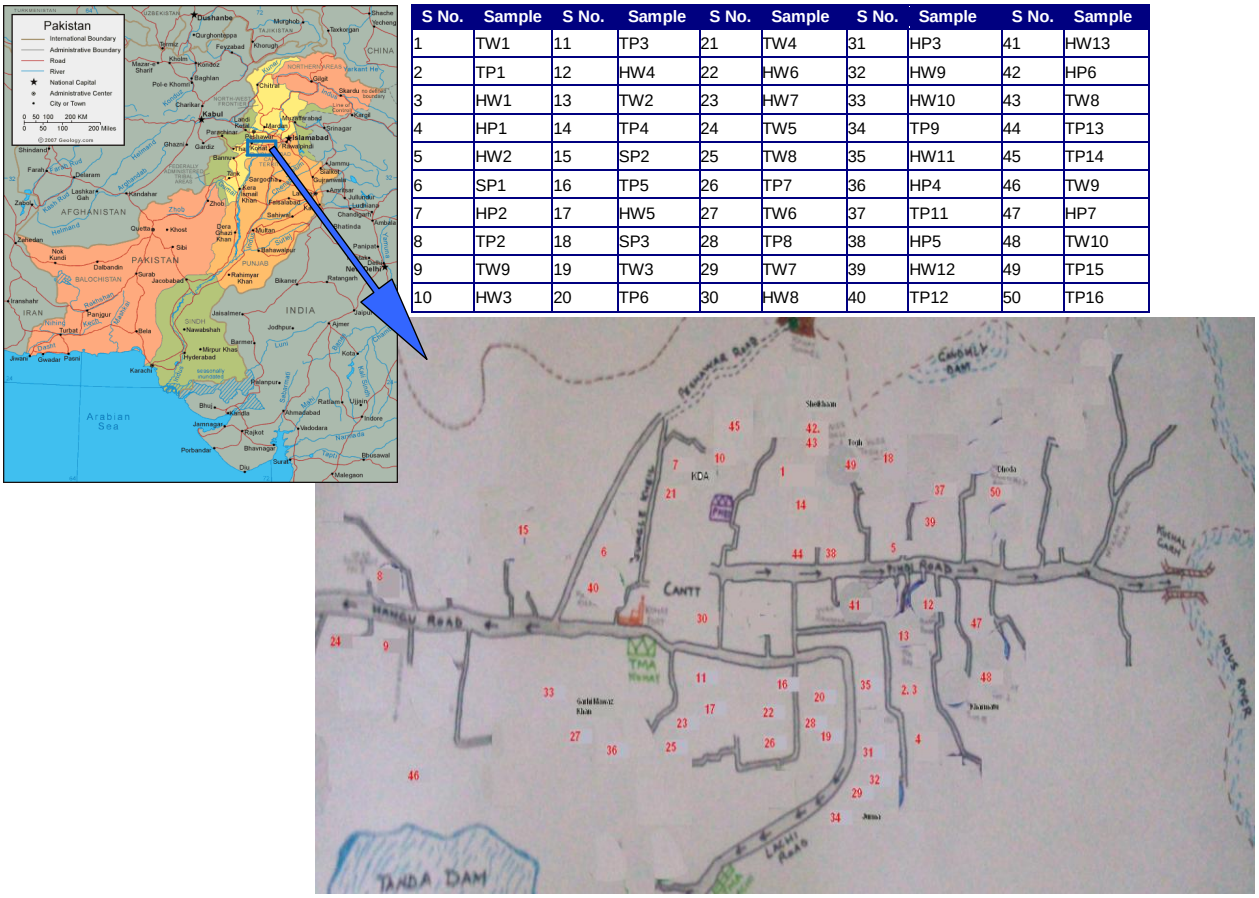
Site description

Kohat is a medium sized town with a population of around 0.5 million people according to the last census data in 1998. The annual population growth rate is 3.25%. It is located in Khyber-Pakhtunkhwa Province of Pakistan at 33°35'13N 71°26'29E with an elevation of 489 metres (1,607 ft). The total area of the district is 2,545 square kilometres (983 sq mi). The river Indus forms the eastern boundary of the district, which separates it from the province of Punjab. Kohat Toi is a principal stream, which enters from Hangu district and flowing to east and southeast, drains into river Indus. Source of water for drinking purpose is mainly municipal water that is tube well water, while some people also use home well water and spring water in some locations. Kohat features a semi-arid climate with very hot summers and mild winters. The mean maximum temperature in summer is over 40°C (104°F) and the mean minimum temperature is 25°C (77°F). The mean minimum temperature during winter is 4°C (39°F) and maximum is 18.35°C (65.03°F). The rainfall is received throughout the year. The monsoon rain is received from May to October. August is the rainiest month, with an average of about 111 mm. The winter rain occurs from November to April. The highest winter rainfall is received in the month of March. The average annual rainfall is about 546 mm.

Water sample collection and physiochemical characterization

Sampling was performed during the period of October (2010) to March (2011) from different areas of District Kohat, Pakistan. A total of 50 Samples were collected from wells, tube wells, spring, hand pumps, and tap water from different areas across the District Kohat (Figure 1). Samples were collected aseptically in 500 ml sterile autoclavable plastic (polypropylene) bottles and transferred in an icebox to the laboratory. These were immediately stored at 4°C. Microbiological analyses were carried out within 24 h of sampling.

The physicochemical parameters including pH, temperature, turbidity, electrical conductivity, and total dissolved solids were measured for all samples using standard methodologies. The pH was measured with pH Meter Model 370 (Jenway, U.K). The temperature was measured on the site of sampling by dipping a standard thermometer inside water for few minutes. Turbidity of natural water arises due to the presence of suspended matter such as clay, silt, organic matter, phytoplankton and other microscopic organisms. It was measured by Hach® 2100Q Handheld Turbidity. The electrical conductivity (EC) of water and the total dissolved solids (TDS) sample were measured by using Jenway Conductivity/TDS meter Model 470.



S No.	Sample	S No.	Sample	S No.	Sample	S No.	Sample	S No.	Sample
1	TW1	11	TP3	21	TW4	31	HP3	41	HW13
2	TP1	12	HW4	22	HW6	32	HW9	42	HP6
3	HW1	13	TW2	23	HW7	33	HW10	43	TW8
4	HP1	14	TP4	24	TW5	34	TP9	44	TP13
5	HW2	15	SP2	25	TW8	35	HW11	45	TP14
6	SP1	16	TP5	26	TP7	36	HP4	46	TW9
7	HP2	17	HW5	27	TW6	37	TP11	47	HP7
8	TP2	18	SP3	28	TP8	38	HP5	48	TW10
9	TW9	19	TW3	29	TW7	39	HW12	49	TP15
10	HW3	20	TP6	30	HW8	40	TP12	50	TP16

Figure 1. Sampling plan: the numbers indicate the 50 sampling location. TP, Tap water; SP, spring; HW, home well; TW, tube well; HP, hand pump.

Isolation and purification of bacterial isolates

Isolation of bacteria was performed on Tryptic Soya Agar (TSA) medium by using spread plate technique. The TSA plates were incubated for 24 h at 37°C after inoculation with 100 µl of the sample and subsequently observed for bacterial growth and isolation. In a given plate, all the isolates with differential colony morphology were selected. After isolation, the isolates were purified and characterized by Gram stain examination.

Phenotypic characterization

The phenotypic characterization of all isolates studied were performed and compared to phenotypic data of known organisms described in the Bergey's Manual of systematic Bacteriology. The phenotypic features characterized are as follows:

Cultural and Morphological characteristics:
The colony morphology, cell morphology, and the motility of bacterial isolates from fresh cultures were evaluated.

Physiological characteristics

Bacterial isolates were tested for growth at different pH (4, 5, 7, 8, 9, 10 and 11), different temperature ranges (4, 10, 15, 25, 35, 45 and 50°C) and salt ranges (0, 1, 2, 3, 4, 5, 8, 10, 13, 15, 20, 25 and 32%NaCl). The optical density at the wavelength of 600 nm was used for evaluating bacterial growth after 6 and 13 h.

A number of biochemical tests were performed for the identification of bacterial isolates with the help of Bergey's Manual and also using ABIS 7 online software. The principal tests used for this purpose are Lactose Fermentation Test (LAC), Indole Test (IND), Methyl Red Test (MR), Voges-Proskauer Test (VP), Citrate Utilization Test (CIT), Urease Test (URE), Nitrate Reduction Test (NIT), Oxidase Test (OXI), Catalase Test (CAT), Hydrogen Sulphide Production (H2S), Aerobic and Anerobic Test (Ae/An).

For LAC test Lactose broth was inoculated and incubated at 37°C for 24 h. After incubation, a positive result was noted as change of color to yellow while no color change was observed in negative results. IND test was performed by culturing the microorganisms in peptone water medium containing tryptophan in a screw capped tube, incubated for 24 h at 37°C and then Kovac's reagent (0.5 ml) was added where the positive results were indicated by the formation of pink red layer on the broth within seconds of adding Kovac's reagent. MR test was performed by inoculation of the glucose phosphate peptone water in a screw capped tube, incubation for 24-48 h and then addition of 5 drops of methyl red where the change in color of the medium to cherry red was considered as positive. VP test was performed by inoculating glucose phosphate peptone water with the microbial isolates in a screw capped tube, incubating for 24-48 h, then adding of 0.6 ml of alpha-naphthol solution and 0.2 ml of Potassium Hydroxide solution. The tubes were then allowed for 5-10 min after shaking well. The red color formation was taken as the positive result. For the CIT test, the Simons citrate agar slants were inoculated and incubated at 37°C for 24-48 h. The positive slants were noted to

change color from green to blue. For URE test, Urea broth was inoculated and incubated at 37°C for 24 to 48 h. The change of color of the broth from yellow-orange to bright pink was considered as positive.

For NIT test, Nitrate broth was inoculated and incubated at 37°C for 24-48 h. After incubation, 5 drops of Sulfanilic Acid and 5 drops of *N*,*N*-dimethyl-1-naphthylamine were added. The change of color of broth to deep red within 5 min meant that the bacteria had produced nitrate reductase. If color did not change, the results were indecisive. Small amount of Zinc was added to the broth. If the solution remained colorless, then both nitrate reductase and nitrite reductase were present. If the solution turned red, nitrate reductase was not present. OXI test was used to assess the bacteria which produce the enzyme cytochrome Oxidase. Filter paper was moistened with a few drops of 1% tetramethyl-p-phenylenediamine dihydrochloride. With a wooden applicator, growth from TSA plate was smeared on the paper. A positive result was the development of purple color. No color change indicated a negative result. CAT test was performed by adding a small amount of bacterial isolate into freshly prepared 1% hydrogen peroxide, and the bubbles of oxygen if appeared the isolate was considered as positive for CAT test. For Ae/An test, TSA was inoculated and incubated at 37°C in anerobic jar. After 24-48 h growth was observed. H_2S test was used to differentiate species of the family *Enterobacteriaceae.* This test was used to determine the ability of an organism to reduce sulfur into H_2S. SIM media was used for the H_2S production test. SIM media contains the sulfur containing amino acid, sodium thiosulfate, cysteine, and ferrous sulfate. The SIM media was inoculated with bacterial cultures by stabbing SIM media with inoculating needle. The tubes were then incubated at 35°C for 24 h. After incubation, a positive result was indicated by a black precipitate formed because of the reaction of H_2S with the iron or ferrous sulfate; while the negative result was indicated by no black precipitate.

Besides manual biochemical tests, Analytical Profile Index 20E (Bio Murex) was also used for further identification. API20E is a micro standardized identification system for Enterobacteriaceae and other non-fastidious; Gram-negative rods, in which 21 miniaturized biochemical test and a database are included.

RESULTS

Physicochemical parameters

Temperature of drinking water samples was found to be in the range of 25.5 to 29.5°C. The lowest value of temperature was 25.5°C for sampling point HW11, TW8 and the highest value was 29.5°C at sampling point TP1. The pH values of water samples ranged between 6.45 to 8.6. The lowest value of pH was 6.45 at sampling point of TW9 in Mohamad Zai area. The highest pH was 8.6 at sampling point HP3 of Jerwanda area.

Turbidity of different samples ranged from 0.2 to 5.2NTU. The highest value was found for TP13 (College Town) that is 5.2NTU. The lowest value of turbidity was for sampling point TW6 (Garhi banoorian) that is 0.2 NTU. EC values of water samples were in the range of 213 to 1229 µs/cm. The lowest value of EC was 213 µs/cm at sampling point TW6 of Garhi Banoorian area. The highest EC was found for the sampling point, TP1 (Billi Tank). TDS value varied from 102 to 561 mg/l. The sampling point HP4 showed the highest TDS value (561 mg/l). The lowest value of TDS was observed at sampling point TW8 (Mian Kheil, 101 mg/l).

Cultural characteristics of bacterial isolates

The total number of colonies appeared and the number of isolates selected are shown in Table 1. A total of 79 isolates were selected. Colony morphology of all the isolates was examined. Characteristics including form, texture, color, margin, elevation and opacity were studied (Table 2). It was observed that the forms of the colonies of bacterial isolates were mostly round while filamentous and irregular form was also observed. The surface characteristics of bacterial isolates were found to be smooth, rough, dry, glistening and shiny. Most of the colonies, which were selected visually based on differences with naked eye, were of whitish and cream colour while yellow colour is also observed. Margin of colonies of bacterial isolated were found to be entire, undulate, filliform, lobate and convex. Most of the bacterial isolates had convex colonies while flat and umbolate margins were also observed. Opacity of the colonies was observed mostly to be opaque or transparent.

Out of the 79 strains, 82% were Gram negative. Most of the strains were rod shaped (92.5%). It was observed that only 10% strains were Gram-positive rods and 7.5% strains were Gram-positive cocci (Table 3). The strains which were appeared to be Gram positive were then analysed for spore staining. Majority of the Gram positive strains (64.2%) were spore forming (Table 3).

Physiological characterization

The selected bacterial strains based on their biochemical characterization along with some of Gram positive strains were then checked for their optimized pH (Table 4). The growth was observed in the range of pH 4 to 10, with four isolates able to grow up to pH 10. The optimum pH for most of the strains was between 7 and 8 with the exception of strain TW3-1 which showed its optimum growth at pH 6. The strain TW9-2 (*B. subtilis*) which was able to grow at pH 10 also showed the highest tolerance to NaCl (10%).

Additionally, the optimum temperature for all the strains was recorded (Table 4). At 4 and 50°C none of the bacteria showed any growth. Most of the bacterial strains showed optimum growth at 0, 1 or 2% NaCl concentration, while only one strain TP10-2 showed optimum growth at 3% NaCl (Table 4). The bacterial isolates showed tolerance range of 0 to 13% NaCl concentrations.

A variety of biochemical assays were carried out to have a comprehensive view of the phenotypic characteristics of bacteria and to identify them (Table 2). Sixteen different genera could be identified out of the 79 strains. The API 20E (Biomurex) Kit was also used to further validate the identification of different bacterial isolates (Table 2). *Pseudomonas* sp. were the most

Table 1. Bacterial density and the number of selected isolates from the different drinking water samples from TP (Tap water), SP (Spring water), HW (Home well), TW (Tube well), HP (Hand pump) in Kohat Pakistan.

Sample	Bacterial cfu in 100µl of sample	Selected isolates	Sample	Bacterial cfu in 100µl of sample	Selected isolates	Sample	Bacterial cfu in 100µl of sample	Selected isolates
TP1	3	2	SP2	4	3	TW3	1	1
TP2	3	3	SP3	3	3	TW4	1	1
TP3	4	4	HW1	3	3	TW5	2	2
TP4	2	2	HW2	3	3	TW6	2	2
TP5	1	1	HW3	2	2	TW7	0	
TP6	3	3	HW4	1	1	TW8	0	
TP7	4	3	HW5	1	1	TW9	2	2
TP8	3	1	HW6	2	1	TW10	1	1
TP9	3	2	HW7	2	2	TW11	0	
TP10	3	3	HW8	3	1	HP1	1	1
TP11	2	2	HW9	0		HP2	2	2
TP12	4	1	HW10	2	2	HP3	1	
TP13	4	1	HW11	2	1	HP4	3	2
TP14	2	1	HW12	1	1	HP5	1	1
TP15	0		HW13	0		HP6	3	2
TP16	2	2	TW1	0		HP7	5	3
SP1	4	4	TW2	0				

frequently identified species followed by *Enterobacter* sp and *Bacillus* sp. A number of coliform bacteria were identified including *E. coli* (Table 5). Some other potential human pathogenic bacteria could also be identified but less frequently including *Salmonella, Enterococcus, Staphylococcus* sp in different samples.

DISCUSSION

Analyzing a total of 50 samples collected from different drinking water sources of Distt. Kohat yielded 101 isolates out of which 79 isolates were selected for further characterization. Unexpectedly high bacterial diversity was evident from this study where 16 different genera could be identified. Such a high bacterial diversity in drinking water has never been reported so far to of our knowledge. The reason for this high biodiversity may be the multiple chances of contaminations from environment due to lack of care and awareness.

The most frequent genera were *Pseudomonas* followed by *Bacillus* and *Enterobacter*. These bacteria are generally the predominant bacterial genera in drinking water system (Block et al., 1997; Berry et al., 2006; Liguori et al., 2010). The Gram-negative and non-fermenting bacteria isolated from drinking water such as from genera *Acinetobacter*, and *Aeromonas* are found commonly in water, soil, and in sewage (Salem et al., 2008).

Pseudomonas sp, *Bacillus* sp, *Acinetobacter* sp, and *Aeromonas* sp exhibit adaptations to environments with low nutrient concentration

Table 2. Morphological and biochemical observations of the different isolates from the different drinking water sources.

Strain	Form	Surface	Colour	Margin	Elevation	Opacity	Cat	Oxi	Cit	Lac	H2S	MR	VP	Nit	Ind	Ure	Mot	Ae/An	Microbe Identified
TP1-2	Circular	Glistening	Cream	Entire	Raised	Transparent	+	-	+	-	+	+	+	+	-	+	+	F	*Proteus mirabilis**
TP1-3	Circular	Smooth	Cream	Entire	Raised	Transparent	+	+	-	-	-	+	-	+	+	-	+	F	*Vibrio parahaemolyticus*
TP2-1	Circular	Smooth	Cream	Entire	Raised	Transparent	+	+	-	+	+	-	+	+	+	-	+	F	*Aeromonas hydrophila /salmonicida* sp.
TP2-2	Circular	Smooth	Yellow	Entire	Flat	Transparent	+	-	+	-	+	+	-	+	+	-	+	F	*Salmonella* sp.
TP2-4	Filamentous	Glistening	White	Lobate	Umbonate	Rough	+	+	+	+	-	+	-	+	-	-	+	A	*Pseudomonas* sp.
TP3-1	Circular	Smooth	Oval	Entire	Convex	Rough	+	+	+	-	-	-	-	+	-	-	+	A	*Pseudomonas* sp.
TP3-2	Contoured	Smooth	Cream	Lobate	Flat	Transparent	+	-	-	-	+	+	+	+	+	+	+	F	*Proteus vulgaris**
TP3-3	Circular	Dry	Cream	Undulate	Flat	Opaque	+	-	+	-	-	-	+	+	-	-	+	F	*Serratia marcescens*
TP3-4	Smooth	Shiny	White	Entire	Convex	Moist	+	-	+	+	-	-	+	+	-	+	+	F	*Enterobacter aerogenes*
TP4-1	Smooth	Shiny	White	Entire	Convex	Moist	+	-	+	+	-	-	+	+	-	+	+	F	*Enterobacter* sp.
TP4-2	Irregular	Rough	Creamy	Undulate	Flat	Opaque	+	+	+	-	+	-	+	+	-	-	+	A	*Bacillus subtilis*
TP5-1	Circular	Rough	Off white	Convex	Slighlty raised	Rough	+	-	+	+	+	+	-	+	-	-	+	F	*Citrobacter freundii*
TP6-1	Circular	Glistening	Yellowish	Entire	Raised	Opaque	+	+	+	-	-	-		+	-	-	+	A	*Pseudomonas flourescens/ P. putida* sp.*
TP6-2	Irregular	Glistening	Cream	Swarming	Flat	Opaque	+	-	+	-	-	+	-	+	+	+	+	F	*Providencia stuartii**
TP6-3	Irregular	Glistening	Creem	Entire	Raised	Opaque	+	-	+	+	-	-	+	+	-	+	-	F	*Klebsella. pneumonia*
TP7-1	Circular	Moist	Circular	Swarming	Slighlty raised	Opaque	+	-	+	+	-	-	+	+	+	+	-	F	*Klebsiella oxytoca*
TP7-2	Circular	Smooth	Colourless	Lobate	Slighlty raised	Opaque	+	-	+	-	+	+	-	+	-	-	-	F	*Salmonella* sp.*
TP7-3	Circular	Smooth	White	Irregular	Flat	Rough	+	+	-	+	-	-	-	+	-	+	-	F	*Staphylococcus epidermidis*
TP8-3	Circular	Smooth	Whitish	Entire	Convex	Translucent	+	-	-	+	-	+	-	+	+	-	+	F	*Escherichia coli*
TP9-2	Circular	Smooth	Non pigmented	Muciod	Slighlty raised	Opaque	+	-	+	-	-	-	-	-	-	-	-	A	*Acinetobacter* sp.
TP9-1	Circular	Smooth	Whitish	Entire	Convex	Translucent	+	+	+	-	-	-	-	+	-	-	+	A	*Pseudomonas aeruginosa**
TP10-1	Circular	Smooth	Colourless	Lobate	Slighlty raised	Opaque	+	-	+	-	+	+	-	+	-	-	-	F	*Salmonella* sp.
TP10-2	Circular	Shiny	White	Entire	Convex	Moist	+	-	+	+	-	-	+	+	-	+	+	F	*Enterobacter aerogenes**
TP10-3	Irregular	Smooth	Creem	Entire	Raised	Opaque	+	+	-						-			A	*Proteus mirabilis*
TP11-1	Circular	Smooth	Cream	Undulate	Raised	Opaque	+	+	+	-	+	-	+	+	-	-	+	A	*Bacillus subtilis*
TP11-2	Circular	Shiny	White	Entire	Convex	Moist	+	-	+	+	-	-	+	+	-	-	+	F	*Enterobacter aerogenes*
TP12-4	Circular	Smooth	Oval	Entire	Convex	Rough	+	+	+	-	-	-	+	-	-	-	+	F	*Pseudomonas* sp.*
TP13-4	Circular	Glistening	White	Entire	Flat	Rough	+	-	+	-	-	-	+	+	-	+	+	F	*Serratia sp*
TP14-2	Circular	Smooth	Cream	Entire	Raised	Transparent	+	+	-	+	+	-	+	+	+	-	+	F	*Aeromonas hydrophila /caviae/sobria 2*
TP16-1	Circular	Smooth	Cream	Undulate	Raised	Opaque	+	-	+	+	-	+	-	-	-	-	-	A	*Bacillus* sp.
TP16-2	Filamentous	Glistening	White	Filiform	Undolate	Opaque	+	+	+	+	-	+	-	+	-	-	+	A	*Pseudomonas* sp.

Table 2. Continued

SP1-1	Circular	Dry	Cream	Entire	Convex	Opaque	+	+	-	-	-	+	+	-	-	-	-	F	*Aeromonas hydophila /salmonicida* sp.
SP1-2	Irregular	Glistening	Cream	Entire	Raised	Opaque	+	-	+	+	-	-	+	+	-	+	+	F	*Enterobacter cloacea**
SP1-3	Circular	Smooth	Cream	Undulate	Raised	Opaque	+	+	+	-	+	-	+	+	-	-	+	A	*Bacillus subtilis*
SP2-1	Circular	Smooth	Oval	Entire	Convex	Rough	+	+	+	-	-	-	-	+	-	-	+	A	*Pseudomonas* sp.
SP2-3	Circular	Smooth	Whitish	Entire	Convex	Translucent	+	+	+	-	-	-	-	+	-	-	+	A	*Pseudomonas* sp.
SP2-4	Circular	Smooth	Cream	Undulate	Raised	Opaque	+	+	+	-	+	-	+	+	-	-	+	A	*Bacillus subtilis*
SP1-4	Round	Smooth	Cream	Entire	Raised	Opaque	+	-	+	-	-	-	-	-	-	-	-	A	*Acinetobacter sp**
SP3-1	Circular	Glistening	Yellowish	Entire	Raised	Opaque	+	-	+	-	-	+	-	+	+	+	+	F	*Providencia retegeri**
SP3-2	Circular	Dry	Cream	Undulate	Flat	Opaque	+	-	+	-	-	-	+	+	-	-	+	F	*Serratia marcescens**
SP3-5	Irregular	Glistening	Creem	Entire	Raised	Opaque	+	-	+	-	-	+	-	+	+	+	+	F	*Providencia retegeri*
HW1-2	Circular	Dry	Cream	Entire	Flat	Opaque	+	-	+	-	+	+	-	+	+	-	+	F	*Salmonella* sp.
HW1-3	Circular	Smooth	Off white	Entire	Slighlty raised	Rough	+	-	+	+	+	+	-	+	-	-	+	F	*Citrobacter freundii*
HW1-1	Circular	Smooth	White	Entire	Flat	Rough	+	-	+	-	-	-	+	+	-	+	+	F	*Serratia odorifera I**
HW2-2	Circular	Smooth	Yellowish	Entire	Raised	Opaque	+	-	-	+	-	+	-	+	-	+	-	F	*Staphylococcus auerous*
HW2-3	Filamentous	Glistening	Yellowish	Filiform	Umbonate	Opaque	+	+	+	-	-	-	-	+	-	-	+	A	*Pseudomonas pseudomallei**
HW2-1	Circular	Shiny	White	Entire	Convex	Moist	+	-	+	+	+	-	+	+	+	-	+	A	*Bacillus* sp.
HW3-2	Filamentous	Glistening	Whitish	Filiform	Umbolate	Opaque	+	+	+	-	-	-	-	+	-	-	+	A	*Pseudomonas* sp.
HW3-1	Circular	Smooth	Off white	Convex	Slighlty raised	Rough	+	-	+	+	+	+	-	+	-	-	+	F	*Citrobacter freundii*
HW5-2	Circular	Shiny	White	Entire	Convex	Moist	+	-	+	+	-	-	+	+	-	+	+	F	*Enterobacter* sp.
HW6-2	Circular	Smooth	Whitish	Entire	Convex	Translucent	+	-	-	+	-	+	-	+	+	-	+	F	*Escherichia coli*
HW4-1	Circular	Dry	Oval	Undulate	Convex	Rough	+	-	+	-	-	-	+	+	-	+	+	F	*Serratia* sp.
HW7-1	Circular	Wetish	Circular	Swarming	Slighlty raised	Opaque	+	-	+	+	-	-	+	+	-	+	-	F	*Klebsella. pneumonia**
HW7-2	Circular	Smooth	Oval	Entire	Convex	Rough	+	+	+	-	-	-	+	+	-	-	+	F	*Pseudomonas* sp.
HW8-3	Circular	Smooth	Whitish	Entire	Convex	Translucent	+	+	-	+	+	-	+	+	+	-	+	F	*Aeromonas hydophila/ Salmonicida* sp.
HW10-1	Circular	Smooth	Yellow	Entire	Flat	Transparent	+	-	+	-	+	+	-	+	+	-	+	F	*Salmonella* sp.*
HW10-2	Circular	Smooth	Whitish	Entire	Convex	Translucent	+	-	+	+	-	-	+	+	-	+	-	F	*Klebsella. pneumonia*
HW11-2	Circular	Smooth	Whitish	Entire	Convex	Translucent	+	-	-	+	-	+	-	+	+	-	+	F	*Escherichia coli**
HW12-1	Circular	Smooth	Cream	Undulate	Raised	Opaque	+	+	+	-	-	-	+	+	-	-	+	A	*Bacillus* sp.
TW3-1	Circular	Smooth	Cream	Entire	Raised	Opaque	+	+	+	-	-	-	+	+	-	-	+	A	*Pseudomonas fluorescens/ P. putida**
TW4-1	Circular	Smooth	Cream	Undulate	Raised	Opaque	+	+	+	-	-	-	+	+	-	-	+	A	*Bacillus* sp.
TW5-1	Circular	Smooth	Cream	Convex	Raised	Opaque	-	-	+	+	-	+	+	+	-	-	-	F	*Enterococus faecalis*
TW5-2	Irregular	Smooth	Creem	Entire	Raised	Opaque	+	+	-	+	-			-	-	+	-	A	*Aeromonas hydophila/ /A. caviea**

Table 2. Continued

TW6-1	Circular	Smooth	Whitish	Entire	Convex	Translucent	+	-	-	+	-	+	-	+	+	-	+	F	*Escherichia coli*
TW6-2	Circular	Smooth	Whitish	Entire	Convex	Rough	+	-	+	+	-	-	+	+	-	-	+	F	*Enterobacter coloacae*
TW9-2	Circular	Smooth	Cream	Undulate	Raised	Opaque	+	+	+	-	+	-	+	+	-	-	+	A	*Bacillus subtilis*
TW9-1	Circular	Shiny	White	Entire	Convex	Moist	+	-	+	+	-	-	+	+	-	-	+	F	*Enterobacter aerogenes*
TW10-2	Circular	Smooth	Cream	Convex	Raised	Opaque	-	-	+	+	-	+	+	+	-	-	-	F	*Enterococus faecalis*
HP1-1	Filamentous	Smooth	Convex	Lobate	Slighlty raised	Opaque	+	+	+	-	-	-	-	-	-	-	-	A	*Micrococcus halobius*
HP2-1	Circular	Moist	Circular	Swarming	Slighlty raised	Opaque	+	-	+	+	-	-	+	+	+	+	-	F	*Klebsiella oxytoca*
HP2-2	Circular	Shiny	White	Entire	Convex	Moist	+	-	+	+	-	-	+	+	-	-	+	F	*Enterobacter aerogenes*
HP4-1	Filamentous	Glistening	Yellowish	Filiform	Umbolate	Opaque	+	+	+	+	-	+	-	+	-	-	+	A	*Pseudomonas flourescens/ P. putida**
HP4-2	Circular	Smooth	Off white	Convex	Slighlty raised	Rough	+	-	+	+	+	+	-	+	-	-	+	F	*Citrobacter freundii**
HP5-1	Filamentous	Glistening	Yellowish	Filiform	Undolate	Rough	+	+	+	-	-	-	+	+	-	-	+	F	*Pseudomonas sp*
HP6-1	Circular	Moist	Circular	Swarming	Slighlty raised	Opaque	+	-	+	+	-	-	+	+	-	+	-	F	*Klebsella. pneumonia*
HP6-3	Circular	Dry	Cream	Entire	Convex	Opaque	+	+	-	-	-	+	+	-	-	-	-	F	*Aeromonas salmonicida**
HP7-1	Circular	Smooth	Cream	Entire	Raised	Transparent	+	+	-	-	-	+	-	+	+	-	+	F	*Vibrio parahaemolyticus**
HP7-3	Circular	Wetish	Circular	Swarming	Slighlty raised	Opaque	+	-	+	+	-	-	+	+	+	+	-	F	*Klebsiella oxytoca**
HP7-5	Circular	Smooth	Oval	Entire	Convex	Rough	+	+	+	-	-	-	-	+	-	-	+	A	*Pseudomonas spp*

TP, Tap water; SP, spring water; HW, home well; TW, tube well; HP, hand pump. The species or genus name of the microbe ending with * signifies that the identification was confirmed by using API 20E kit. Cat, Catalase test; Oxi, oxidase test; Cit, citrate utilization test; Lac, lactose fermentation test; H_2S, hydrogen sulfide production test; MR, methyl red test; VP, Voges-Proskauer test; Nit, nitrate reduction test; Ind, indole test; Ure, urease test; Mot, motility test; Ae/An, aerobic and anerobic test.

(Thereza et al., 2002). *Aeromonas* is widespread in aquatic environment and its presence in our water samples was not surprising (Belt et al., 2007). *Bacillus* sp have also been isolated previously from drinking water systems (Lee et al., 2009; Zhang et al., 2006). *Pseudomonas aeruginosa, Acinetobacter*, *Klebsiella*, *Serratia*, *Aeromonas* and certain “slowgrowing” bacteria may be naturally present in the environment (WHO, 2011). They may be able to cause disease in vulnerable subpopulations: the elderly or the very young, patients with burns or extensive wounds, those undergoing immunosuppressive therapy or those with acquired immunodeficiency syndrome (AIDS). If water used by such persons for drinking or bathing contains sufficient numbers of these organisms, they can produce various infections of the skin and the mucous membranes of the eye, ear, nose and throat (WHO, 2011). Hence, these microorganisms may be considered as opportunistic pathogens.

Physicochemical parameters such as pH, TDS, Turbidity and Electrical Conductivity have key influence on the growth of bacterial population. For example, pH values ranging from 3 to 10.5 could support growth of indicator and pathogenic bacteria (Aydin, 2006). At extreme pH (<4.5 or >8.2) cells die-off can be expected but generally, as in our case, the pH range for the most drinking water sources was close to 7 not inhibiting the growth of bacteria (Million, 2008). However, pH of drinking water less than 7.0 causes corrosion of water pipes so metal thus releases into the drinking water causing metal contamination and deteriorating the water quality although the pH of drinking water usually ranges from 5.5 to 9. Moreover, turbidity and temperature may also affect the microbial population of drinking water. The higher values of turbidity are often associated with higher population of microorganisms as well as the suspended particles (Environmental Monitoring and assessments, 2006). Turbidity of TP13 was greater than the limits prescribed by WHO and ISI 10500-91 (Patil, 2009). The highest EC was found for the sampling point, TP1 (Billi

Table 3. Spore staining of Gram positive isolates.

Strain	Shape	Spore Staining
TP4-2	Bacilli	Sporing
TP7-3	Cocci	Nonsporing
TP11-1	Bacilli	Sporing
TP16-1	Bacilli	Sporing
SP1-3	Bacilli	Sporing
SP2-4	Cocci	Sporing
TW4-1	Bacilli	Sporing
TW5-1	Cocci	Nonsporing
TW9-2	Bacilli	Sporing
TW10-2	Cocci	Nonsporing
HP1-1	Cocci	Nonsporing
HW2-1	Bacilli	Sporing
HW2-2	Cocci	Nonsporing
HW12-1	Bacilli	Sporing

Tank) indicating the presence of high amount of dissolved inorganic substances in ionized form. TDS value varied from 102 to 561 mg/l. The sampling point HP4 showed the highest TDS value (561 mg/l) which is greater than the given range of WHO and ISI 10500-91 (Patil, 2009). However, the overall physicochemical analysis showed that parameters including pH, turbidity and TDS of all the water samples were within the permissible limits of WHO for drinking water quality standards. Thus, it seems that the actual pH, turbidity, and temperature of drinking water had immediate direct effect neither on health of human beings nor on the microorganisms (Jehan et al., 2009).

Colony morphology and Gram staining is one of the basic microbial techniques used to group the bacteria. The present results revealed that 82% of the strains were Gram negative which is in agreement with previous studies. It is generally observed that most of the bacteria isolated from water are Gram negative (Berry et al., 2006; Block et al., 1997). Moreover, all the bacterial strains isolated in the present study belonged to class *Proteobacteria* which reinforce the previous studies conducted by Williams et al. (2004). It may be concluded that *Proteobacteria* are among the most predominant microorganisms found in drinking water systems around the world.

The present results clearly indicated that there may be fecal contamination of drinking water in Kohat Distt. of Pakistan which is a serious health concern. The data showed that most of the drinking water sources are contaminated with coliforms and pathogenic bacteria. The bacterial species isolated were mostly the members of the family *Enterobacteriacea*. The members of this family are mostly Gram negative and non spore forming bacilli (Ashbolt, 2004). Although other coliforms were also present but it is important to understand that coliform bacteria are widely distributed in nature and it is not necessary that they come from fecal pollution (Griffith et al., 2003). However, the species isolated in this study also included *E. coli*, *Citobacter* and *Klebsiella* which are the known indicators of fecal contamination. Of course, the presence of these bacteria in drinking water systems is quite alarming. The Gram positive cocci isolated such as *Staphylococcus aureus* are also known as fecal streptococci. While the strains of *S. feacalis* isolated are enterococci present in man and in various animals (Valeria, 2005). Nonetheless, the public health problems may arise from these water-born pathogen contaminations which necessitate more awareness about such contaminations as well as water treatment before using for drinking purpose.

Bacteria such as *Proteus* sp isolated from water samples are also of public health importance. The species of *Proteus* generally belong to the intestinal flora but these are also widely distributed in water and soil (Schlegel, 2003). *E. aerogenes* strains were also isolated from the water samples included in group of non-fecal coliforms. The *E. aerogenes* can be found in soil and vegetation which serve as a source through which pathogenic bacteria enters into the water (Shittu et al., 2008). The consumption of drinking water contaminated with pathogenic microbes of fecal origin is a major risk to health of human beings (Chatterjee et al., 2007).

Some of the strains isolated in the present study can

Table 4. Physiological growth optimization of the selected isolates.

Strain	Species name	pH[a]	NaCl[b] (%)	Temperature (°C)						
				4	10	15	25	35	45	50
TP1-2	*Proteus mirabilis*	5-(8)-9	0-(0)-5	NG	NG	NG	MG	OG	NG	NG
TP3-2	*Proteus vulgaris*	4-(8)-9	0-(1)-5	NG	NG	NG	MG	OG	LG	NG
TP6-1	*Pseudomonas sp*	5-(7)-9	0-(0)-5	NG	LG	LG	MG	OG	NG	NG
TP6-2	*Providencia stuartii*	5-(8)-9	0-(1)-8	NG	NG	NG	MG	OG	LG	NG
TP7-2	*Salmonella* sp.	5-(8)-9	0-(0)-8	NG	NG	NG	MG	OG	LG	NG
TP7-3	*Staphylococcus epidermidis*	4-(7)-9	0-(2)-10	NG	NG	LG	MG	OG	LG	NG
TP9-1	*Pseudomonas aeruginosa*	5-(7)-9	0-(0)-6	NG	NG	NG	MG	OG	NG	NG
TP10-2	*Enterobacter aerogenes*	4-(7)-9	0-(0)-8	NG	NG	NG	MG	OG	NG	NG
TP12-4	*Pseudomonas* sp.	5-(7)-9	0-(0)-5	NG	LG	LG	MG	OG	NG	NG
SP1-2	*Enterobacter cloacea*	6-(7)-9	0-(0)-10	NG	NG	LG	MG	OG	LG	NG
SP1-4	*Acinetobacter* sp.	6-(8)-9	0-(0)-10	NG	NG	NG	MG	OG	NG	NG
SP2-4	*Bacillus subtilis*	5-(7)-9	0-(1)-8	NG	NG	LG	MG	OG	NG	NG
SP3-1	*Providencia retegeri*	5-(7)-9	0-(3)-10	NG	NG	NG	MG	OG	NG	NG
SP3-2	*Serratia marcescens*	6-(7)-9	0-(2)-8	NG	NG	NG	MG	OG	NG	NG
HP1-1	*Micrococcus halobius*	6-(7)-10	0-(0)-8	NG	NG	NG	MG	OG	NG	NG
HP4-1	*Pseudomonas* sp.	6-(8)-9	0-(0)-8	NG	NG	NG	MG	OG	NG	NG
HP4-2	*Citrobacter freundii*	5-(7)-9	0-(2)-13	NG	NG	NG	MG	OG	NG	NG
HP6-2	-	4-(7)-9	0-(1)-3	NG	NG	NG	MG	OG	NG	NG
HP7-1	*Vibrio parahaemolyticus*	5-(7)-10	0-(2)-8	NG	NG	NG	MG	OG	LG	NG
HP7-3	*Klebsiella oxytoca*	5-(7)-9	0-(2)-8	NG	NG	NG	MG	OG	LG	NG
TW3-1	*Psuedomonas* sp.	5-(6)-10	0-(0)-5	NG	NG	NG	MG	OG	NG	NG
TW4-1	*Bacillus* sp.	4-(7)-9	0-(2)-10	NG	NG	NG	MG	OG	NG	NG
TW5-1	*Enterococcus faecalis*	4-(7)-10	0-(2)-8	NG	NG	LG	MG	OG	LG	NG
TW5-2	*Aeromonas sp*	5-(8)-9	0-(1)-5	NG	NG	NG	MG	OG	NG	NG
TW9-2	*Bacillus subtilis*	5-(8)-10	0-(1)-10	NG	NG	LG	MG	OG	LG	NG
HW1-1	*Serratia odorifera*	5-(7)-9	0-(1)-8	NG	NG	NG	MG	OG	NG	NG
HW2-1	*Bacillus* sp.	5-(7)-9	0-(1)-8	NG	NG	LG	MG	OG	LG	NG
HW2-2	*Staphylococcus auerous*	4-(7)-9	0-(2)-8	NG	NG	LG	MG	OG	LG	NG
HW2-3	*Pseudomonas pseudomallei*	5-(8)-9	0-(1)-8	NG	NG	LG	MG	OG	NG	NG
HW7-1	*Klebsiella pneumonia*	4-(7)-9	0-(1)-8	NG	NG	NG	MG	OG	LG	NG
HW10-1	*Salmonella* sp.	5-(7)-8	0-(0)-8	NG	NG	NG	MG	OG	MG	NG
HW11-2	*Escherichia coli*	4-(7)-9	0-(1)-10	NG	NG	NG	MG	OG	LG	NG

have some beneficial aspects. The present study showed that various strains are able to grow at wide range of pH and NaCl concentration. Salinity being one of the core issues for the agriculturists and a lot of work is being done to increase the salt tolerance of the crops as well as the bioremediation of the saline areas (Munns and Tester, 2008). In the present study, *B. subtilis* strain TW9-2 could grow even at a salt concentration of 13%. Such salt resistant strain may have potential salt tolerant genes. Moreover, *P. fluorescens* and *P. putida* have been identified as useful biocontrol agents (Weller, 1988). Similarly *C. freundii* has been shown to be potential microorganism for the bioaccumulation of heavy metals (Montgomery et al., 2009). Such organisms may be used for the bioremediation of heavy metals to clean up the environment (Macaskie et al., 2006). These isolates will be studied for their use as biocontrol and bioremediation which is an important perspective.

Conclusion

It can be concluded from the study that the drinking water in the studied region is considerably contaminated with

Table 5. Bacterial prevalence in different drinking water samples.

Microbe identified	No. of isolates	Samples contaminated
Pseudomonas sp	15	TP3, TP2, TP6, TP9, TP12, TP16, SP2, HW2, HW3, HW7, TW3, HP4, HP7
Enterobacter sp	10	TP3, TP4, Tp10, TP11, SP1, HW5, TW6, TW9, HP2
Bacillus sp	9	TP4, TP11, TP16, SP1, SP2, HW2, HW12, TW9, TW4
Klebsiella sp	7	TP6, TP7, HW7, HW10, HP2, HP6, HP7
Aeromonas sp	6	TP2, TP14, SP1, HW8, TW5, HP6
Serratia sp	5	TP3, TP13, SP3, HW1, HW4
Salmonella sp	5	TP2, TP7, TP10, HW1, HW10
Citrobacter freundii	4	TP5, HW1, HW3, HP4
Escherichia Coli	4	TP8, HW6, HW11, TW6
Acinetobacter sp	2	TP9, SP1
Providencia retegeri	3	TP6, SP3
Proteus sp	3	TP1, TP3, TP10
Enterococcus feacalis	2	TW5, TW10
Vibrio parahaemolyticus	2	TP1, HP7
Staphylococcus epidermidis	1	TP7
Micrococcus halobius	1	HP1

TP, Tap water; SP, spring water; HW, home well; TW, tube well; HP, hand pump.

fecal originated microorganisms. The area under study has unsatisfactory drinking water supply system. Enormous biodiversity of bacteria in drinking water was quite surprising on one hand and on other hand resulted into isolation of some bacterial species which may be of interest to agriculture and environment.

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