ISOLATION AND IDENTIFICATION OF PATHOGENIC BACTERIA FROM DRINKING WATER OF KHAIRPUR, SUKKUR AND ROHRI

BY

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A THESIS

Submitted in the partial fulfillment of requirements for the degree of

DOCTOR OF PHILOSOPHY

DEPARTMENT
MICROBIOLOGY
SHAH ABDUL LATIF UNIVERSITY
KHAIRPUR

May 2010

Dedication

I should like to dedicate this thesis to my respected parents for their enthusiasm and interest in my education.

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Declaration

This thesis is the original work of author: Abdul Hussain Shar Baloach

Acknowledgements

I would like to take this opportunity to thank Professor Dr, Yasmeen Faiz Kazi for giving me opportunity to do research under her kind supervision and efforts to get funds to complete my Ph.D. I am most grateful to the Dean faculty of Natural sciences Professor Dr. Miandad Zardari for his kind support, providing facilities, and stimulating atmosphere in my home Department 'The Department of Microbiology Shah Abdul Latif university Khairpur Sindh Pakistan. My deepest gratitude to Professor Dr Mohd Hanif Noomrio without his strong recommendation for financial support from Shah Abdul Latif University Khairpur Sindh Pakistan this work was not possible. I am very much thankful to Syed Maqsood Zia for evaluating the data statistically and helping me to apply statistics on my research data using SPSS. Thanks are also due for Mr. Roger Parton senior lecturer, Division 1 Infections & Immunity Institute of Biomedical & Life Sciences University of Glasgow UK and Dr. Anna K. Harding Prof. and Graduate Coordinator Department of Public Health, Health and Human Sciences Oregon State University (USA) for reading, commenting and valuable suggestion for the manuscript of thesis as foreign external examiner. I am also thankful to Dr. Aqeel Ahmed Professor Department of Microbiology University of Karachi and Dr. Safia Amed Associate Professor Department of Microbiology Quaid-e-Azam University Islamabad for final evaluation of my thesis. My friends deserve thanks: Irshad Hussain Soomro, Dr. Nisar Ahmed Kanher, Dr. Pir Bux Ghumro, Dr. Fateh Mohd soomro, Mr. Syed Zafar Ali Shah Mr. M. Saleem Rahpoto Mr. Mumtaz Ali Soomro and Mr. Abdul Latif Solangi for their moral encouragement for PhD research. I can't hesitate to say thanks to my parents and my children Ather, Marvi, Azhar, Afzal, Amjad and Arslan and my life partner Zawar Katoon for their assistance regarding completion of my Ph.D thesis. I am also thanking to the staff of Microbiology Department for their help. I would like to acknowledge the financial support provided by Shah Abdul Latif University Khairpur Sindh Pakistan. Without its contribution this project would not have been completed. And at last a great thanks to my family for all their love, which inspired me with the feeling that any thing is possible.

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Abbreviations

AOC **Assimilable Organic Carbon**
API **Analytical Profile Index**
ASM **American Society for Microbiology**
ACE **Accessory Cholera Enterotoxin**
AWWARF **American Water Works Association Research Foundation**
APHA **American Public Health Association**
AWWA **American Water Works Association**
CT **Cholera Toxin**
CFSAN **Centre for Food Safety and Applied Nutrition**
CDC **Centers for Disease Control**
CSO, **Combined Sewer Overflows**
DOM **Dissolved Organic Matter**

DAEC	**Diffusively Adherent *E. coli***
DNA	**Deoxyribonucleic Acid**
DPD	**Diethyl-Para-Phenylenediamine**
DCA	**Deoxycholate Citrate Agar**
DOH	**Department of Health**
DVC	**Direct Viable Count**
EPA	**Environmental Protection Agency**
EIEC	**Enteroinvasive *Escherichia coli***
EPEC	**Enteropathogenic *Escherichia coli***
ETEC	**Enterotoxigenic *Escherichia coli***
EMB	**Eosine Methylene Blue Agar**
FDA	**Food and Drug Administration**
FACS	**Fluorescently Activated Cell Sorting**
GIT	**Gastrointestinal Tract**
HAV	**Hepatitis A Virus**
HAI	**Hospital Acquired Infection**
IUCN	**International Union on Conservation of Nature**
ISO	**International Organization for Standardization**
ICU	**Intensive Care Unit**
IMS	**Immunomagnetic Separation**
LT	**Heat Labile Toxin**
MST	**Microbial Source Tracking**
MMWR	**Morbidity-Mortality Weekly Report**
MAC	***Mycobacterium avium* Complex**
MGD	**Million Gallons per Day**
MOR	**Municipal Office Rohri**
MOK	**Municipal Office Khairpur**
MPN	**Most Probable Number**
MFT	**Membrane Filter Technique**
NCV	**Non-Cholera Vibrios**
NRCC	**National Research Council Canada**

NAG	**Non-Agglutinable Vibrios**
NDWP	**National Drinking Water Policy**
PFGE	**Pulsed-Field Gel Electrophoresis**
PCRWR	**Pakistan Council of Research and Water Resources**
RCCS	**Rates of Correct Classification System**
ST	**Heat Stable**
SES	**Sanitary Epidemiologic Services**
SMC	**Sukkur Municipal Corporation.**
TSIA	**Triple Sugar Iron Agar**
UNICEF	**United Nations International Children Emergency Fund**
USAID	**United States Agency for International Development**
USA	**United States of America**
WHO	**World Health Organization**
WSC	**Water System Council**
WEF	**Water Environment Federation**
XLD	**Xylose Lysine Desoxycholate**
TCBS	**Thiosulphate Citrate Bile Salt**
ZOT	**Zonula Occludens Toxin**

ABSTRACT

Little information is available on water borne diseases in Pakistan. This probably is due to absence of an infrastructure for detection and recording such infection and its source. There is no reason to believe that the risks of water born diseases are any different from those in the rest of the world. This possibility is supported by data presented in this study which show correlations of enteric infections in various cities to address the condition of sanitation, standards of living and education. The data reflects the incidence and public health impact of these diseases in the area under study. This research project describes the fecal indicator bacteria, different waterborne bacterial pathogens, pH, temperature and chlorine residual in municipal water intended for drinking and other domestic uses from Khairpur Sukkur and Rohri Sindh Pakistan. The work was done by collecting water samples twice in a month for three years of study period from 2005-2007. Drinking water microbiological quality was primarily determined by enumeration of "indicator organisms", whose presence indicates faecal contamination. The presence of the indicators is often a key in assessing potential public health risks due to bacterial pathogens and is used in drinking water quality regulations and guidelines in many countries. The enumeration of fecal coliform specifically of *Escherichia coli* (indicator of fecal contamination) from human as well as animal sources per 100 ml of municipal water used for drinking purposes has been recommended by World Health Organization (WHO) worldwide to monitor the quality of drinking water. According to World Health Organization (WHO) guideline standards for drinking water total and fecal coliform the indicator of fecal contamination must not be detectable in any 100 ml samples.

In present study the enumeration of fecal coliform bacteria by membrane filtration method revealed that the number of fecal coliform (*E. coli*) in water was higher than standard set by World Health Organization (WHO) and Pakistan. The number of fecal coliform (*E. coli*) was varying in different seasons. In winter months it was 2.69-3.17 log cfu/100 ml and in summer months it was 3.30-3.47 in more than 70% drinking water samples collected from municipal water of Khairpur. The drinking water samples collected from Sukkur were found with highest number of faecal coliform bacteria ranging from 3.30-3.45 log cfu/100 ml in winter months to 3.60- 3.84 log cfu/ 100 ml summer months in 75% water samples, and in the drinking water collected from Rohri the number of fecal coliform bacteria were also higher in summer months. In winter months the number of fecal coliform was 2.0 3.30 log cfu/ 100 ml and in summer

months the number of fecal coliform was 3.39-3.45 log cfu/100 ml in water samples. The presence of fecal indicator bacteria (*E. coli*) in higher number in more than 75% water samples investigated was the indicative of possible presence of etiologic agents of other waterborne bacterial infectious diseases. The enumeration of total and fecal coliform bacteria showed that the municipal water of area under study was of inferior quality. The water sample tested were contained thousand of total and fecal coliform bacteria per 100 ml. the results of study suggested that the level of fecal contamination in municipal water was very high. The health impact of that unsafe drinking water could be the prevalence of waterborne diarrhoeal diseases in the population. A moderate correlation was observed between the Densities of fecal coliform and diarrhoeal cases in the in the area under study. Such water with a high number of total and fecal coliform could be the potential sources of waterborne bacterial pathogens. As it is evident from the study of 216 drinking water samples 12 species from 8 genera were isolated and identified. In this research different waterborne bacterial pathogens were isolated and identified with varying frequency by using the membrane filtration technique and Analytical Profile Index system for Enterobacteriaceae (API 20E). As the membrane filter technique (MFT) is highly reproducible, it can be used to test large volumes of samples and yield numerical results more rapidly than the multiple tube procedure; this technique was extremely useful in monitoring drinking water quality. In 1950s the membrane filter technique came into practice as a substitute for MPN method. The membrane filter technique is highly suitable for drinking water and different natural waters quality assessment. The API 20E system is considered the gold standard for identification of *Enterobacteria.* It has been reported in many studies that API 20 is a good method in its sensitivity with genus level identification (92%) and at the species level (72%) while working on genus *Yesinia.* The likelihood values from 62.5% to 99% of *Salmonella enterica* by API 20E has also been reported. In present study the species from family *Enterobacteriaceae, Vibrionaceae, Aeromonadaceae, Pseudomondaceae* were isolated. Some opportunistic bacteria like *C. meningosepticum* and *Non- Fermenter* species were also isolated from drinking water of three cities. In drinking water of Khairpur city the percentage of isolation rate was i.e. *E. coli,* 64%, *P. mirabilis,* 67% *P. rettgeri* 60%, *P. stuarti* 67%, C *youngae* 64%, *K. oxytoca* 49%, *V. cholerae* 29.16% *and V. mimicus* 47.22%, *A. hydrophila* 53%, *P. aeruginosa* 69.44%, *C. meningosepticum* 57% and *Non- Fermenter* species 61% were isolated. In drinking water of Sukkur city the *E. coli,* 69.44%, *P. mirabilis,* 65.28% *P. rettgeri* 71%, *P. stuarti* 61%,

C. youngae 60%, *K. oxytoca* 54.16%, *V. cholerae* 22.22% and *V. mimicus* 50%, *A. hydrophila* 50%, *P. aeruginosa* 76%, *C. meningosepticum* 51.38% and *Non- Fermenter* species 57% were isolated. In drinking water of Rohri the *E. coli,* 76.38%, *P. mirabilis,* 68% *P. rettgeri* 69.44%, *P. stuarti* 67%, C *youngae* 69%, *K. oxytoca* 44.44%, *V. cholerae* 32% *and V. mimicus* 54.16%, *A. hydrophila* 51.33%, *P. aeruginosa* 78%, *C. meningosepticum* 50% and *Non- Fermenter* species 60% were isolated. In this study some new bacterial pathogens was isolated and identified from water samples tested for example *C. meningosepticum P. rettgeri P. stuarti*, *C. youngae*. These bacteria are not included in the list of bacterial pathogens of water previously. These findings could be valuable or a breakthrough in the inclusion of these species as waterborne pathogens. The isolation of waterborne bacterial pathogens at such a high percentage is of a grave concern, because contaminated water plays crucial role in the prevalence of waterborne and water related bacterial diseases outbreaks in the urban and rural population in Khairpur, Sukkur and Rohri. It is important to note that the municipal water of Khairpur and other cities were found contaminated with multi-drug resistant thermo-tolerant *E. coli*. The diseases i.e. Diarrhea, Dysentery, Gastroenteritis, Typhoid fever and Cholera and other water related nosocomial infection in health care setting may be the result of consumption of such polluted water with. The temperature of water samples was also determined to observe the seasonal effect on the bacterial population in fresh water environments. It was observed that when the temperature of water ranged from 25- 32^{o}C the isolation rate of bacterial species in drinking water was 68-77% and when the temperature of water samples ranged from 17-22^{o}C the isolation rate was 34-42.61%. The temperature plays an important role in the microbial growth in biofilms where bacteria and other organism colonize, establish themselves and then produce permanent source of contamination in water distribution systems. The pH of water samples was also measured because the pH imparts its effect on water quality. Our result indicates that pH was in limits (6.5-8.5) of WHO guideline standard for drinking water. When the residual chlorine in drinking water samples was determined, a very high number of drinking water samples were found without residual chlorine. In water samples collected from Khairpur, more than 80% of water samples were found without chlorine. More than 90 % water samples from drinking water of Sukkur were found without residual chlorine and also the same percentage of water samples was found without chlorine in city of Rohri Sindh Pakistan. It is evident from these results that the bacteriological quality of drinking water was not according to the standard of World Health

Organization guidelines; for drinking water. The presence of high rate of waterborne pathogens in our study may be due to this reason. The disinfection treatment of drinking water has important role in reducing the waterborne epidemics. The inadequate disinfection treatment of municipal water results in the provision of unsafe drinking water to the people which can pose a great threat and risk of waterborne epidemics by bacterial pathogens to the population consuming it. Such negligence may result in a catastrophic disaster in the area. Water supplying authorities should take account of this situation and take measures for the provision of contamination free drinking water to prevent waterborne disease outbreaks by bacterial pathogens.

1. **INTRODUCTION**

Water covers approximately 70% of surface of earth and remaining volume is found in the environment, out of which only 2% of the world's water is drinkable (Lim, 1998). Chemically water is a molecule containing importance of feature of life. As water is a universal solvent it dissolves salts, inorganic and organic compounds and gases that take part in metabolic reactions, maintain the macromolecular framework, stabilize plasma membrane, thermoregulation, transport nutrients, and maintain hemostasis and body volume/weight (Armstrong *et al*. 2007; Bourne and Seager, 2001; Buyckx, 2007; Charney, 2008; Kleiner, 1999; Sawka *et al.* 2005).Water is an important component of all cells and is prerequisite of life on earth. The water composition of a cell varies from 45% to 95%, according to Anthony and Elizabeth (1980), water comprises just about 80% of weight of a microorganism and above 70% of human body weight is water. Water helps regulate human body's temperature, works as a nature's air conditioner inside our body. An average body contains 42 liters of water. With a loss of 2.7 liters, one can suffer from dehydration, weakness, and headaches and consequently reach a state of pathology. It is one of most critical of all resources, equally for human and for the natural environment. The accessibility of potable water remains a key issue of development. Enough water is necessary for development. The demand and supply of water use-cycle puts pressure on human needs for fresh water. Reservoirs (dams), irrigation canals, wells and other withdrawal activities show that human have a manipulating and important impact on water cycle (Solley *et al*. 1998). The environmental effects of these activities are noticeable. For example, the dams can cause the loss of land, cultural and biological resources in that area. Displacements of people, loss of wild life and continuous alteration in river ecology and hydrology is the another effect (Botkin and Keller 2005). The fecal contamination of the fresh water sources also poses a major threat to the mankind. The sewage pollution in tropical Asian regions is severe health risk to people that live near rivers and water ways. Direct discharge of domestic wastes leaching from poorly maintained septic tanks and improper management of farm wastes are suspected as the major sours of waterborne diseases (Huttly 1990). Many developing regions suffer from the lack of safe drinking water for their population. About 800 billion people in Asia and Africa are living

without access to safe drinking water. Consequently this has caused many people to suffer from various waterborne diseases (Tanwir *et al.* 2003)

1.1 Sources of Water

1. 1.1 Surface Water (rivers, lakes, storage reservoirs)

1.1.1.1 River or stream: Rivers comprise the major source of water supply. Some rivers are recurrent (water available through out the year) and some are non-perennial (water available in rainy season only)

1.1.1.2 Ponds or lakes: The natural and man-made depressions, where surface runoff is collected in rainy seasons

1.1.1.3 Storage reservoir: An artificial lake which shaped by constructing dam across a river valley

1.1. 2. Ground Water: (infiltration wells, springs, hand pumps and tube wells).

1.1.2.1. Infiltration well: For tapping water from sandy river beds

1.1.2.2. Infiltration gallery: For tapping water from sandy river bed sometimes flat tunnels are constructed in the beds

1.1.2.3. Springs: When underground water reappears at the ground surface by percolation or by underground pressure.

1.1.2.4. Wells: A simulated hole made into the ground for taping underground water.

1.1.2.5 Hand pumps: Hand pumps are the source of water in the underdeveloped and developing countries.

1.1.2.6. Tube wells: A Tube well as the name implies is basically a tube or pipe bored into the underground pool, fitted with a filter at the lower end and worked at the top (Basak 2003)

1.2 Uses of Water

According to water cycle for water use water is taken from source to the place of consumption e.g. industry, home or farm, where from it is disposed off or seldom re-used. In most countries water is directly disposed off in open atmosphere or disinfected prior to sending it back to environment (Solely *et al.* 1998). The U.S Geological survey has classified the usage of water keeping in view the current and future demand.

1.2.1. Commercial use.

1.2.2. Domestic use.

1.2.3. Industrial water use.

1.2. 4. Livestock use.

1.2.5. Mining use.

1.2.6. Public supply.

1.2. 7. Thermoelectric power.

1.2. 8. Agricultural use (Botkin and Keller 2005).

1.3 Sources of Water Pollution

1.3.1. Surface runoff

1.3.2. Human

1.3.3. Animals

1.3.4. Industrial

1.3.5. Agricultural

1.3.6. Sewage

1.4 Types of Water Pollution

1.4.1. Physical contamination (pH, temperature, odor, turbidity and color)

1.4.2. Chemical contamination (organic and inorganic chemicals)

1.4.3. Biological contamination (bacteria, viruses and protozoa).

Water contamination refers to degradation of water quality from a public health or ecological view point. A pollutant is any biological physical and chemical substance that is present in an identifiable surplus and is known to be injurious to other desirable living organisms. Water pollutants include heavy metals, sediments, certain radioactive isotopes, phosphorus, nitrogen, sodium, arsenic, heat, fecal coliforms bacteria, other pathogenic bacteria, virus and protozoan pathogens. The pollution of municipal water by human and animal sources is the major threat to the public health in poor countries. Water contaminated with excreta from animal or anthropogenic sources, which may be the carrier or active cases of infectious diseases serve as the vector of disease. Consumption of that water in any respect may cause the fatal disease (Gadgil 1998). Fresh water is precious resource that must be conserved and closely monitored for chemical pollutants and microbial contamination (Levy *et al.* 1998); because surface waters are uncovered to environmental elements like wild life droppings, urban and agriculture run-off and they require extensive treatment (ASM 2002). When the rain water flows as run-off, it passes through the ground surface and gets collected in rivers, lakes and ponds. On its way, the water gets polluted by dangerous salts, acids, minerals, pathogenic bacteria and radioactive

substances (Gillespie 1994; Basak 2003). Polluted water is a major vehicle of a number of water borne diseases that have shaped history in the past and will surely continue to do so in the future. In world today the waterborne diseases are the major health concern. Approximately 4 billion cases of diarrheal diseases reported which represent 5.7% of the worldwide disease burden in the year 2000 (WHO 2000). Post-collection pollution may results in the failure of measures taken to prevent contamination at the source level. To avoid this problem care must be taken during collection and storage of drinking water and to ensure the safe quality of water at point of use. Further to control this problem of contamination the protected sources i.e. boreholes, stand pipes or wells should be established for water quality improvement. It has observed that the drinking water gets more contaminated at household level than sources, which indicates the contamination during collection of water (Lindskog & Lindskog 1988). The polluted water can result into major epidemics of waterborne disease by waterborne bacterial pathogens (Table 1). Human excreta are the major source of waterborne infection. Bacteria capable of growing in clean water include 1.*Burkholderia pseudomallei,* 2. *Naegleria fowleri* 3. Atypical *Mycobacteria* and 4. *Legionella* (WHO 2004)

1.5 Indicator organisms

Since it would be practically impossible to test water for each of the wide variety of pathogens that may be present, microbiological water quality monitoring is primarily based on the tests for indicator organisms' http:// www.who.Int/water_ sanitation_ health

. There is no single indicator organism that can universally be used for all purposes of water quality surveillance. Each of the wide variety of indicator available for this purpose has its own advantages and disadvantages, and the challenge is to select the appropriate indicator, or combination of indicators, for each particular purpose of water quality assessment.

Indicators most commonly used are of faecal or sewage origin and the following are some of the most important requirements of such indicators:

1 Present whenever pathogens are present.

2 Present in same or higher numbers than pathogens

3 Specific for faecal or sewage pollution.

4 At least as resistant as pathogens to conditions in natural water environment, and water purification and disinfection processes.

5 Non- pathogenic

6 Detectable by simple, rapid and inexpensive methods.

Ideally, various other properties are desirable, such as counts which are directly related to those of pathogens. However, the fundamental and most important requirement is that pathogens should be absent or inactivated whenever indicators are absent or inactivated http:// www.who.Int/water_ sanitation_ health

Many indicators have been studied and recommended for water quality assessment (ISO, 1990; Standard Methods 1992). Evaluation of the reliability of indicators is carried out by comparison of their incidence and survival in the water and treatment processes with that of selected pathogens, by epidemiological studies, on the consumers of the water supplies, by the calculations based on the minimal infectious dose of pathogens, and by experiments with human volunteers (Regli *et al.* 1991). The following is a summary of the most important features of commonly used indictors:

1.5.1 Escherichia coli. This species is a member of the group of faecal coliform bacteria. *Escherichia* coli have the important features of being highly specific for the faeces of man and warm-blooded animals. For all practical purposes these bacteria can not multiply in natural water environment and they are therefore used as specific indicator for fecal pollution. They generally distinguished from other thermo-tolerant coliform by the ability to yield a positive indole test within 24 hours at 44^0C http:// www.who.Int/water_ sanitation_ health

1.5.2 Thermo-tolerant coliform bacteria

This term refers to certain members of the group of total coliform bacteria which are more closely related to fecal or sewage pollution which generally do not redially replicate in water environment. This group of bacteria is also known as fecal coliform, presumptive *E. coli* fecal *E. coli,* etc http:// www.who.Int/water_ sanitation_ health. Thermo-tolerant coliforms are primarily used for the assessment of fecal pollution in waste water and raw water sources. They are detectable by simple and inexpensive tests and widely used in water quality monitoring. The test methods used are the multiple tube and membrane filtration using membrane Faecal Coliform (mFC) medium and incubation for 24 hours at 44 ^{0}C. In the membrane filtration the individual colonies can be identified and the presence of *E. coli* provides strong evidences of fecal pollution http:// www.who.Int/water_ sanitation_ health

1.5.3 Coliform bacteria (total coliform)

The term total coliform refers to a vaguely defined group of gram negative bacteria which have long history in water quality assessment. In outdated literature these bacteria go by all sorts of names including coliform, colis etc. some of the bacteria included in this group are almost conclusively of fecal origin while other members may also replicate in suitable water environment. These bacteria which can be determined by simple and inexpensive test are primarily used for assessment of general sanitary quality of finally treated and disinfected drinking water. Methods used are multiple tubes, membrane filtration using Lawrence Experimental Station (LES) Endo agar and incubation for 24 hours at 35-37^{0}C. More recently coliform bacteria are also identified by their passion of the enzyme beta-D_galctosidase which hydrolyses chromogenic substrates such as orth-nitrophenyl beta-D-galactopyranoside (ONPG) resulting in release of chromogen and a color change in liquid media http:// www.who.Int/water_ sanitation_ health.

1.6. Fecal Indicator Organisms of Water Quality

Historically, improvement of principles, rules and regulations associated to drinking water quality targeted water distribution systems. In the nineteenth century in Europe, this was to find out the solution to problems of waterborne epidemics of infectious disease (Tollon 2005). For the provision of safe drinking water, the guideline standards for drinking water were established. The filtration and, later, disinfection techniques were adopted to control the pollution of drinking water. The term indicator was emerged for indication of the occurrence of fecal coliforms as well as pathogenic bacteria in drinking water. This technique was adopted for analysis of possible presence of bacterial pathogens (Tollon 2005). World Health Organization (WHO) has promoted this idea to control drinking water quality (WHO 1993), quality of recreational water (Bartram and Rees 2000) and the quality of wastewater reused in agriculture (Mara and Cairncross 1989). Assessment, safety and water treatment procedures are given priority. Suitability of water quality is established by turbidity, pH, indicator bacteria, and free residual chlorine for drinking waters and for wastewater reuse the enteric helminths count and trematode eggs are used as a standard method (Bluementhal *et al.* 1999). In several countries, risk assessment is done to establish drinking water treatment needs on the basis of disease from reference pathogens (Regli *et al.* 1991; 1993; Briscoe 1996). Isolation and control of indicator bacteria have played effective roles in reducing the incidences of epidemics by waterborne bacterial pathogens (Payment *et al*. 1991). However, the volume of water examined by taking

irregular 100 ml water samples from a large water body frequently only represents a millionth of 1% of total water body. Hence the absence of indicator in these little samples may not report their actual concentration in the water (Gale 1996). To determine the fecal contamination of water, the organisms used are known as indicator organisms which become the indication of fecal contamination. This kind of contamination by any pathogenic microorganisms that occur in intestinal tract of the human and animals may too be present in water (Tasneem 1997). The coliform bacteria are mostly used for this purpose. These bacteria can grow aerobically and as facultative anaerobes, are Gram negative rods and ferment lactose with acid and gas (Monica 1984, Johnson 1995). Thermotolerant bacteria are best to fulfill the indicator criteria. Hence they are present in significant numbers in the fecal material, unable to grow in natural waters (WHO 2000). Historically, fecal indicator bacteria (FIB) including total and fecal coliforms and enterococci have been used in many countries as a monitoring tool for microbiological impairment of water and for prediction of presence of bacterial, viral and protozoan pathogens. These microorganisms are of fecal origin from higher mammals and birds, and their presence in water may indicate fecal pollution and possible association with enteric pathogens. (McFeters *et al.*, 1974; McFeters, 1990), Currently, there are very few review papers critically evaluating the relationships between conventional and alternative fecal indicators and the presence of bacterial, viral and protozoan pathogens (Horman *et al*., 2004; Noble and Fuhrman, 2001; Payment *et al.* 2000).

1.6.1. Alternative indicators of fecal pollution

Alternative fecal indicators such as fecal anaerobes (genera *Bacteroides* and *Bifidobacterium*, spore-forming *Clostridium perfringens*), viruses (*B. fragilis* phage, coliphages (FRNA phage)), and fecal organic compounds (coprostanol) have been increasingly applied. It seems that the use of alternative indicators together with conventional fecal markers is very promising to identify the source of fecal pollution and associated pathogens. However, replacement or combination of any indicators requires adequate epidemiological studies to support their use.

1.6.2 Fecal anaerobes

Fecal anaerobes account for a significant portion of fecal bacteria (Matsuki *et al*., 2002; Wood *et al.*, 1998) and are limited to warm-blooded animals (Fiksdal *et al.* 1985; Franks *et al.* 1998; Macy, 1979). The primary weakness of fecal anaerobes as indicator is short survival in non-host environments due to their low oxygen tolerance. The degree of their tolerance to atmospheric

oxygen appeared to be genus-related characteristics (Avelar *et al.* 1998). A comparison of oxygen tolerance for fecal isolates demonstrated a broad spectrum among species and revealed general patterns. *Bacteroides adolescentis* and *Bacteroides infantis* showed intermediate tolerance and survived 48 h in the presence of oxygen. When aerated, no viable cells of *Bifidobacterium* spp. were detected after 48 h (Rolfe *et al*. 1977). Of the fecal isolates, *C. perfringens* showed the highest oxygen resistance, surviving 72 h or more in the presence of oxygen (Rolfe *et al.* 1977). Therefore, since fecal anaerobes generally cannot survive long in aerobic conditions, detection of large numbers of these microorganisms in water samples indicates recent or extensive fecal contamination.

1.6.3 Bacteroides spp.

Until now, the need to maintain anoxic conditions for cultivation, isolation and biochemical identification limited the usage of anaerobic *Bacteroides* species as fecal indicator. However, the increasing use of recent molecular methods overcomes this problem (Kreader, 1995).Importantly, since certain *Bacteroides* species are highly host-specific, it is possible to identify the source of fecal contamination by tracking host-specific *Bacteroides* species (Kreader, 1995; Simpson *et al*. 2004). Researches have recently differentiated *Bacteroides–Prevotella* species originated from human and cow feces by using terminal restriction fragment length polymorphism (T-RFLP) and length heterogeneity PCR (LH-PCR) analyses, and then successfully applied these host-specific genetic markers to real water samples (Bernhard and Field, 2000a and Bernhard and Field, 2000b; Boehm *et al.* 2003). Only few studies using this indicator have been conducted, indicating the need for future studies at larger scales including studies on geographical distribution of host-specific *Bacteroides* genetic markers (Bonjoch *et al.* 2004).

1.6.4 Bifidobacterium spp.

Ecological distribution of *Bifidobacterium* spp. is highly variable in animals (Bonjoch *et al*. 2004). Although feces from humans, chickens, cows, dogs, pigs, horses, cats, sheep, beavers, goats, and turkeys were investigated, *Bifidobacterium* spp. was isolated from only feces of humans and swine (Resnick and Levin, 1981). They were also frequently detected mainly from raw sewage and septic tanks (human feces), even though there was no significant difference in the concentrations of fecal coliforms, enterococci, or clostridia between human and animal fecal samples (Bonjoch *et al.* 2004; Resnick and Levin, 1981). Therefore, unlike *Bifidobacterium* spp.,

none of these conventional parameters can discriminate the origin of fecal pollution. This conclusion has been also confirmed for tropical freshwaters (Carrillo *et al.* 1985). PCR, multiplex PCR and real-time PCR approaches with strain-specific primers have been recently developed and applied for detection and quantification of host-associated *Bifidobacterium* spp. (Bonjoch *et al.,* 2004; Matsuki *et al*., 2004; Nebra *et al*., 2003). Relatively short survival time is presently a problem in terms of recovery of these microorganisms from water body. High background levels of predators and Gram-positive rods and cocci could prevent growth and/or detection of *Bifidobacterium* spp. in the aquatic environment (Rhodes and Kator, 1999).

1.6.5 Clostridium perfringens

C. perfringens has been successfully used as fecal indicator for sewage-contaminated streams, ocean environments (Hurst et al., 2002) and sea water (Roll and Fujioka, 1997). As the majority of clostridia population forms spore, they are extremely resistant to the environmental stress and persist for longer time than other indicator bacteria (e.g., fecal coliforms and fecal streptococci) and most of pathogens do (Davies *et al.,* 1995; Horman *et al.,* 2004; Medema *et al*., 1997). Temperature and predators did not significantly affect the survival rates of this microorganism. Therefore, spores of *C. perfringens* represent one of the most conservative indicators of fecal pollution. Spore-forming bacteria are especially useful to determine the ultimate fate of sewage or storm water released into water body. *C. perfringens* may be ideal microorganisms to evaluate the completeness of disinfection in drinking water treatment processes (Payment and Franco, 1993; Payment *et al*., 2000). A criticism of proposed *C. perfringens* usage as an indicator of pollution is that they have extended viability and wide distributions in aquatic sediments. Spores of *C. perfringens* can be detected even in long distance from contamination sites, indicating remote or old fecal pollution (Desmarais *et al*., 2002; Sorensen et al., 1989). Additionally, their concentrations vary among different animal species. Feces of cattle, horse and sheep contain less *C. perfringens* than human feces do (Sorensen *et al*., 1989). Similar to many alternative fecal indicators, *C. perfringens* standards have not yet been evaluated based on epidemiological studies on the acceptable risk associated with fecal pollution.

1.7. Bacterial Pathogens in Drinking Water

Most bacterial pathogens potentially transmitted by water as indicated in table 1. These bacteria communicate diseases and are discharged in the excreta of infected humans and other animals. Some waterborne bacterial pathogens, such as *Legionella and* atypical *Mycobacteria* that can

grow in water and soil reach the host by inhalation or direct contact where they can cause infections of respiratory tract, skin or brain tissues (WHO 2006).

Table 1

Examples of Bacterial Pathogens Considered in the WHO Guidelines for Drinking Water

Pathogen	Health significance	Persistence in water supplies	Resistance to chlorine	Relative infective dose	Important animal reservoir
Bacteria					
C. jejuni	High	Moderate	Low	Moderate	Yes
Pathogenic E coli	High	Moderate	Low	High	Yes
S. typhi	High	Moderate	Low	High	No
Salmonella sp.	High	Short	Low	High	No
Shigella sp.	High	Short	Low	Moderate	No
V. cholerae	High	Short	Low	High	No
Y. enertocolitica	High	Long	Low	High (?)	Yes
P. aeuginosa	Moderate	May multiply	Moderate	High (?)	No
Aeromnas species	Moderate	May multiply	Low	High (?)	No
H. pylori	?	?	?	?	?

Source: WHO seminars pack for drinking water Quality 2004

1.7.1 *Salmonella*

Salmonella, a very large group of rod shaped Gram negative bacteria comprising more than 2000 known serotypes that are members of family *Enterobacteriaceae*. These serotypes are virulent to humans and can cause a variety of symptoms from mild gastroenteritis to severe disease or death (Maier *et al.* 2000). There are substantial territorial changes in the occurrence of the serogroups (Leminor 1981). The majority of *Salmonella* are of enteric origin of animals particularly of pigs, cows, goats, sheep, rodents, hens, ducks and other poultry. Although *S typhi* and *S. paratyphi* are usually restricted to humans (Monica 1984). The pathogenicity of *Salmonella* species varies in terms of serotype, number of organism and on host status. *S. typhi* is a typical human pathogen and infective dose for *Salmonella* in humans is 1×10^5 organisms (Levinson& Jaw 2000). *S. typhi*, *S. paratyphi* A, and *S. paratyphi* B, cause septicemia with high temperature without diarrhea, a condition known as enteric fever. Many serogroups can cause a transitory enteric infection like gastroenteritis with diarrhea in susceptible individuals. However certain serotypes can cause asymptomatic infection (Maier *et al.* 2000; Lloyd 1983).

1.7.1.1. Significance in Drinking Water

Surface water getting household sewage discharge, meat processing wastes or stockyard wastes are likely to be contaminated with *Salmonella*. By ingestion of food or water contaminated with *Salmonella* the infection may develop within 12-36 hrs (Maier *et al.* 2000). Waterborne epidemics are primarily linked with *S. typhi*. The *S. paratyphi* B or other *Salmonella* are much less linked (LIoyd 1983). The humans and animal that are infected with *Salmonellae* can be the major source of transmission of infection through contaminated water. Due to fecal pollution of water, waterborne diseases can occur more frequently by *Salmonella spp*: The ecological sharing of major diseases is often strongly related with the design of waterworks pipeline networks. *Salmonellae* can be transmitted in open wells through drainage or flooding of polluted surface water into insecure well shafts. *Salmonellae* are rarely detected from piped water supplies, whether disinfected or untreated. However; the presence of this organism indicates the major fault in design or management of the system (Chiu *et al.* 2006; LIoyd 1983). Turbidity should be low in drinking water if proper chlorination is required. The low concentration assimilable organic carbon (AOC) in drinking-water can inhibit the growth of *Salmonella* in water. Numerous outbreaks are caused by contaminated sediments in the distribution system (WHO 1996). The crop grown in water which is fecally contaminated may harbor *Salmonella*. In the same way livestock and poultry infected with *Salmonella* can cause salmonellosis. In fertilizers *Salmonella* could be found in large numbers if they are prepared from animal products. All the above sources of food can disseminate *Salmonella* and can cause mild to severe infection. In developing countries the practice of dumping of slaughterhouse waste in water bodies is a key source of salmonella infection (Kamplemacher 1977; Mersch 1987).

1.7.1.2. Disease Caused by Salmonella:

In the last 20 years *Salmonella* infections in human have increased significantly in the developed world (Kapperud *et al.* 1995). In United States, about 2-4 million cases of salmonellosis (diarrhea) occur each year (Maier *et al*., 2000), 70% of these are occurring during travelling. Enteric fever is widespread in poor countries affecting around 12.5 million persons annually (CDC 2005). In 1997, the WHO reported an estimated 17 million cases with 0.6 million deaths occurring each year. During 1977-1986, Chile experienced a significant typhoid fever epidemic; the causes were utilization of vegetables irrigated with contaminated wastewater (Fica *et. al.* 1996, Cabello and Springer, MMWR 1998). In Karachi (Pakistan) during 1994, 100 cases of typhoid fever linked with contaminated drinking water were reported (Luby *et al.* 1998).

1.7.2. *Escherichia coli*

The human and warm-blooded animals contain *E. coli* in their excreta.. This organism is a Gram-negative rod, aerobic or facultative anaerobic, cannot produce spores, is motile by peritrichous flagella and ferments lactose and glucose with production of acid and gas. All strains are catalase positive, nitrate positive and oxidase negative (Monica 1984; Jawetz 1998; Bailey and Scott 1990; Prescott *et al.* 1999). There are six classes of pathogenic *E. coli* 1. Enteropathogenic (EPEC) 2. Enteroinvasive (EIEC) 3. Enterotoxigenic (ETEC) 4. Enterohemorrhagic verocytotoxin-producing *E. coli* (EHEC) 5. Enteroaggregtive (EaggEC) and 6. Diffusively adherent *E. coli* (DAEC). the members of the first four classes are implicated to cause waterborne diseases (FDA 2002).

1.7.2.1. Significance in Drinking Water

Coliform bacteria including *E. coli* occur in a variety of environmental sources. The plants and water slimes may be a colonizing habitat for *E. coli.* A pathogenic *E. coli* may enter into the water body by dumping of sewage that can cause infection. However the presence of thermo-tolerant *E. coli* referred to as fecal coliforms is a indication of water pollution either by human or animal sources (WHO 2000).

1.7.2.3. Diseases Caused by E. coli

Enterohemorrhagic *E. coli* O157:H7 has emerged as a new threat to public health. Over 20,000 cases of infection and around 250 deaths annually are reported in the United States (Koutkia *et al.* 1997). In 2000 seven people were killed and more than one thousand were affected in Walkerton Ontario. Hundreds of children were affected by *E. coli* and two died in Atlanta in 1998 (CDC 2002; Hrudy *et al.* 2003). 2088 cases of diarrhea were reported in New Caledonia in 1990 and 1991. Enteric pathogens i.e. *Salmonella, Shigella, Escherichia coli* were isolated from diarrheal patients. The contaminated public water supply resulted in an outbreak of *Escherichia coli* O157 and Campylobacteriosis in a Glenrothes, fife village where 711 cases of gastroenteritis were reported (Germani *et al.* 1994; Jones and Roworth 1996; Weintraub 2007). The diarrheal disease by pathogenic *E. coli* is the main cause of morbidity and death amongst children in third world countries. In Kenya 239 patients were reported with bacterial diarrhea. Diarrhoeagenic *Escherichia coli* including; enteropathogenic, enterotoxigenic and enterohemorrhagic strain were isolated from 119 patients, 63 cases of *Salmonella* spp. and 56 cases of *Shigella* spp were

reported. The main source of infection was consumption of polluted drinking water (Saidi *et al.* 1997). Another outbreak in 1993-1994 was reported in Kenya. The diarrhoeagenic agents were found from 767 of 1362 patients. The main causative agents were diarrhoeagenic *Escherichia coli* (DgEC), Rotavirus, *Shigella spp., Salmonella spp*, *Entamoeba histolytica*, *Giardia lamblia*. It was reported that the disease was caused by consuming fecally contaminated water (Aihara 1997; Yatsuyanagi *et al.* 1996). In Bangladesh, Qadri *et al.* (2000) isolated enterotoxigenic (ETEC) *E. coli* from the patients with diarrhoeal disease.

The detection of *E coli* O157:H7 in environmental samples is a major public health concern, since presence of pathogenic strains of this organism in tainted meats, poultry, and still in drinking water, can pose serious health threats (Aljaro *et al.* and William *et. al.* 2005). Gastrointestinal infection resembles cholera, and gastroenteritis caused by *Salmonellae* could be caused by *E. coli* (Feachem 1982; James *et al.* 2005). Outbreaks of gastroenteritis have been attributed to Enteropathogenic subtypes of *E. coli* strains. Dysentery- syndrome has been reported due to infection by Enteroinvasive strains of *E. coli* (EIEC) (Scotland 1989). Since *Shigella* and *E. coli* are closely related, their pathogenicity similar and Enteropathogenic or Enteroinvasive strains can cause severe infection. ETEC produces enterotoxin and is capable of colonizing the small intestine. Other factors include adhesion which helps the bacteria for attachment and colonization. Enterohemorrhagic *Escherichia coli* are the fourth class also called verocytotoxic *E. coli*, and include O157:H7. Production of a cytotoxin (verotoxins) is a feature that has an effect on Vero (monkey) cells (Koutkia 1997). These toxins are also called as Shiga-like toxin because they are very similar to those produced by *Shigella* species (Warren and Jawetz 2000; Faith *et. al.* 1996; Bouvet *et al.* and, Kumar *et al.* 1999). Symptoms include mild diarrhea that may lead to hemorrhagic colitis, abdominal pain but no fever. In infants and young children, hemolytic uraemic syndrome is common and can cause anemia. Enteroaggregative *E. coli* (EAggEC) are a group of *E. coli* characterized by the ability to adhere to cultured cell monolayer with an aggregative or stacked brick adhesion phenotype. Epidemiological studies suggest that the strains of EAggEC are a major cause of protracted diarrhea in children and may cause diarrhea in adults (Law and Chart 1998; Qadri *et al.* 2000; Duque *et al.* 2002).

1.7.3. *Vibrio cholerae*

Vibrio species are motile, possessing monotrichous flagellum, non-sporing, Gram negative, comma shaped morphology and are the members of family *Vibrionaceae.* The members of this

family are aerobes and facultative anaerobes. They are catalase and oxidase positive and nitrates positive. From the medical point of view, special consideration has been given to the uniqueness of those strains which are linked to cholera. Previously, *Vibrio cholerae* were classified on the basis of O1 antigens (WHO 1996), but later the Vibrio was divided into two i.e. 1. "classical" and 2. "El Tor" biotypes. The El Tor produces a heat-sensitive haemolysin, active against sheep and goat RBCs (Attridge *et. al.* 1990). The O1 antigen lacking *Vibrio* are known as non-cholerae vibrios (NCV) or non-agglutinable (NAG) *Vibrios* (WHO 1996). This subtype is now designated as "non-O1 *V. cholerae*". Study of DNA/DNA homology between *Vibrio cholerae*, NCVs, and NAG vibrios established a strong relationship (WHO 1996). *Vibrio cholerae* possesses around 80 serological types. *V. cholerae* serogroup O1 is mainly responsible for cholera. However, O 139 also causes cholera. The Ogawa (factors A and B) and Inaba (factors A and C) are serotypes of O1 group (WHO 1996).

1.6.3.1 Significance in Drinking Water

Surface water is natural habitat for *V. cholerae* where it reproduces and lives in relationship with other organisms. Cholera as a disease can expand either by fecal pollution of food and drinking water by independent proliferation of the sequence of *V. cholerae.* There is growing proof that Ecology plays a key role in regulation of its virulence genes. *Vibrio cholerae* become a pathogen by horizontal gene transfer due to environmental stress. Ecological factors such as light, saline content and heat influence propagation of bacteriophage which harbors the cholera toxin genes (Shears 2001, Reidle and Klose 2002). Therefore the isolation of *V. cholerae* O1 from drinking water has an alarming significance (WHO 2002). It is a well established fact that cholera is a waterborne disease (WHO 2000), however food borne nosocomial infection and person to person transfer is also reported. Miller *et al.* (1985) and Attridge *et al.* (1990) found that in several ecological conditions *V. cholerae* as well as *V. cholerae* serotype O1 is part of the indigenous flora of waters.

1.7.3.2. Diseases Caused by Vibrio spp:

Infections due to *V. cholerae* occur by the ingestion of contaminated water or food. The organism colonizing small intestine by producing a toxin co regulated pilus (Taylor *et al.* 1987). *Vibrio cholerae* serogroups O1 causes disease by producing an enterotoxin that causes a sudden onset of profuse watery diarrhea of "rice water" stools. Non-O1 *Vibrios* can cause bloody watery diarrhea. This organism can also cause wound infections and bacteremia (Neill 2005; Seas

2005). Cholera has re-emerged as potential infectious diseases in the recent past with a worldwide increase in its occurrence. In 1991 after a hundred years absence the re-appearance of cholera epidemic caused by *V. cholerae* O1 El Tor in Latin America staggered the health authorities (Tauxe and Blake 1992). Another unprecedented incident of cholera occurred by *V. cholerae* non-O1 known as *V. cholerae* O139 Bengal in 1992 in India and spread rapidly in several south Asian countries (Ramamurthy *et al*. 1993). The occurrence of cholera was reported in ninety-four countries in 1994, which was the largest ever number of countries in a single year (WHO 1995). In 2004, cholera epidemics killed 297 persons and 14000 other became ill in various African countries. In Chad during 2003-2004 the Ministry of Health reported 131 cholera cases and 11 deaths (case fatality rate 8%). The outbreak was started through consumption of contaminated water from Logone River which was believed to be contaminated with fecal material from human sources (WHO 2004). These incidences witness that old enemies like cholera are still a potential threat to the public health worldwide particularly in developing countries (WHO 2004).

1.7.4. *Shigella spp.*

The members of genus *Shigella* are non-motile, non-spore-forming rods. The Gram reaction is negative and they are capable of growing in aerobic and anaerobic conditions. Biochemically these bacteria ferment sugars with production of acid but no gas, lactose fermentation is very rare and, excluding *Sgigella dysetriae* serotype 1, all members are catalase and nitrate positive, oxidase test is negative in all members except *Shigella dysenteriae* type 1. O antigen is the basis of serogroups among Shigellae (WHO 1996). In certain reports it is mentioned that among *Shigella flexneri* the lysogenic bacteriophage is the source of antigenic specificity determination. Serological typing has a reliable application for all species excluding *Shigella sonnei* (Monica 1984; Levinson & Jawetz 2000).

1.7.4.1. Significance in Drinking Water

This organism is usually found in water contaminated with human feces (Saha *et al.* 2009). The detection of *Shigellae* from drinking water suggests current human faecal pollution. Due to extreme pathogenicity, this organism is of essential public health importance. Outbreaks of bacillary dysentery have been linked to water supplies when disinfection of water supply has not been instituted or has become faulty. Such water-borne outbreaks are frequently spectacular in

the large number of people concurrently infected and in the speed with which they can be ended when the water supply is sufficiently treated (Lewis 1992).

1.7.4.2. Disease Caused by Shigella sp

Approximately 10% to 20% of diarrhea cases in 0.3 million people in the United States annually are caused by *Shigella* spp. Thirty nine *Shigella* species transmit a disease to an expected 200 million people and causes 0.65 million deaths worldwide annually, mostly in the developing world (Guerrant and Lima 2000). The majority of children below ten years age are most susceptible for *Shigella* infection. The shigellosis may develop after 24-72 hr of ingestion of food or water contaminated with this bacteria (WHO 2000).The infective dose is as few as 10-100 cells linked to the age and condition of host. Outbreaks of bloody diarrhoea due to Sd1 are most common in overcrowded, impoverished areas with poor sanitation, inadequate hygiene practices, and unsafe water supplies. Refugees and internally displaced persons are at especially high risk. In the past two decades major outbreaks have occurred in Africa, South Asia and Central America. Between 1993 and 1995, outbreaks were reported in several central and southern African countries. In 1994, an explosive outbreak among Rwandan refugees in Zaïre caused approximately 20,000 deaths during the first month alone. Between 1999 and 2003, outbreaks were reported in Sierra Leone, Liberia, Guinea, Senegal, Angola, the Central African Republic and the Democratic Republic of Congo. In 2000, outbreaks of bloody diarrhoea due to Sd1 that were resistant to the fluoroqunoline occurred in India and Bangladesh (WHO 2005). In 2001, sixty nine people, several of them children, became ill with this enteric bacterium transmitted through an improperly disinfected pool in Atlanta, GA (ASM 2002).These bacteria secrete shiga toxin with enterotoxic, cytotoxic and neurotoxic properties and attack the colonic epithelium which results in a powerful but superficial destructive process. Bacteremia and spreading are exceptional; signs and symptoms include bloody diarrhea, high fever, malaise, headache and abdominal pain (Guerrant 2000, Dupont 2005). *Shigella* species are present in counts of 10^3 to 10^9 per gram of stool initially and then drop off rapidly in the post-convalescent period. Exudative colonic hemorrhagic diarrhea is the hallmark of the illness. Stool culture on xylose-lysine-desoxycholate agar identifies the pathogen. Antibiotics can shorten the course of illness (Dupont 2005, Gore 2003).

1.7.5. *Pseudomonas aeruginosa*

P. aeruginosa belongs to the family *Pseudomonadaceae*. It is a Gram negative rod, motile with monotrichous flagella. The organism ferments sugar by oxidation. *Pseudomonas aeruginosa* is oxidase, catalase and citrate positive. It is also capable of growing on higher temperatures ranges from 41-42^{O}C. Blue-green fluorescent color (pyocyanin) is the mark of identification for *P. aeuginosa* (Gillespie 1994).

1.7.5.1. Significance in Drinking Water

Pseudomonas are able to grow in water containing only traces of nutrient, e.g. tap water, and this favors their persistence in the hospital environment (Levinson &Jawetz 2000). Because of the ability of *P. aeruginosa* to live in both inanimate and human environments it has been characterized as a ubiquitous microorganism. In inanimate environments *P. aeruginosa* is frequently detected in water reservoirs polluted by animal and human waste such as sewage and sinks inside the hospital. It is also found in faeces, soil, water (swimming pools and whirlpools), sewage, plants, animals and humans (Botzenhardt and Doring 1993). *P. aeruginosa* grows preferably in a moist environment. In waters enriched with organic material, *P. aeruginosa* grows and its presence indicates the quality regarding cleanliness of the water distribution system and the quality of bottled waters (Pollack 1995). The presence of *P. aeruginosa* contributes to the alteration in odor, color, turbidity and taste of the drinking water (WHO 2003). Most precisely the infections caused by *P. aeruginosa* are water related. Contaminated water when come into contact with human, food, drinks and pharmaceutical products can be the source of infection. *P. aeruginosa* colonizes sinks, tap fittings, drains and shower heads, especially in health care settings (Pollack 1995).

1.7.5.2. Diseases Caused by P. aeruginosa

In the second half of the last century, *P. aeruginosa* has become an important hospital pathogen. This microorganism is prevalent in the hospital environment. Disease onset increases among indoor patients proportionally with duration of hospitalization (Chastre and Trouillet 2000). That is why it is a major nosocomial microorganism obtained from clinical research material and causing hospital-acquired infections (HAI). According to the data of Center for Disease Control (USA), *P. aeruginosa* is the fifth most common pathogen among hospital microorganisms and causes 10% of all hospital-acquired infection (HAI). The illnesses caused by *P. aeruginosa* include rashes, pustules and ear infections (Otitis externa) especially in swimmers using whirl

pools with insufficient disinfection **(**Jones *et al.* 1985, Caldern and Mood 1982). In water supplied to hospital and in the manufacture of pharmaceutical preparations, *Pseudomonas aeruginosa* gets access to surgical wounds and it can contaminate eye drops from where it can cause serious infection (Parker 1984).

1.7.6. *Helicobacter pylori*

The causative agent of gastritis and peptic ulcer, *H. pylori* exhibits spiral or curved rod-shape morphology. It is a Gram negative, motile bacterium. This human pathogen may be found on the gastric mucosa of man (Warren and Marshall 1983) and spread through the gastrointestinal tract (Wisniewska *et al.* 2002). The transmission may be facilitated by saliva or gastric fluids between the members of a population (Leung et al 1999; Hulten *et al.*1996 Mazri-Hiriart *et al.* 2001). However epidemiological data show the two routes 1.Oral-oral and 2. Fecal-oral of transmission (Brenner *et al.* 2001; Lu *et al.* 2002; Gramley *et al.* 1999). Presently, the waterborne transmission is a key objective of research (Engstrand 2001)

1.7.6.1. Significance in Drinking Water

H. pylori is able to survive in tap water for many days in its pathogenic cylindrical form (Hulten *et. al.*1996). Vincent (1995) and Shahamat *et al.* (1993) reported that *H. pylori* can survive in river water for several months. The transmission through water has also been reported by numerous researchers who have conducted studies in several countries in which the water sources related risk from *H. pylori* has been concluded (Zhang *et al.* 1996; Goodman *et. al.* 1996; Klein *et al.*1991). The source of drinking water had a strong effect on the prevalence of *H. pylori* infection. The prevalence of infection among river water drinkers was 97% compared with 71% in those who drank tap water. Control measures including the prevention of fecal contamination of drinking water from human and animal sources and proper disinfection are necessary to protect drinking water supplies from this virulent microorganism. However, for the determination of presence/absence of this organism the *E. coli* is no reliable index (Queralt *et al.* 2005; Nurgalieva *et al.* 2002)**.**

1.7.6.2 Diseases Caused by H. pylori

H. pylori associated with peptic and duodenal ulcer and stomach cancer may be transmitted via contaminated drinking water or crop washed or irrigated with contaminated water. Diagnostic surveys suggest that this organism causes infection in the majority of the world's population (CDC, 1998). The organism has been detected in biofilms in distribution pipes even disinfected

with chlorine, and is disseminated to the consumers. This results in gastritis and if not treated can lead to peptic ulcer and ulcer of duodenum. In some cases, especially in immunocompromised hosts, complications may lead to gastric cancer. As described previously for other waterborne pathogens, the presence of *H. pylori* increases in cases of overcrowding and poor living conditions (Park *et al.* 2001; Queralt *et al.* 2005).

1.7.7 *Aeromonas.* *Aeromonas* are Gram-negative, non-spore-forming, rod-shaped, facultative anaerobic bacilli belonging to the family Aeromonadaceae. Although *A. hydrophila* is the focus of this section, other aeromonads, such as *A. caviae* and *A. sobria*, have also been isolated from human faeces and from water sources (Havelaar *et al.,* 1992; Janda and Abbott, 1998; Villari *et al.,* 2003). Morphologically, aeromonads are indistinguishable from members of the Enterobacteriaceae family, such as *E. coli*. They also share many biochemical characteristics, with the differentiation being that aeromonads are oxidase positive and Enterobacteriaceae are oxidase negative.

1.7.7.1 Significance in Drinking Water

A. hydrophila is a wide spread representative of *Aeromonas* found in water, water habitants, doemestic animals and foods (fishes shellfish poultry and raw meat). The microorganism has the potential to food and waterborne pathogens especially strains from hyberdization group(HG1) associated with clinical cases of illness (Daskalov 2006). Species, mainly *A. caviae*, *A. hydrophila* and *A. veronii* subspecies *sobria*, have been isolated from patients (particularly infants) with diarrhoea (Sow *et al*. 1977; Chatterjee *et al*. 1989; Krovacek *et al*. 1989; Ashiru *et al*. 1993), There is no doubt that *Aeromonas* can frequently be detected in a variety of waters, and several investigators have described its detection in potable water (LeChevallier *et al*. 1980, 1982; Clark *et al.* 1982; Millership and Chattopadhyay 1985; Havelaar *et al*. 1990) at levels of up to 1900 cfu/ml. However, even with modern tools for discrimination between bacterial isolates, it is difficult to determine if the types of organisms present in drinking-water are the same as those found in patients with diarrhoea. *Aeromonas* is also frequently found in foods, and many studies have been carried out to determine its incidence. In all published studies, *Aeromonas* was found in a variety of foodstuffs with isolation rates of up to 84% and concentrations of up to 105/g, with ready-to-eat foods being frequently contaminated (Fricker and Tompsett 1989; Palumbo *et al*. 1989; Knochel and Jeppesen 1990; Hanninen 1993).

1.7.7.2 Diseases caused by Aermonas

The common routes of infection suggested for *Aeromonas* are the ingestion of contaminated water or food or contact of the organism with a break in the skin (Schubert, 1991). In recent years, *A. hydrophila* has gained public health recognition as an opportunistic pathogen. It has been implicated as a potential agent of gastroenteritis, septicaemia, cellulitis, colitis, and meningitis, and is frequently isolated from wound infections sustained in aquatic environments (Krovacek *et al.*, 1992; Gavriel *et al.*, 1998). It has also recently been implicated in respiratory infections (Janda and Abbott, 1998). Individuals at the greatest risk of infection are children, the elderly, and the immunocompromised (Merino *et al.*, 1995). For *A. hydrophila*, the virulence of the organism is, at least in part, thought to result from the production of specific enterotoxins (Schubert, 1991). The primary toxins are haemolysins(Janda, 1991). In addition, some aeromonads produce a range of cell surface and secreted proteases that may enhance their virulence (Janda, 1991; Gosling, 1996). It has been demonstrated that a significant proportion of the *A. hydrophila* isolated from water (chlorinated and unchlorinated supplies) contained genes responsible for enterotoxigenic or cytotoxic activity (Ormen and Ostensvik, 2001). Expression of virulence factors has been shown to be influenced by environmental temperature. *A. hydrophila* isolated from the environment produced significantly less enterotoxins when grown at 37°C compared with 28°C, whereas the clinical isolates tested produced more enterotoxins at 37°C than at 28°C (Mateos *et al.*, 1993). The temperature of the human body is approximately 37°C; therefore, strains that produce virulence factors at this temperature are likely to be more important as pathogens.

1.7.8 Yersinia

The genus *Yersinia* is classified in the family Enterobacteriaceae. They are heterotrophic bacteria with 11 recognized species, some of which cause disease in humans, and both pathogenic and non-pathogenic strains of *Yersinia* have been found in surface water and un-chlorinated drinking water (Lassen 1972; Caprioli *et al*. 1978; Cafferkey *et al*. 1993). The source of the organism is the environment or non-human hosts, such as wild animals and birds. The species *Y. pestis, Y. pseudotuberclosis* and certain serotypes of *Y. enterocolitica* are pathogens for humans. *Yersinia pestis* is the cause of bubonic plague through contact with rodents and their flies. *Yersinia* spp. Are Gram-negative rods that are motile at 25^{o}C and not at 37^{o}C. *Yersinia* genus of

1.7.8.1 Significance in drinking water

Although most *Yersinia* sp. detected in water are probably non-pathogenic, circumstantial evidence has presented to support transmission of *Y. entercolitica* and *Y. pseudotuberclosis* to humans from untreated drinking water. The most likely source of pathogenic *Yersinia* spp. is human or animal waste. The organisms are sensitive to disinfection processes. Within a water safety plan (WSP), control measures that can be used to minimize the presence of pathogenic *Yersinia* spp. in drinking-water supplies including protection of raw water supplies from human and animal waste, adequate disinfection and protection of water during distribution. Owing to the long survival and/or growth of some strains of *Yersinia* spp. in water, *E. coli* (or, alternatively, thermotolerant coliforms) is not a suitable index for the presence/absence of these organisms in drinking-water (WHO 2000).

1.7.8.2 Diseases caused by Yersinia

Yersinia enterocolitica penetrates cells of the intestinal mucosa, causing ulcerations of the terminl ilium. Yersiniosis genrally presents as an acute gastroenteritis with diarrhoea, fever and abdominal pain. Other clinical manifestation includes greatly enlarged painful lymph nodes referred to as "buboes." The disease seems to be more acute in children than in adults (WHO 1997). *Yersinia enterocolitica* that occur in the environment are considered to be pathogenic for humans. This depends on the possession of virulence factors associated with pathogenesis of infection. Serotypes O:3, O:4,32, O:5,27, O:6,30, O:6,31, O:8, O:9 and O:21 are thought to be pathogenic for humans and cause diarrhoea or mesenteric adenitis, a disease that often mimics appendicitis. Other serotypes have been isolated from patients with infection, but their role is uncertain. The most common serotype of *Yersinia enterocolitica* associated with human infection is serotype O:3. (WHO 2000)

1.7.9 ***Campylobacter.*** Campylobacter are mainly spiral-shaped, S-shaped or curved rod-shaped bacteria. There are 16 species and six subspecies assigned to the genus *Campylobacter*, of which the most frequently reported in human disease are *C. jejuni* (subspecies jejuni) and *C. coli. C. laridis and C. upsaliensis* are also regarded as primary pathogens, but are generally reported far less frequently in cases of human disease. Most species prefer a micro-aerobic (containing 3-10% oxygen) atmosphere for growth. A few species tend to favour an anaerobic environment, although they will grow under micro-aerobic conditions also (WHO 2000)

1.7.9.1 Significance in drinking water

In addition to food there are also several environmental *Campylobacter* sources including water, for drinking and recreation, and contact with food production animals and pets, and exposure to soil. How significant the different risk factors for *Campylobacter* infections are in different countries is hard to evaluate. Surface drinking water supplies seems to be a risk factor for high *Campylobacter* incidence. Disinfection of drinking water at the water works is, as mentioned earlier not always sufficient, and improvement of the water pipelines or the control of pressure drops are interventions that should be implemented. Modern equipment for control of pressure drops is available on the market (LeChevallier *et al*. 2003).

1.7.9.2 Disease caused by Campylobacter

Campylobacteriosis is the disease caused by the presence of campylobacters. The onset of disease symptoms usually occurs two to five days after infection, but can range from one to ten days. The most common clinical symptoms of campylobacter infections include diarrhoea (frequently with blood in the faeces), abdominal pain, fever, headache, nausea, and/or vomiting the symptoms typically last three to six days (WHO 2000).

Campylobacter are the most common cause of human bacterial gastroenteritis in the United Kingdom, with *Campylobacter jejuni* being the predominantly isolated species. They are widespread in the environment and occur very commonly in the intestinal tracts of animals, including birds. *Campylobacter* can easily be isolated from surface waters, and a number of outbreaks in the United Kingdom have been associated with private water supplies. An outbreak of gastroenteritis associated with contamination of a public water supply occurred in Wales in September 2000. Two hundred and eighty-one people out of a population of 1215 served by the supply developed gastroenteritis following an incident of influx of surface water into a holding tank for treated water. Godoy *et al.* (2002) reported the overall attack rate by *C. jejuni* 18.3% transmitted through untreated drinking water.

1.8. Emergence of New Pathogens

Health authorities, with the passage of time, face the problems of new emerging pathogens. These pathogens overcome the conventional procedures used for water purification and distribution. It has been reported that previously unknown microorganisms in water were found responsible for outbreaks of waterborne diseases (US DHHS 1998; WHO, 1998 CDR 1998)**.** *Caliciviruses, E coli O157:H7, Helicobacter sp. Mycobacterium avium complex,* (MAC), are the candidates for new emerging pathogens (EPA 2002). This problem needs regular attention as to

what may pose a "new threat" and also by regular monitoring and development of detection techniques to control the newly emerging pathogens noted by LeChevallier *et al.* (1999), thereby emphasizes that "knowledge is the first line of defense towards safe drinking water."

1.9. Environmental Transmission of Infectious Diseases

Infectious diseases are contagious. There are various routes of exposure. Feces containing pathogens join conventional cycle end up in sewage. Although the levels of microorganisms are reduced through sewage treatment procedures by 90-99.9%, sewage still contains the substantial quantities of the pathogens. In distribution waters, the pathogenic bacteria reach humans using such waters for domestic and recreational purposes. Ideally, municipal water treatment should safeguard against transmission of disease in the population. Sewage sludge can also transfer pathogens, when used in agriculture as a fertilizer. Pathogens could also transmit through animals to humans as zoonoses (Wahlström *et al.* 2003; Hutchison *et. al.* 2004). Pathogens in water could enter by snow melting to water courses with slurry manure or sludge (Hansen and Ongerth, 1991; Kistemann *et al.* 2002; Ferguson *et al*. 2003). Sewer overflows (SO) causes wastewater combined with water, sometimes untreated or partially-treated to reach the consumer. Microbial pollution to watercourses by leaking septic tanks, waterfowl etc is common. Environmental transmission can clearly cause infection and diseases. It is evident that this cycle in the environment goes on (Wahlström *et al.* 2003; Hutchison *et al.* 2004)

1.10. Effect of Temperature on Drinking Water Quality

Environmental factors usually affect living organisms, including pathogens. The growth, occurrence and potential of causing infection may also be influenced by chemical and physical factors. The temperature can influence the occurrence ad transmission of a pathogen associated with phytoplankton. As high temperatures cause evaporation which results in increased humidity that helps pathogens to grow better than in a dry climates. Many diseases common to tropical climate are waterborne e.g. cholera (Brinkman 1994). The growth of coliforms can also be increased in water with elevated temperature (WHO 2004). With sufficient nutrient contained in water and with increased temperature, the growth of microorganisms can be more rapid than in water at lower temperature (Donlan and Pipes, 1988; LeChivallier *et al.* 1996). Thus elevated temperature can also support the prevalence of opportunistic pathogens in biofilms. In tropical and subtropical areas, the warm temperature in combination with organic matter in water

provides favorable conditions similar that in the gut of man and other animals. In such water, *E. coli* grows rapidly (Solo-Gabriele *et al.* 2000; Jimenez *et al.* 1989)

1.11. Effect of pH on Drinking Water Quality

The measure of acid-base equilibrium is known as pH of a substance. In natural waters this balance of acid and base is maintained through carbon dioxide- bicarbonate-carbonate equilibrium phenomenon (WHO 2007). The pH of water decreases when carbon dioxide concentration increases; where as decreased carbon dioxide will cause pH to rise (WHO 2007). The pH of most raw water ranges between 6.5-8.5. Extreme pH can cause irritation to skin, eyes and mucous membranes. Water with pH values 10 & 12 has reported to cause hair fibers to swell. Irritation of gastrointestinal tract has been reported in some individuals. The pH of water should preferably be less than 8 for proper chlorination. If the pH of water before water distribution in pipes is not maintained, it can result in corrosion of pipes and contamination of drinking water and cause bad taste, odor and color (Haleem 1996)

1.12. Importance of Chlorine Disinfection of Drinking Water

Chlorine was first accepted as an important disinfectant for water treatment when John Snow used it to disinfect water causing cholera. After that, the use of chlorine as a chemical disinfectant was adopted on a large scale in the UK for the treatment of public water, which rapidly decreased the number of death from typhoid (Christman 1998). Chlorine disinfection treatment of drinking water is believed be extending the average life of humans from 45 year in early 1990s to 76 years today (CCC 2005). Approximately 98% of municipal water disinfection facilities now use chlorine as disinfectant of choice. In U.S. around 200 million people use chlorinated drinking water (Rutala and Weber 1997, Christman 1998). The killing effect of chlorine has been evaluated through research on bacteria in drinking water and chlorine dioxide (ClO_2) is recommended as good disinfection for water treatment (Junli *et al.* 1997). Rutala and Weber 1997 reported that *H. pylori* can enter into distribution system through a break in disinfection or accumulation into the system itself, it can survive within distribution system where the oxidizing disinfectant is not used in the required concentration (Katherine *et al.* 2002). To control waterborne disease epidemics, chlorine is an essential chemical disinfectant. Although a health risk from chlorine by-products exists, the risk from microbial pathogens cannot be compromised by omitting the chlorine disinfection of drinking water (Galal-Gorchev 1996).

1.12.1 Application of chlorine

Chlorine may be applied in any one of the following form

1.12.1.1 Chlorine

Chlorine may be applied in two ways: Gaseous form or Liquid form

In gaseous form chlorine gas is dissolved in water and a solution is formed. This solution is mixed with water according the dose ascertained in the laboratory test. In liquid form chlorine gas is converted to liquid by applying a pressure of 7 kg-11 kg/ cm^2 in a cylinder specially made for this purpose. The liquid chlorine is added to the water by device which is known as chlorinator. (Bassak 2003). The use of free chlorine is favored universally for the following advantages.

1. Chlorine is most powerful for destroying the bacteria absolutely. 2. The process of application is easy. 3. It can be stored easily. 4. It can be stored easily. 5. The optimum dose can be easily found by break point chlorine. (Bassak 2003)

1.12.1.2 Chloamines. The free chlorine is not stable in water (Bassak 2003). To make this stable, some amount of ammonia is mixed with water along with chlorine. As a result of chemical reaction some compound are formed which are known as chloramines. The following chloramines are formed:(i) Mono-chloramines. NH_2CL. It is effective for pH value above 7. (ii) Dichloramine ($NHCL_2$). It is effective for pH value between 5 and 7. (iii) Nitrogen trichloride (NCL_3). It is effective for pH value 4 (Bassak 2003). The ammonia may be mixed with water in gaseous or solution form. The following are the benefits of adding ammonia along with chlorine. It makes chlorine stable in water. It reduces the amount of chlorine necessary for treatment. It becomes more powerful in killing bacteria. It reduces the irritating effect of chlorine (Bassak 2003)

1.12.1.3 Chlorine dioxide. Sometimes chlorine dioxide (ClO_2) is used for removal of bacteria. It is produced by passing chlorine gas through sodium chlorite in a closed container. It is very unstable and should used very quickly. It can remove test and odor. It is not suitable for large scale, it may used for small instillation like housing estate, factories etc (Bassak 2003)

1.12.1.4 Bleaching powder. Bleaching powder is also known as calcium hypochlorite [Ca $(OCl)_2$] when it is mixed with water hypochlorite ions (OCl) are formed. These ion again

combine with hydrogen ions (H+) present in water thus hypochlorous acid is formed. This phenomenon is known as hypo-chlorination. The hypochlorous acid and hypochlorite ions are both responsible for the killing of bacteria. The bleaching powder is available in white powder which contains usually 35 percent chlorine. Before application the bleaching powder is dissolved in water and a solution is prepared. This solution is added to the water according predetermined dose. Generally the dose for bleaching powder is 2-4 ppm. Bleaching powder is not recommended for public water supply but it is suitable for disinfecting the water of swimming pools, ponds etc. (Bassak 2003).

In Chlorine is effective, affordable, and widely available for disinfection. Chlorine is used to destroy disease-causing organisms in water, an essential step in delivering safe drinking water and protecting public health. Chlorine is by far the most commonly used disinfectant in all regions of the world. Where widely adopted, chlorine has helped to virtually eliminate water-borne diseases such as cholera, typhoid and dysentery (WCC 2008). Chlorine also eliminates slime bacteria, molds and algae that commonly grow in water supply reservoirs, on the walls of water mains and in storage tanks (WCC 2008). Only chlorine-based disinfectants leave a beneficial "residual" level that remains in treated water, helping to protect it during distribution and storage. When chlorine is added to water, some of the chlorine reacts first with organic materials and metals in the water and is not available for disinfections (this is called *chlorine demand* of water). The remaining chlorine concentration after the chlorine demand is accounted for is called total chlorine. Total chlorine is further divided into: 1) the amount of chlorine that has reacted with nitrates and is unavailable for disinfection which is called *combined chlorine* and,2) the *free chlorine* which is the chlorine available to inactivate disease causing organisms and thus to measure to determine the potability of water. World Health Organization (WHO) reported, "Chlorine is most widely and easily used, and the most affordable of the drinking water disinfectants. It is also highly effective against nearly all waterborne pathogens (WCC 2008)

1.13. Water and Sanitation Problems in Pakistan.

Population Reference Bureau (2006) has reported that the population of Pakistan would rise to 228.8 million by the year 2025 and it would reach up to 295 million by the year 2050. By population the Pakistan is on sixth number in the world. Population rise has a direct relation to water usage; in Pakistan the quality of (ground and surface) water is unsatisfactory because of the deposition of untreated wastewater and unchecked usage of fertilizers and insecticides. It is

reported that around 38.5 million people have no safe drinking water available and about 50.7 million do not have access to sanitation in Pakistan (Khan 2007). Drinking water qual**ity in** Pakistan ranks as 80th of 122 Nations. Bacteriological contamination of drinking water has remained one of the major problems in the country in rural as well as urban areas (Abid & Jamil, 2005; Kahlown, Tahir, & Sheikh, 2004). Such contamination is due to leakage of pipes, pollution from sewerage, biofilm formation in the distribution system, intermittent water supply, and human activities. (Tahir1989; Tahir & Bhatti 1994; Sajjad & Rahim, 1998; Latif, Akram, & Altaf, 1999 and Chandio,1999). The excessive monsoon rains, floods, herbicides, fungicides, untreated municipal waste, and coastal water pollution due to waste discharges and oil spills are extremely hazardous. To protect the consumers health it is of crucial importance to establish drinking water quality standards and criteria that are chemically balanced and medically safe (Health Services Academy 2008). General public, in Pakistan, use subjective quality criteria like brackish, foul smelling, bad tasting, turbid or colored water to determine that it is not suitable for drinking. The agencies responsible for monitoring of water quality perform periodic checks of the basic water parameters against certain recommended standards. In order to ensure that consumers throughout the country are receiving quality water, research-based standards and guidelines for quality drinking water must be available to monitoring agencies. In 1999, Hashmi & Shahab recommended to establish standards and guidelines for quality drinking water. In 2002, the Pakistan Standards Institute compiled the preliminary standards for quality of drinking water. In 2004, Pakistan Council of Research in Water Resources prepared a report related to water quality in Pakistan with recommendations for establishing standards as shown in table 3 (Johri 2005). In March 2005, Health Services Academy, the Ministry of Health, Government of Pakistan in collaboration with World Health Organization (WHO) reviewed current standards implemented in Pakistan for quality control of drinking water and updated these standards in accordance with the quality standards of World Health Organization (WHO). Through a combination of lectures, discussions, intense work sessions, and utilization of reading literature provided by WHO and Ministry of Health, quality standards for drinking water in Pakistan were finalized. During all sessions, a careful attention was given to the following considerations: (1) all modifications in standards remain in correspondence with the social, cultural, geological, economic, technical and other significant conditions specific to the regional areas. (2) A review of existing national research-based data related to drinking water quality should be conducted.

(3) The work done by individual experts and by specialists from different agencies throughout the country should be coordinated and utilized in the finalization of standards. (4). In addition to WHO guidelines and standards (Table 2), USEPA standards, Malaysian standards, and Indian water quality standards were to be utilized for further benefits. (5) The standards must have a long range positive impact on human health in Pakistan. (6) Recommendations should be made based on the finalized standards for future plans of action. This report includes review of the available literature and proposed guidelines and standards for Pakistan. The Ministry of Health is playing its due role in the overall effort of the Government of Pakistan as a safeguard against water borne diseases (Ministry of Health 2008)**.**

1.14. Waterborne Diseases in Pakistan.

The government of Pakistan estimated with regard to diarrhea that this mainly water-related disease accounts for 14% of illnesses for children under five and 7% of all disease in people age of five and older (Ministry of Economic Affairs and Statistics 2004). The Pakistan Council of Research and Water Resources (2005)) assessed that 40% of all reported illnesses are water-related. It is estimated that 200,000 children in Pakistan die every year due to diarrheal diseases alone (United Nations Industrial Development Organization 2003). Unsafe water affects mainly rural and urban poor, who suffer above the average from sickness and water related diseases (Pakistan National Human Development Report 2003). Despite clear guidelines by Pakistan standard quality control authority for drinking water (Table 3) the quality of drinking water is inferior. In Pakistan around 30-40% infections and deaths are linked to the unsafe water quality. As the population exerts pressure on water quantity and quality, this results in deterioration of drinking water quality. Where population is dense it is susceptible to have more pollution. In Karachi more than 10,000 people die annually of renal failure due to polluted drinking water (Luby *et al.* 2001). In Pakistan the major diseases linked to drinking water are diarrhea, gastroenteritis, typhoid, cryptosporidium infection, giardiasis, intestinal worms and some strains of hepatitis. 60% of child's deaths are related to waterborne diarrhea in Pakistan (NDWP 2005). Despite the high mortality rate especially in infants, no proper attention is given to the drinking water quality. However in some places some remedial measures such as filtration and chlorination have been taken but these are quite in-sufficient and inefficient and confined only to urban areas which account for only 32% population of the country**.** An outbreak of hepatitis-E was reported in Islamabad due to consumption of contaminated drinking water (Haleem 1996).

According to quarterly report of infectious disease of District Health Office Khairpur (2007) the average number of people affected by diarrheal diseases was 6000-10000 per year in Khairpur city. The Sukkur is a thickly populated city of Pakistan with 1 million populations that faces the water-related problems. The drinking water is not of good quality; consequently waterborne diseases are very common. Number of people affected is very high ranging from 0.07- 0.08 million per year (District Health Office Sukkur 2007). Close to Sukkur city, on the left bank of River Indus the Rohri city is being supplied drinking water from River Indus. The water is stored in open reservoirs. After giving minor chlorine disinfection treatment, the water is supplied to consumers. In Rohri city the waterborne diseases are also common. 6000-7000 cases of diarrhea per year were reported during 2002-2007 by District Health Office Sukkur (2007).

Table 2
WHO Microbial Standards for Quality of Drinking Water

Properties /Parameters	WHO Standards
Bacterial	
All water intended for drinking (*E. coli* or thermo-tolerant coliform bacteria)	Must not be detectable in any 100ml sample
Treated water entering the distribution system *(E. coli/* thermo-tolerant coliform and total coliform bacteria)	Must not be detectable in any 100ml sample
Treated water in the distribution system (*E. coli*/thermo-tolerant coliform and total coliform bacteria)	Must not be detectable in any 100 ml sample. In case of large supplies, where sufficient samples are examined, must not be present in 95% the samples taken throughout any 12-Month period

Source: WHO Seminar Pak 2004

Table 3
Microbial Standards for Quality Drinking Water in Pakistan

Properties /Parameters	Standards for Pakistan	WHO Standards	Remarks
Bacterial			
All water intended for drinking (*E.coli* or Thermotolerant Coliform bacteria)	Must not be detectable in any 100ml sample	Must not be detectable in any 100ml sample	Most Asian countries also follow WHO standards
Treated water entering the distribution system *(E. coli/* thermo tolerant coliform and total coliform bacteria)	Must not be detectable in any 100ml sample	Must not be detectable in any 100ml sample	Most Asian countries also follow WHO standards
Treated water in the distribution system (*E. coli*/thermo tolerant coliform and total coliform bacteria)	Must not be detectable in any 100ml sample. In case of large supplies, where sufficient samples are examined, must not be present in 95% of the samples taken throughout any 12- Month period.	Must not be detectable in any 100 ml sample In case of large supplies, where sufficient samples are examined, must not be present in 95% the samples taken throughout any 12- Month period.	Most Asian countries also follow WHO standards

PSQCA: Pakistan Standards Quality Control Authority.2008

1.15 Drinking Water Quality Assessment Methods

A wide range of bacteria having health significance in drinking water has been reported. The researchers have outlined the methods currently used for the isolation and identification of important indicator bacteria including various pathogenically significant bacteria. A limitation of the judgment of quality of drinking waters is usually the presence of very insignificant number of bacteria. . It is therefore necessary to highlight the different techniques. These are absorption, enhancement, identification and quantification. The following section gives as description of the techniques in water quality monitoring from the micrbiological point of view http://www.who.int/water_sanitation .

1.15.1 Filtration Technique

1.15.2 Most Probable Number Technique

1.15.3 Immunocapture

1.15.4 Centrifugation

1.15.5 Flow cytometry

1.15.6 Pre-enrichment and Enrichment Techniques

1.15.7. Cultivation Techniques

1.15.8 Chromogenic Media-based Detection Methods

1.15.9 Other Techniques

1.15.9.1 Hydro-extraction

1.15.9.2 Molecular Methods Targeting Nucleic Acids

1.15.9.3 Polymerase Chain Reaction (PCR)

1.15.9.4 Fluorescence in Situ Hybridisation (FISH)

1.15.10 Emerging procedures

1.15.10.1 Laser Scanning Analysis

1.15.10.2 DNA – Chip Array

1.15.10.3 Biosensors

1.15.1 Filtration technique

In this method bacteria are collected on a membrane filter of 47 mm diameter with range of pore size of 0.22 to 0.45 µm. The filters are either placed on appropriate media or pads impregnated liquid media and bacteria are enumerated after incubation at appropriate media and temperature. Generally touching only the outer side of holder, the sterile filter holder assembly is opened and

the funnel is removed from the top. A sterile filter is placed on the filter support screen. The funnel assembly is replaced tightly. The sample is poured into the funnel wetting the filter for smaller samples size, 20-30 ml of sterile buffer can be poured first to give an even distribution of the sample. The sample is sucked through the filter using a vacuum pump. The funnel is rinsed with 20-30 ml of sterile buffer once or twice and the rinse too is sucked through the filter. The filtration apparatus is then disassembled using a sterile forceps and then membrane filter is transferred onto the nutrient medium in the Petri dish. Then the Petri dish is incubated for the given period of time at the prescribed temperature. After incubation the formed on the filter are counted. One of primary advantage of this method is its speed. Definitive results can be obtained with in 22-26 hours. Larger volumes of sample with low bacterial content can be analyzed. Since a direct count is made, results re precise. The instrument is portable, hence useful for the field studies (htt://www.microbiologyproceedure.com).

1.15.2 Most Probable Number Technique

The most probable number (MPN) technique is an important technique in estimating microbial population size in soils waters, and agricultural products. The technique does not rely on quantitative assessment of individual cells instead it relies on specific qualitative attributes of microorganism being counted. The important aspect of MPN methodology is the ability to estimate a microbial population size based on a process- related attributes. The MPN technique estimates microbial population size in a liquid substrate. The methodology for MPN technique is dilution and incubation of replicated cultures across several serial dilution steps. This technique relies on the pattern of positive and negative test results following inoculation of suitable of suitable test medium (usually with pH indicator dye). The results are used to drive a population estimate based on the mathematics of Halverson and Ziegler (BIOL/CSES4684). Despite the numerous advantages of using MPN technique there are many disadvantages to the method i.e. procedure takes very long time for the confirmed test results. In MPN the results are probability calculations and can not be accurate, it requires more glass wares and media and the false positive results are of common occurrence (htt://www.microbiologyproceedure.com).

1.15.3 Immunocapture

Immunomagnetic separation (IMS) are said to be competent for separation of prokaryotic pathogens from various samples including blood, food, water and fecal samples (Olsvik, ***et. al.*** 1994). This technique involves magnetic beads covered with corresponding antibody of interest.

The target microorganism are recovered by mixing water samples and coated beads and superintendent separation by magnetic particle divider. Immunoaffinity techniques together with magnetic beads containing appropriate antibody are used to detect a number of diverse target cells in water samples, including *E. coli* O157:H7 (Biswas *et. al.* 1994., Muir *et. al.* 1993). Enrichment of water bacteria followed by Immunomagnetic separation and incubation on selective media can be used as an alternate. The immunological methods have shown limited success in case of isolation of indicator bacteria from drinking water because of lacking sensitivity or had high rates of false positives due to cross-reaction (Obst *et. al.* 1989; Hubner *et. al.* 1992; Fayer *et. al.* 2000)

1.15.4 Centrifugation

It is a frequent separation technique using differential centrifugation (pelleting). However the limitation is the small volume of water that can be processed. Continuous-flow rotors are alternative to this shortcoming for processing large volumes of water http://www.who.int/water_sanitation .

1.15.5 Flow Cytometry

By using flow cytometry method, a range of particulate matter including bacteria suspended in a water sample can be isolated. In the sensing region of a flow chamber the particles flow through a light beam (laser beam). The sensing region of a flow chamber characterizes these particles on basis of shape, size and density. The light scatters by the photomultiplier collects the light scattered through fluorescence or labeled particles http://www.who.int/water_sanitation. An important feature of flow cytometry is its potential of swift analysis: within three to five minutes the assay itself can be completed. However in water monitoring system the use of flow cytometry has limitations and it is not cost effective. (Deere *et al.* 2002)

1.15.6 Pre-enrichment and Enrichment Techniques

It could be impractical to go after each pathogen in water analysis, therefore the indicator parameter are reliable to be used. However in some circumstances the hunt for virulent bacteria could be necessary, for example evaluation of a new water supply system and during an outbreak (WHO 1984). However the density of virulent bacteria may be low (Emde *et al.* 1992) and the chances of detection are low due to stressed circumstances, pre-enrichment is necessary to increase the chances of recovery of these virulent bacteria. (Emde *et al.* 1992)

1.15.7 Cultivation Techniques

Cultivation of Bacteria

Selective media has been used for quick isolation of bacteria in water samples. Different researchers have observed that certain selective agents may have a bacteriostatic effect on those coliform bacteria which are environmentally or oxidatively injured (McFeters *et al.* 1986). A medium excluding selective agent (T7 medium of LeChevallier *et al.*, 1982) allow a significant increase in the detection level of target bacteria (Hurst *et. al.* 2001). In high value samples presence/absence test is occasionally used when the likelihood of the wanted microorganisms are very low. However this method does not enumerate the extent of pollution even when result is positive. Good accuracy could be achieved by using sufficient replicates. There are computer programs for the computation of MPN tables (Gonzales, 1996). The results become significant when total colonies are counted in replicate http://www.who.int/water_sanitation.

1.15.8 Chromogenic Media-based Detection Methods

Use of specific enzymes in media and excluding selective agents allow most important improvement in isolation and identification of wanted bacteria. It is called defined substrate method for the isolation of coliforms and *E. coli*, designed by Edberg *et al.* (1991). Commercially this has been converted as the Colilert® technique that correlate significantly with membrane filter and MPN techniques when used to analyze freshwater (Fricker *et al.* 1997; Eckner, 1998). The main feature of Colilert® technique is the change in color, that is (the color of water) the sample turns the medium yellow, indicating the action coliforms with β-galactosidase reaction on the substrate ONPG (O-nitrophenyl- β -D-galactopyranoside), and fluorescence under long-wavelength UV light if the substrate MUG (5-methylumbelliferyl- β -D-glucuronide) is used by β-glucuronidase +ve coliforms. The analytical technique depends on addition of commercially dehydrated indicator nutrients having two distinct substrates. (APHA, AWWA, WEF 1998). A main problem of any assay relying on enzyme activity is false positive results, because other members of *Enterobacteriaceae* also possess the β-glucuronidase enzyme. This could result in the isolation of false positive organisms. Conversely a few strains of *E. coli* (including pathogenic strains) are β-glucuronidase negative and cannot be identified by this method http://www.who.int/water_sanitation .

1.15.9 Other Techniques

1.14.9.1 Hydro-extraction

In this procedure water sample is placed into a dialysis bag which is made up of cellulose, then placed in hygroscopic material. This extracts water but viruses/ phages and other macro solutes remain inside the membrane. For the isolation of viruses from low quantity water samples this procedure is recommended as an option (APHA, AWWA, WEF 1998; Padan *et. al.* 1967).

1.14.9.2 Molecular Methods

Hybridization is a reaction which involves two matching nucleic acid strands, they combine together to form a double -stranded molecule. When denatured, one of the nucleic acids fragment work as a probe. (e.g cloned DNA fragment, artificial oligonucleotide. The probes are labeled with Non-radioactive label, and can be detected by an immuno-enzymatic reaction. Oligonucleotide probes can be bound to filter, microtiter plates, and micro-chips and unknown DNA is detected (Grimont, 1988). When nucleic acid amounts are controlled, this technique works well but is frequently questionable on field samples with unknown bacterial species. Furthermore, probes targeting DNA could not distinguish between live and dead bacteria http://www.who.int/water_sanitation .

1.14.9.3 Polymerase Chain Reaction (PCR)

Polymerase Chain Reaction (PCR) is the most important molecular biology technique. The polymerase chain reaction (PCR) revolutionized molecular biology to a similar extent as the discovery of plasmids and restriction endonucleases (Wassenegger 2007).

The PCR protocol allows the detection of bacteria in different types and sources of water samples (Shaban & Malkawi 2007). This method can be performed *in vitro* in a thermocycler, Using DNA probes as templates and two oligonucleotide primers that bind to corresponding sequences of target; this method permits the exponential duplication of nucleic acid fragments. http://www.who.int/water_sanitation. After the separation of amplicons, in an electrophoretic step, it can be concluded which microorganisms were present in the original water sample. Hence, this technology permits precise detection of large groups of organisms by using primers which attach to more preserved fragment encoding for 16S ribosomal RNA or DNA fragments which are responsible for virulence markers. The limitation of PCR reaction is that it does not differentiate between live and dead organisms. As compared to DNA, messenger RNA (mRNA) complementary to that DNA is very short lived and half-life of only a few minutes (Wassenegger

2007) Reverse transcriptase (RT) PCR however assesses viability and mRNA could be a better choice http://www.who.int/water_sanitation. Usually the PCR is useful but in drinking water quality testing by detection of *E. coli* further reliability evaluation is required (Rompre *et al.* 2002).

1.15.9.4 Fluorescence in Situ Hybridization (FISH)

By using *in situ* hybridization method organisms can be isolated in their natural habitat so the pre-enrichment culture technique not is required. For isolation of bacteria by (FISH) a typical *in situ* hybridization would have the following steps (1) Filtration of a water sample through a membrane, (2) Fixation of bacterial cells, (3) Permeation of cells, (4) Hybridization with a fluorescent probe, (5) Washing to remove unbound probe, and (6) Microscopic examination. rRNA molecules are perfect target for *in situ* hybridization because their presence in bacteria with conserved segments of their sequences occur in about 30000 copies per cell. Databases contain many rRNA sequences. As the fluorescent signal varies frequently depending on the sequence position of the target on the rRNA, a special care should be taken for target accessibility while the probe is designed (Fuchs *et al.* 1998). The fluorescent signal is stronger when the probe is longer with various labels (Trebesius *et al.* 1994) or when a peptide nucleic acid (PNA) is used as a probe (Prescott and Fricker, 1999).

1.15.10 Emerging procedures

1.14.10.1 Laser Scanning

By improving test procedures based on chromogenic, and fluorogenic substrates for the detection and enumeration of coliform bacteria and *E. coli,* the investigation can be completed in 24-48 hours. By the help of various instruments as a substitute of visual approach many ideas have been analyzed to enhance the sensitivity of enzymatic reactions. It has been observed that spectrophotometry is useful in reducing the 24hr Colilert® test by 6 hours (Rice *et al.* 1993).

1.15.10.2 DNA – Chip Array

In future there are great chances of the development and application of DNA –Chip-array technology for the characterization of fecal indicators and pathogens. Biosensors based on gene recognition, are looking very suitable in the microarray format for investigating microorganisms. The method was developed at Stanford University (Ekins and Chu, 1999). There are two variants of the DNA microarray technology, in terms of the property of arrayed DNA sequence with known identity: 1. Probe cDNA (500~5 000 bases long) is immobilized to a solid surface such

as glass using robot spotting and exposed to a set of targets either separately or in a mixture. 2. An array of oligonucleotide (20~25-mer oligos) or peptide nucleic acid probes is synthesised either *in situ* (on-chip) or by conventional synthesis followed by on-chip immobilization. The array is exposed to labeled sample DNA, hybridized, and the identity/abundance of complementary sequences is determined. This method, originally called GeneChip arrays or DNA chips, was first developed at Affymetrix Inc. (Lemieux *et al*., 1998; Lipshutz *et al*., 1999).

Microarrays using DNA/RNA probe-based rRNA targets may be coupled to adjacent charged couple device detectors (Guschin *et al*., 1997). Eggers *et al*. (1997) have demonstrated the detection of *E. coli* and *Vibrio proteolyticus* using a microarray containing hundreds of probes within a single well (1cm2) of a conventional microtiter plate (96 well). The complete assay with quantification took less than one minute. The microarray under development by bioMerieux (using Affymetrix Inc. Gene Chip technology) for an international water company (Lyonnaise des Eaux, Paris, France) is expected to reduce test time for faecal indicators from the current average of 48 hours to just four hours. In addition, the cost for the standard water microbiology test is expected to be ten times less than present methods. The high resolution DNA chip technology is expected to target a range of key microorganisms in water. The prototype Gene Chip measures about 1 cm2, on which hybridization occurs with up to 400 000 oligonucleotide probes.

1.15.10.3 Biosensors

The optics, immunoassays and other chemical tests that can be used for the detection microorganisms are the basis for biosensor. Mostly isolation of the bacterial pathogens is the major goal of this work (Wang *et al.* 1997). There is an immunoaffinity procedure to isolate bacteria on membrane beads, fiber optics or probe tips, followed by detection using laser excitation of bound fluorescent antibodies, acoustogravimetric wave transduction, or surface plasmon resonance. Various kinds of biosensors presently are in the development process, especially to detect and identify pathogens from food, e.g. meat and poultry and could be used for the water quality assessment for detection of waterborne pathogens (Georgia Tech: Research Institute, 1999). The biosensor operates with three primary components – integrated optics, immunoassay techniques and surface chemistry tests. It indirectly detects pathogens by combining immunoassays with a chemical sensing scheme. In the immunoassay, a series of antibodies selectively recognize target bacteria. The ‘capture’ antibody is bound to the biosensor

and captures the target bacteria as it passes nearby. A set of 'reporter' antibodies (which bind with the same target pathogen) contain the enzyme urease, which breaks down urea that is then added resulting in the production of ammonia. The chemical sensor detects the ammonia, affecting the optical properties of the sensor and signaling changes in transmitted laser light. These changes reveal both the presence and concentration of specific pathogens in a sample at extremely minute levels. The method is currently unable to distinguish viable from non-viable microbes, and it will be necessary to increase the sensitivity in order to apply this technique to water testing. Nonetheless this methodology has a great potential for future application, especially as it is extremely fast http://www.who.int/water_sanitation.

Objectives of Research

- To assess the level of fecal pollution in the drinking water (municipal water) of Khairpur, Sukkur, and Rohri cities Sindh, Pakistan, (by determining) the number of fecal coliforms found in drinking water sources.
- To evaluate the effect that water storage procedures has on the number of *E. coli* in drinking water consumed in households.
- To determine the association between cases of diarrhea and number of fecal coliform in municipal drinking water
- To determine the association between season and prevalence of waterborne bacterial pathogens in drinking water.
- To determine the prevalence of various waterborne bacterial pathogens in municipal water of Khairpur, Sukkur and Rohri Sindh Pakistan.
- To evaluate the quality of drinking water of Khairpur, Sukkur and Rohri in the light of World Health Organization guideline values for drinking water.

2. MATERIALS AND METHODS

2.1 Sampling Sites

The present study was carried out to investigate the municipal water of urban areas of interior Sindh Pakistan. In these areas there was no such type of research carried out before. In urban areas, the municipal water from river water sources is stored in the main storage reservoirs and supplied to the population. Keeping in view the public health risk from drinking water three cities i.e Khairpur, Sukkur and Rohri were selected to investigate the quality of drinking water. The source of drinking water in these cities is surface water from River Indus for drinking and other domestic uses. Indus River is the main river in Pakistan which flows from north to south. The total length of the river is 3200 kilometers (2000 miles). The river has a total drainage area exceeding 1,165,000 square kilometers (450,000 square miles). Its estimated annual flow stands at around 207 cubic kilometers (http://www.newworldencyclopedia.org/entry/Indus.

The Khairpur water works which was selected for sampling has the capacity of 2.75 (MGD) million gallons per day. The drinking water of Khairpur is being taken from surface water from Mirwah *Canal* to the storage reservoirs where it is subjected to sedimentation using alum. No further information regarding chlorination or other disinfection treatment was available. The total population of Khairpur city is 0.15 million but only five thousand (3.30%) people have the piped water supply (Municipal Office Khairpur 2006). Sukkur city was also selected for the water quality testing. Sukkur Municipal Corporation is providing 10 million gallons per day (10 MGD) drinking water to the residents of Sukkur city. The daily need is much greater, that is 16 million gallons per day (16 MGD), for the population of the city. Sukkur city a thickly populated but the sanitation facilities are inadequate. The city has the population of 1 million people and only twenty five thousand (3.12%) people have a piped water facility (Sukkur Municipal Corporation 2006). The available source of drinking is river Indus where from water is stored in reservoirs sedimentation by alum is made further treatment data are not available. Adjacent to Sukkur Rohri city was also selected for present study. The source of drinking water is via river Indus from where water is collected in reservoirs sedimented by using alum but no data on further treatment by chlorination are available This city is facing the similar problems, in Rohri the population is 0.13 million and only four thousand (3.07%) people have a piped drinking water supply. The city has limited sanitation facilities, and the municipal authorities provide 2 million

gallons/day (Municipal Office Rohri 2006). In the light of microbial standards for drinking water quality by Pakistan and WHO (Table 2&3) the water under study ranked poor quality.

Color plate 1 Storage reservoirs of municipal water of Khairpur city

Color plate 2 Storage reservoir of municipal water of Sukkur city

Color plate 3 Storage reservoirs of Rohri city.

2.2 Materials

In present study the materials used for the isolation and identification of pathogenic bacteria from drinking water of Khairpur, Sukkur and Rohri are given as under

2.2.1 Glassware used

- Petri plates 15 ×100 mm
- Petri plates 12×47 mm
- Test tubes 18×150 mm
- Sugar tubes 12×100 mm
- Measuring cylinder 100 ml
- Conical Flasks 500 ml
- Conical Flasks 250 ml
- Microscope slides
- Reagent bottles
- Pastew pipette

2.2.2 Equipment used

- Electrical Balance (Shimadzu Japan)
- Oven (Gallenkamp)
- Incubator(Memmert)
- Autoclave (OSK 8869 Autoclave Type C OGAWA SEIKI CO LTD)
- Microscope(Osaka Japan)
- Magnifying glass
- Filtration assembly Vacuum presser pump (Barant USA)
- Membrane filter (Micropore USA)

  i. Pore size 0.45 micrometer and 0.2 micrometer

  ii. Color White

  iii. Diameter 47 mm

  iv. Surface Plain
- Wire loop
- Smooth-tipped forceps

2.3 Methods used in Present Study

2.3.1 Membrane Filtration Technique

Bacteriological analysis using the Membrane Filtration Technique was used by applying the standard method: Method for the Examination of waters and Associated Materials (E.A 2002). In present study using this method the waterborne bacterial pathogens were isolated and identified with different isolation rates. When necessary the water samples were diluted to 1:10and 1:100 1:1000 (Pettibon 1998). The isolated colonies were independently identified using standard microbiological methods. A 100 ml aliquot of water samples were filtered through membrane filter of pore size 0.45 μm and the diameter 47 mm (Micropore). When samples form different sampling sites were analyzed then to avoid the contamination filtration flask were sterilized after each sample filtered. Further details of the technique will be given in detail in each section of pathogen isolation in following pages.

Table 4.

Selective, Differential and Basic Culture Media used in the Study

BACTERIA	CULTURE MEDIA
E. coli *Non-fermenter, Proteus .*	Eosin Methylene Blue (EMB OXOID England) Agar
Providencia ,Klebsiella *Citrobacterr,Chryseobacteriun.*	Eosin Methylene Blue (EMB OXOID England) Agar Eosin Methylene Blue (EMB OXOID England) Agar, Buffered Peptone Water(OXOIDEngland),
Salmonella.	Rapaport Vassiliadis Soya Enrichment Broth(OXOID England), Xylose Lysine-Deoxycholate (XLD, BioM, USA), Deoxycholate Citrate(DCA BioM, USA) Agar
Aeromonas	Ampicillin Dextrin Agar (BioM)
Vibrio	Alkaline Peptone Water (OXOID) Thiosulfate Citrate Bile Salt Sucrose (TCBS) Agar (BioM, USA)
Pseudomonas.	Pseudomonas Agar F (BioM USA) **Other Culture Media and Reagents** Nutrient Agar (OXOID England) MacConkeys Agar (BioM USA), Gram Staining Reagents, Analytical Profile Index (API 20 E) Kit (BioMerieux Vitek Inc.,Hazelwood, MO 63042)

2.3.2 Analytical Profile Index (API 20E)

The basis of API 20E is classical microbiology, clinically proven and accepted. For the identification of members of *Enterobacteriaceae* the API 20E which can identify 20 different biochemical tests is a most suitable identification system (Koneman *et al.* 1997). In present study the final identification of all isolates was carried out by API 20E. (BioMerieux Vitek Inc. Hazelwood, MO 63042). The likelihood values were calculated on basis of cultural characteristics and morphology on selective media, Gram staining, motility and oxidase test. The isolates having 99% likelihood were subjected to API 20E. (Neubauer *et al*. 1998) For every

isolate API 20E strips in duplicate were inoculated and incubated according to manufacturer's instructions. The identification of isolates was calculated using the manufacturers coding system based on reactions to the reagents in the twenty compartments (Aldridge *et al.* 1978).

2.4 Sampling of Drinking Water

For regular monitoring the drinking water quality the minimum sample number criteria by WHO is given as: for piped drinking water in distribution system i. e. <5000 population served = 1 sample /month, 5000–100000 population served = 1 sample per 5000 population/month >100000 =1 per 10 000 population, plus 10 additional samples/ month (WHO 1997**).** As this study was carried out for the isolation of pathogenic bacteria from water for the determination of trends of the occurrence of these waterborne pathogens in main storage water reservoirs, the sufficient number of samples was collected. The sampling was carried out fortnightly (composite samples) for 3 years, from main storage reservoirs of Khairpur, Sukkur and Rohri cities of Sindh Pakistan. Sampling was carried out at 10. am from main reservoirs of each city according to standard procedure of microbiology The tap of sampling pipe was first disinfected with heat by igniting cotton soaked with sprit to remove the contaminant and then water was allowed to run for five minutes to flush out the contaminants from the neck of the tap. Samples were collected in the sterilized screw caped, Erlenmeyer flask of 500 ml (Pyrex). Physical parameters i.e. pH, temperature, and chlorine were investigated on-site prior to the addition of sodium thiosulfate pentahydrate. To neutralize the residual chlorine, the solution of sodium thiosulfate pentahydrate ($Na_2S_2O_3.5H_2O$) at concentration of 18 mg/l was added to the flask (Niemela *et al.* 2003). The collected samples were kept in ice box (-2^oC) and transported to laboratory with in one hour. Duplicate samples were collected from each site and for each sampling period.

2.5 pH Analysis

For pH analysis 216 drinking water samples were analyzed. The pH of water samples was analyzed by using PAL High Accuracy Electrochemistry Test Pen. The cap was removed and the tester was calibrated with buffer solution at pH 7, then tester was dipped up to immersed level, in water sample and after one minute results were recorded, the same procedure was repeated for other water samples.

2.6 Chlorine Analysis

For chlorine analysis 216 drinking water samples were analyzed. The free residual chlorine was measured by using HI 3831 F Free Chlorine Test Kit (HANNA chemical test kit Italy), via a color cube. The addition of water produces hydrochloric acid and hypochlorus acid.The hypochlorus acid act as the disinfectant and bleaching agent. These are known as free chlorine which is measured by a colorimetric method. Reaction was buffered at 6.3 pH in that condition DPD (N, N- diethyl1, 4-p-phenylenediamine) was immediately oxidized by chlorine producing a reddish color. The color intensity of the solution determined the free chlorine.

2.7 Temperature Measurement

For temperature measurement 216 drinking water samples were analyzed in different quarters during 2005-2007from each site .The temperature of the drinking water samples was determined at site of collection using electronic, digital thermometer (QUARTZ OREGON SCIENTIFIC)

2.8 Bacteriological analysis

2.8.1 Isolation, Identification and Enumeration of Fecal Coliform (*Escherichia coli*) on Eosin Methylene Blue Agar (EMB, OXOID England)

Sample Processing

The method was used according to the published method for the examination of waters and associated materials (E.A 2002). Numerous researchers have used the membrane filtration technique i.e. Kristen *et al.* 1996, Alonso *et al.* 1994. The sterile filtration apparatus was placed in a position and connected with source of vacuum. The funnel was removed and holding the edge of the membrane filter of pore size 0.45 µm and the diameter 47 mm (Micropore) with sterile smooth tipped forceps, the sterile membrane filter was placed, grid-side upward onto the porous disc of the grid base. The sterile funnel was replaced securely on the filter base. The 100 ml water sample in duplicate was poured into the funnel by applying the vacuum of 65 kpa (500 mm of mercury) sample was filtered slowly through membrane filter the stopcock was closed as sample was filter to prevent the air to be drawn through the membrane filter. The funnel was removed and the filter was carefully transferred to 47 mm diameter Petri plates containing appropriate media which were left at appropriate temperatures for 30 minutes prior to use. To remove the air bubbles trapped between the membrane filter and the medium the membrane filter was rolled onto the medium (Table 4). The Petri plates were incubated at 44.5 ^{o}C for 24 hours. After incubation the membrane filters were examined for typical colonies. The 2-3 mm in

diameter, smooth with entire edge and green metallic sheen colonies were counted. The number of tested samples was designated as (NT) and number of positive samples was designated as NP. The suspected colonies were sub-cultured to tube of lactose peptone water (LPW) and incubated at 44.5 ^{O}C for 24 hours one tube of tryptone water (TW), incubated at 37 ^{O}C for 24 for the production of acid and gas. 10 suspected colonies were selected from each plate for identification by cultural characters, Gram staining, motility and oxidase test. Then 99% likelihood qualifying bacteria were subjected to API and the positive number and percentage for each type of bacterial species was calculated. Further the isolates were sub-cultured on nutrient agar for final confirmation by API 20E biochemical kit.

For, fecal coliform (*E. coli*) the colony forming units (cfu) were counted as below.

$$\text{Number of fecal coliform per 100 ml of water} = \frac{\text{Number of } E.\ coli \text{ colonies}}{\text{Volume of sample filtered (ml)}} \times 100$$

(US EPA 2002).

The isolation rate (%) for all bacterial species was determined as under.

$$\text{Isolation rate (\%)} = \frac{\text{Number of samples positive (NP)}}{\text{Number of samples tested (NT)}} \times 100 \text{ (Begum\& Khan 2001)}$$

2.8.2 Antibiotic susceptibility of thermotolerant *E. coli* 2

Antibiotic susceptibility of thermotolerant *E. coli* 2 was performed using a disk diffusion method (Bauer 1966)

2.8.3 Isolation and Identification of *Proteus spp.* on Eosin Methylene Blue Agar (EMB, OXOID England)

Sample Processing

The samples were processed in same fashion as in isolation and identification of *Escherichia coli* the typical mucoid irregular colonies with swarming growth were selected as proteus and sub- cultured on nutrient agar for further identification by oxidase test and number of positive (NP) samples was recorded

2.8.4 Isolation and Identification of *Providencia spp.* on Eosin Methylene Blue Agar (EMB, OXOID England)

Sample Processing

The samples were processed in same fashion as in isolation and identification of *Escherichia coli*. The pink colored colonies were selected and sub- cultured on nutrient agar for further identification by gram staining and oxidase test and number of positive (NP) samples was recorded.

2.8.5 Isolation and Identification of *Klebsiella spp.* on Eosin Methylene Blue Agar (EMB, OXOID England)

Sample Processing

The samples were processed in same fashion as in isolation and identification of *Escherichia coli* The pink colored mucoid colonies were selected and sub- cultured on nutrient agar for further identification by gram staining and oxidase test and number of positive (NP) samples was recorded.

2.8.6 Isolation and Identification of *Citrobacter spp.* on Eosin Methylene Blue Agar (EMB, OXOID England)

Sample Processing

The samples were processed in same fashion as in isolation and identification of *Escherichia coli* The brown colored dark centered colonies were selected and sub- cultured on nutrient agar for further identification by gram staining and oxidase test and number of positive (NP) samples was recorded

2.8.7 Isolation and Identification of *Non-fermenter spp.* on Eosin Methylene Blue Agar (EMB, OXOID England)

Sample Processing

The samples were processed in same fashion as in isolation and identification of *Escherichia coli* The selected colorless colonies were sub- cultured on nutrient agar for further identification by gram staining and oxidase test and number of positive (NP) samples was recorded

2.8.8 Isolation and Identification of *Chryseobacterium spp.* on Eosin Methylene Blue Agar (EMB, OXOID England)

Sample Processing

The samples were processed in same fashion as in isolation and identification of *Escherichia coli* The orange colored colonies were selected and sub- cultured on nutrient agar for further identification by gram staining and oxidase test and number of positive (NP) samples was recorded.

2.8.9 Isolation and Identification of *Vibrio spp.* on Thiosulfate Citrate Bile Salt Sucrose Agar (TCBS, BioM USA)

Sample Processing

After filtration, as indicated before the membrane filter was carefully transferred to alkaline peptone water for *V. cholerae*, incubated at 25 ^{o}C for 2 hours followed by 37 ^{o}C for 16 hours. After incubation a loopful of alkaline peptone water taken from top of the broth streaked onto Petri dishes of Thiosulfate Citrate Bile Salt Sucrose (TCBS) Agar and incubated at 37 ^{o}C for 24 hours. After 24 hours typical flat colonies 1-3 mm in diameter yellow (sucrose-fermenting) and blue green (non-sucrose-fermenting) on Thiosulfate Citrate Bile Salt Sucrose (TCBS) Agar (Table 4) were observed, further the results were confirmed by sub-culturing on nutrient agar incubated at 37 ^{o}C for 24 hours. By using API 20E (BioMerieux) biochemical test kit the isolates were confirmed.

The Petri plates were incubated at 37 ^{o}C for 24 hours. The process was repeated to other replicates of water samples. After incubation the all presumptive colonies were sub-cultured on nutrient agar, number of positive (NP) samples recoded and the final confirmation was made by API 20E biochemical test kit**.**

2.8.10 Isolation and Identification of *Aeromonas spp*. on Ampicillin Dextrin Agar (BioM, USA)

Sample Processing

After filtration, as described before the membrane filter was carefully transferred to 47 mm diameter Petri plates containing Ampicillin dextrin agar (Table 4) which were left at 37 ^{o}C temperatures for 30 minutes prior to use. To remove the air bubbles trapped between the membrane filter and the medium the membrane filter was rolled onto the medium. The Petri plates were incubated at 37 ^{o}C for 24 hours. The process was repeated to other replicates of water samples. After incubation the membrane filters were examined for typical colonies. The colonies 2-3 mm in diameter, smooth with entire edge deep yellow or orange yellow periphery

were transferred to nutrient agar; number of positive (NP) samples recoded and the final confirmation was made by API 20E biochemical test kit**.**

2.8.11 Isolation and Identification of *Pseudomona*s on Pseudomonas Agar F (BioM USA)

Sample Processing

After filtration as described before the membrane filter was carefully transferred to 47 mm diameter Petri plates containing pseudomonas agar F (Table 4) which were left at 37 ^{o}C temperatures for 30 minutes prior to use. To remove the air bubbles trapped between the membrane filter and the medium the membrane filter was rolled onto the medium. The Petri plates were incubated at 37 ^{o}C for 24 hours. The process was repeated to other replicates of water samples. After incubation the all presumptive colonies were sub-cultured on nutrient agar, number of positive (NP) samples recoded and the final confirmation was made by API 20E biochemical test kit**.**

2.8.12 Isolation of *Salmonella:* on Xylose Lysine-Deoxycholate Agar (XLD, BioM, USA)

Sample Processing

After filtration as described before the membrane filter was carefully transferred into 90 ml buffered peptone water mixed well and incubated at 37 ^{o}C for 24 hrs. After incubation for 24 hrs, the growth mixed well and 0.1 ml of buffered peptone water sub-cultured into 10 ml of Rapaport Vassiliadis enrichment broth (Table 4) and incubated at 41.5 ^{o}C for 24 hrs. After that time a loopful of Rappaport Vasiliadis enrichment broth was sub-cultured on XLD, DCA and incubated at 37 ^{o}C 24 hrs (Microbiology of Drinking Water 2002-Part 2.). The number of positive (NP) samples was noted and further identification was made by API 20E biochemical test kit.

2.9 Gram Staining

The smear was prepared and fixed on clean glass slide and stained with crystal violet for 30 seconds then smear washed with distilled water. Gram's iodine was added for 10 seconds then the smear was washed with tap water and decolorized with 95% Acetone alcohol and finally safranin a secondary dye was added to the smear for 30 seconds washed with tap water, air dried and observed under oil immersion objective(100X).

2.10 Oxidase Test

The reagent was prepared according to manufacturer's instruction. 2-3 drops of freshly prepared oxidase reagent (Annex: II) were placed on to the filter paper contained in a Petri dish. With a wooden stick some of the growth of test organism grown on non-selective agar (i.e. nutrient agar) was smeared onto the treated filter paper. Those bacteria which gave the positive result by changing the filter paper in deep blue purple color within 10 second were reported as oxidase positive.

2.11 Distilled Water Motility Test for Vibrio

Loopful of growth suspected of having *Vibrio cholerae* from a nutrient agar plate mixed in a drop of sterile distilled water on one end of slide. On the other end of slide a loopful of growth mixed in drop of peptone water was placed, then each preparation was covered with cover glass seal and was examined under the 40x objective with the condenser iris closed sufficiently for a good contrast (Monica 1984)

2.12 Confirmation of Isolates by (API 20E) Biochemical Test.

Preparation of Bacterial Inoculums

The inoculums were prepared in 5 ml sterilized 0.85 % NaCl solution by touching the well isolated colony of the testing organism with a sterile wire loop.

For biochemical reactions the isolates were first grown on a non-selective medium (i.e. nutrient agar) at 37 ^{o}C for 24 hours. With a sterile wire loop, a well isolated colony was transferred to the 5 ml saline and mixed thoroughly. By pouring the 5 ml of tap water in the corrugated incubation tray the humid atmosphere was provided for the incubation of the test bacteria. Using a sterile Pasteur pipette the tube section of the microtubule was filled with bacterial suspension, the tube and cupules section of the CIT, VP and GEL tubes was filled with bacterial suspension. After inoculation, the cupules section of the ADH, LDC, ODC, H2S and URE tubes were filled with sterile mineral oil in order to create anaerobic condition. Placing the plastic lid on the tray the strips were incubated in duplicate at 37 ^{o}C for 24 hours and the results were recorded according to API 20E (BioMerieux Vitek Inc.,Hazelwood, MO 63042)

L-Tryptophane Deaminase (TDA) Test.

1 drop of 10% ferric chloride was added and the development of brown-red color recorded as positive test.

Voges-Proskauer (V-P) Test

1 drop of V-P reagent II (KOH) followed by 1 drop of V-P reagent I (ά-napthol) was added and results were recorded after 10 minutes as the red color indicates positive Voges-Proskauer (V-P) test.

Indole Test

I drop of Kovacs reagent was added and results were recorded after two minutes. Appearance of red ring is positive indole test

Nitrate Reduction

2 drops of nitrate reagent A (dimethyl-ά-napthylamine) and two drops of nitrate reagent B (Sulfanilic acid) were added to GLU cupules and then results were recorded. The development of red color indicates positive nitrate reduction test. Each positive reaction was indicated with a + in the appropriate compartment of the lab report.

Table 5
Dehydrated Substrates in API 20E System

S.NO.	SUBSTRATE	FULL NAME	S.NO.	SUBSTRATE	FULL NAME
1	OPNG	Ortho-NitroPhenyl-βD-Galctopyranoside	12	GLU	Glucose
2	ADH	Arginine DiHydrolase	13	MAN	Mannitol
3	LDC	Lysine DeCarboxylase	14	INO	Inositol
4	ODC	Ornithine DeCarboxylase	15	SOR	D- Sorbitol
5	CIT	Citrate	16	RHA	L-Rhamnose
6	H_2S	Hydrogen Sulphide	17	SAC	D-Saccharose
7	URE	UREase	18	MEL	L-Melibiose
8	TDA	L-Tryptophane DeAminase	19	AMY	Amygdalin
9	IND	Indole	20	ARA	L-Arabinose
10	VP	Voges-Proskauer			
11	GEL	Gelatinase			

2.13 Statistical Analysis

For Statistical Analysis (SPSS 13.0 for Windows version) and Curve expert 1. 3 software were used. The data were analyzed by one sample T test (p value) for frequency differences and isolation percentage differences in different seasons. Pearson correlation, linear fit and quadratic fit model for correlation were applied.

3. RESULTS

3.1 Enumeration of Fecal Coliform (*Escherichia coli)* per 100 ml of Drinking Water (Municipal Water) of Khairpur, Sukkur and Rohri (2005-2007).

The enumeration of indicator organisms (i.e. Fecal coliform *Escherichia coli*), by membrane filtration technique was carried out from drinking water during study period in three cities (i.e. Khairpur, Sukkur and Rohri of Sindh Pakistan). Very high numbers of fecal coliform per 100 ml of water samples were found throughout the study period. In summer months the bacterial counts were higher as compared to winter months.

3.1.1 Enumeration of Fecal Coliform (*Escherichia coli)* per 100 ml of Drinking Water of Khairpur City

Isolation of fecal coliform (*E. coli*) was carried out from drinking water of Khairpur city as described in the material and methods section. The identification was performed by API 20E (Materials &Methods). The typical biochemical reactions observed after incubation of isolates in API 20E strips as shown in color plates 4 .Very high numbers of fecal coliforms (*E. coli*) and total coliform was isolated, in drinking water samples, collected from municipal water of Khairpur city during 2005-2007. It is evident from the Fig. 1, 2, 3 and table 6 that the number of fecal coliform bacteria (*E. coli*) was thousand times higher than the WHO guideline values for drinking water during consecutive three years of study. The highest number of fecal coliform (*E .coli*) was observed during summer months compared to winter months. Fig 4 and 5 show the seasonal impact on quality of water. The distribution of fecal coliform (*E. coli*) was recorded in each month of study years. Because a large number of total and fecal coliform bacteria in water samples recorded, it was difficult to present these numbers in multi-digit form. Therefore the data was transformed into Log. The Log is a scientific mathematical notation and an easy way to handle large data numbers**.** In 2005, in the month of January the number of fecal coliform (*E. coli*) was 2.9 log cfu, in February 2.8 log cfu, in March 2.9 log cfu, in April 3.1 log cfu, in May, 3.3 log cfu, in June it was 3.3 log cfu, in July 3.4 log cfu, in August 3.3 log cfu, in September 3.2 log cfu, in October 3.3 log cfu, in November 3.1 log cfu and in December 2.6 log cfu/100 ml was observed (Fig. 1). The concentration of *E. coli* was significantly higher in summer months than in cold months ($p < 0.01$). In 2006, in the month of January the number of

fecal coliform (*E. coli*) 100 ml of drinking water samples was, 2.6 log cfu, in February 3.0 log cfu, in March 2.9 log cfu, in April 3.1 log cfu, in May 3.1 log cfu, in June 3.3 log cfu, in August 3.4 log cfu, in September 3.3 log cfu, in October 3.3, log cfu in November 3.1 log cfu and in December the number of fecal coliform (*E. coli*) was 2.8 log cfu/100ml. The concentration of *E. coli* was significantly higher in summer months than in winter months ($p< 0.01$). In 2007 the number of fecal coliform in January was 3.0 log cfu, in February 3.1 log cfu, in March 3.1 log cfu, in April 3.2 log cfu, in May 3.3 log cfu, in June 3.4 log cfu, in July 3.4 log cfu, in August 3.4 log cfu, in September 3.3, log cfu in October 3.3 log cfu, in November 3.3 log cfu and in December the number of fecal coliform (*E. coli*) was 3.0 log cfu/100 ml drinking water counted, as shown in Fig. 3. The concentration of *E. coli* was significantly higher in summer months than in cold months ($p< 0.008$). The number of fecal coliform bacteria per 100 ml in Khairpur municipal water throughout the study period was lowest in the month of December with the minimum 2.6 log cfu, maximum 3.0 log cfu and mean 2.8 log cfu and highest in the month of October minimum 3.3 log cfu, maximum 3.4 log cfu and the mean 3.3 log cfu /100 ml. of water were counted (Table 6).the frequency of contaminated samples of drinking water randomly collected from selected main storage reservoirs, distribution line, and consumers tap was determined. The percentage shown that in summer months the frequency of contaminated water samples and densities of total and fecal coliform were higher as compared to winter months as shown in figure 4 and 5.

3.1.2 Prevalence of Diarrheal Disease in Khairpur City

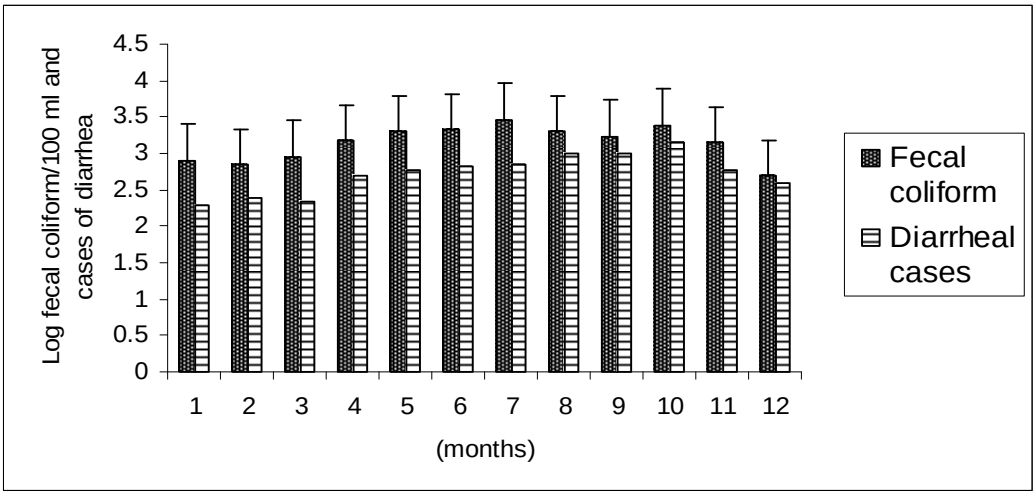
The data of diarrhea cases were provided by District Health Office and from District Hospital monthly reports. The incidence rate of diarrhoeal disease in 2005, 2006 and 2007 remained 0.05, 0.03 and 0.05 respectively (Fig 6). In order to determine correlations with number of *E. coli* in 100 ml of drinking water of the area under study and diarrheal cases in the population using that water during the study period, the data were log transformed According to District Health Authorities the prevalence of diarrheal disease may be due to consumption of contaminated water. According to these data the highest number of diarrheal cases was reported in the months of April- October in 2005. June- October in 2006 and from May - November in 2007 and the number of fecal coliform was roughly directly proportional to diarrheal disease reported in Khairpur district hospitals (Fig 1, 2, and 3) A strong positive correlation was found between number of fecal coliform per 100 ml of water used for drinking in the city of Khairpur and cases

of diarrhea the population using that unsafe water during the study period (*r =0.79*) as shown in fig. 7.

3.1.3 Antibiotic Susceptibility of Thermo-Tolerant *Escherichia coli* 2

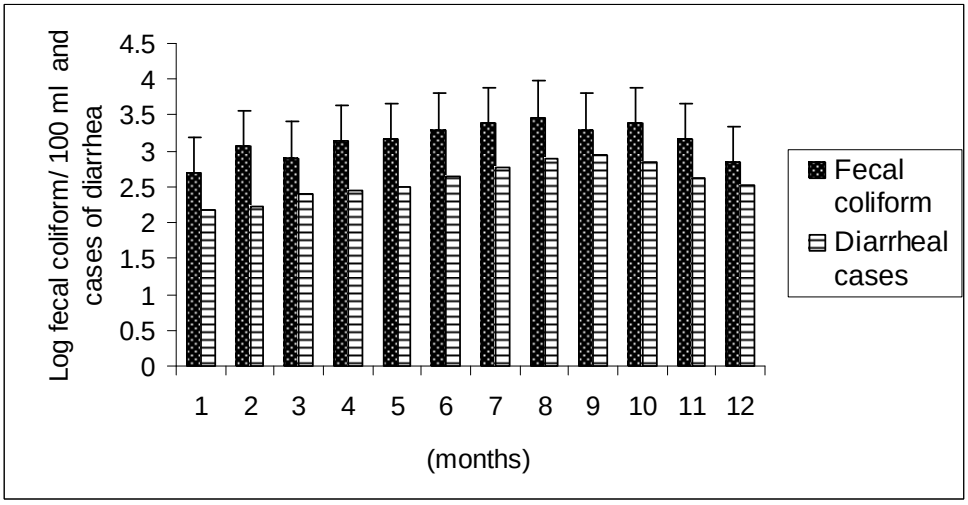
In order to evaluate the multi-drug resistance pattern in *E. coli* 2 which was isolated in present study from drinking water of Khairpur city the disc diffusion method was applied. A total 72 drinking water sample were collected and analyzed by membrane filtration method during 1 year study from various points in Khairpur City. Out of these 58 (80.55%) samples were found to be contaminated with thermo-tolerant indole negative strains of *Escherichia coli* known as *E. coli* 2. This type (indole negative) *E. coli* was also observed by Schets and Havelaar (1991) in a comparative study of indole production and beta glucornidase activity for detection of *E. coli.* They reported that 2% *E. coli* strains were indole negative. The susceptibility of these isolates to 35 antibiotics was studied by disc diffusion method and the organism was highly sensitive to levoflaxin, cefipime, enoxobid, noroxin, tarivid, ciproxin, avelox, amikacin, kanamycin, rocifin, pipenedic acid and slightly sensitive to cravit, naladixic acid, neomycin, cefizox, fortum cefotaxime, cefizox, fortum, tobramycin and cefoperoxone. The resistance against 16 antibiotics such as meropenem, linkomycin, fusidic acid, orbenin, penicillin, streptomycin, bacitracin, minocin, zinacef, amoxil, ceclor, claracid, cephalexin, augmentin, cephradin and dalacin was shown by these isolates (Table 8). We report the presence of multi-drug resistance in thermo-tolerant *Escherichia coli* isolated in municipal water with different levels of prevalence in Khairpur city. In this study a higher number of positive results were obtained in all sampling points indicating more fecally polluted municipal water (Table 9).

Fig. 2
Fecal Coliform per 100 ml Drinking Water of Khairpur City in Comparison with Cases of Diarrhea (2005) (n =24)



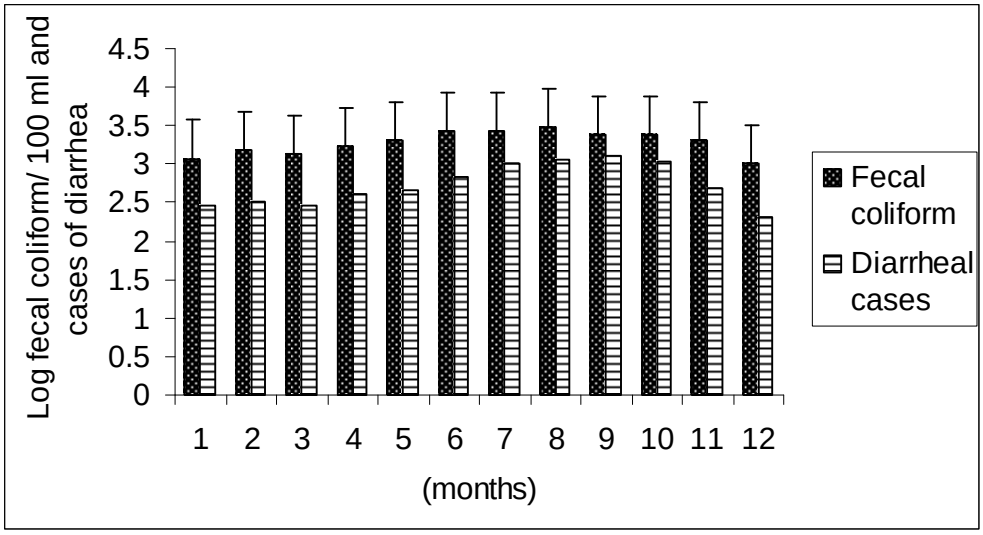
Monthly distribution of fecal coliform (*E. coli*) in 100 ml drinking water sample from municipal water of Khairpur during 2005. Diarrheal cases are also shown during same time period. Error bar indicate the ± SE Log cfu/100ml

Fig. 2
Fecal Coliform per 100 ml Drinking Water of Khairpur City in Comparison with Cases of Diarrhea (2006) (n =24)



Monthly distribution of fecal coliform (*E. coli*) in 100 ml drinking water sample from municipal water of Khairpur during 2006. Diarrheal cases are also shown during same time period. Error bar indicate the ± SE Log cfu/100ml.

Fig.3
Fecal Coliform per 100 ml Drinking Water of Khairpur City in Comparison with Cases of Diarrhea (2007) (n =24)



Month wise distribution of fecal coliform (*E. coli*) in 100 ml drinking water sample from municipal water of Khairpur during 2007. Diarrheal cases are also shown during same time period. Error bar indicate the ± SE Log cfu/100ml

Figure 4
Frequency of Bacteriologically Contaminated Drinking Water Samples from Khairpur City

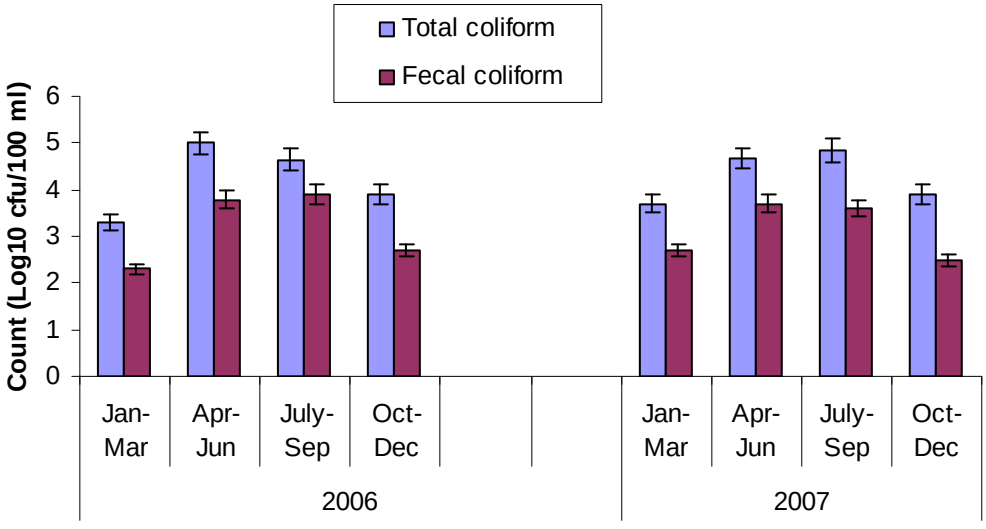


Figure 5

Number of Total and Fecal coliform Bacteria per 100 ml of Drinking Water of Khairpur City

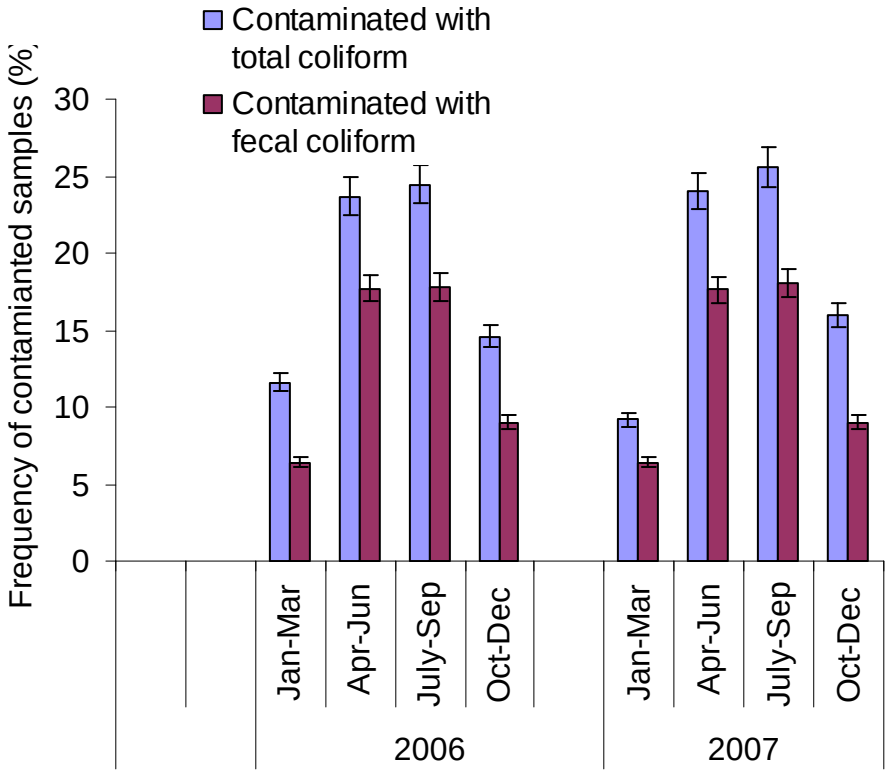


Table 6
Microbiological results (Minimum, maximum and average of Total and *Escherichia coli*) (n = 90)

		Total coliform (Log cfu/ 100ml)			*Escherichia coli* (Log cfu/ 100ml)			
Site	n	Max.	Min.	Av.	Max.	Min.	Av.	WHO limits
Main reservoir	30	3.00	3.94	3.68	1.46	2.47	2.05	00/100ml
Distribution line	30	3.79	4.20	4.06	2.00	3.26	3.00	00/100ml
Consumer tap	30	4.00	4.30	4.15	2.50	3.53	2.99	00/100ml

Table.7
Fecal Coliform Descriptive Statistics of Drinking Water Samples from Khairpur City (2005-2007) (n =72)

MONTH	MINIMUM LOG CFU/100ML	MAXIMUM LOG CFU/100ML	MEAN LOG CFU /100ML	± S.E LOG CFU/100 ML
January	2.6	3.0	2.9	2.3
February	2.8	3.1	3.0	2.3
March	2.9	3.1	3.0	2.2
April	3.0	3.2	3.1	2.2
May	2.9	3.3	3.1	2.5
June	3.0	3.4	3.2	2.7
July	3.0	3.4	3.2	2.6
August	3.2	3.4	3.3	2.6
September	3.2	3.4	3.3	2.6
October	3.3	3.3	3.3	1.5
November	3.0	3.3	3.1	2.3
December	2.6	3.0	2.8	2.1

Fig. 6
Annual Incidence Rate of Diarrhea in Khairpur City during 2005-2007

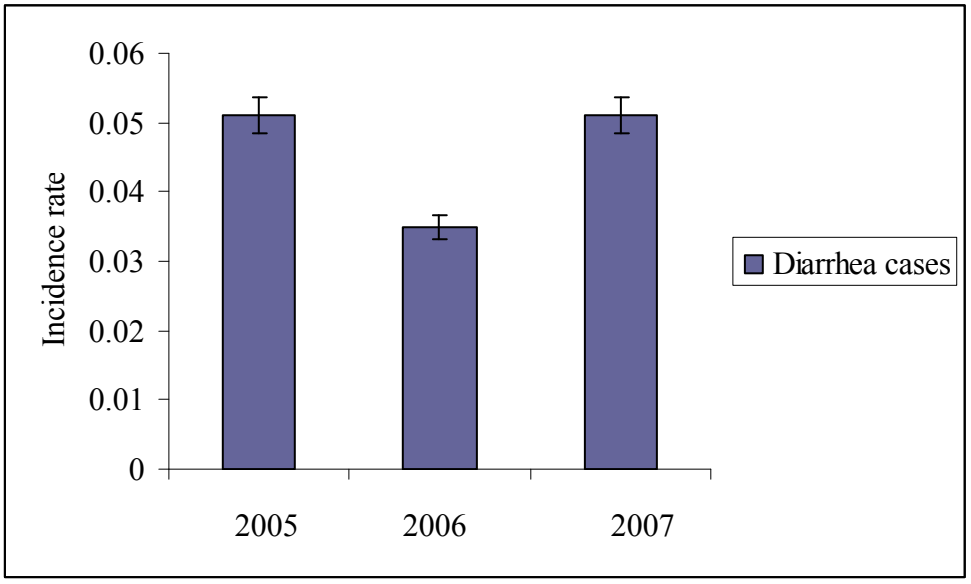
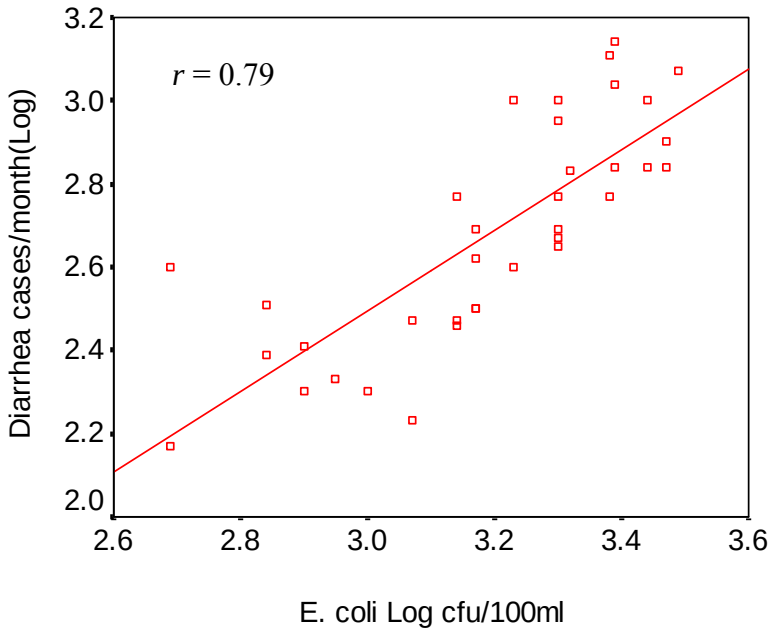


Fig. 7



Correlation between *E. coli* counts per100 ml of drinking water and diarrhea cases per month in Khairpur city.

Table 8

Multi-drug resistance of thermo-tolerant *E. coli 2*

Antibiotic	Quantity (µg)	Sensitivity	Antibiotic	Quantity (µg)	Resistant
Cefipime	5	S	Meropenem	10	R
Emepenem	30	S	Lyncomycin	10	R
Nalidixic acid	10	S	Fusidic Acid	10	R
Neomycin	30	S	Orbenin	5	R
Cefizox	30	S	Penicillin	10	R
Enoxobid	30	S	Streptomycin	10	R
Fortum	30	S	Bacitracin	10	R
Cefotexime	30	S	Monocin	30	R
Noroxin	30	S	Zinacef	30	R
Tarivid	10	S	Amoxil	10	R
Tobramycin	5	S	Ceclor (BBL)	30	R
Ciproxin	10	S	Clarid	15	R
Cefoperoxone	5	S	Cephalexin	30	R
Avelox	75	S	Augmentin	30	R
Amikacin	5	S	Cephradime	30	R
Kanamycin	30	S	Dalacin	2	R
Rocifix	30	S			
Pipenedic Avid	20	S			
Levofloxin	5	S			

Table 9

Prevalence of Thermotolerant *E. coli* 2 in Municipal Water Collected from Different Points in Khairpur City (n= 72)

Source of water	Site	No. of samples	No. of + ve for *E. coli*	*E. coli* (%)
Surface water	Reservoir	24	13	54.00
Surface water	Distribution line	24	22	92.00
Surface water	Consumer's tap	24	23	96.00
Total		72	58	80.55

3.1.4 Enumeration of Fecal Coliform (*Escherichia coli)* per 100 ml of Drinking Water of Sukkur City

The bacteriological quality of drinking water of Sukkur was evaluated by counting the fecal coliform bacteria in 100 ml drinking water samples collected from main reservoirs of municipal water of Sukkur city during 2005-2007.The densities of fecal coliform throughout study period were found higher in the summer months than winter months. In 2005 the number of fecal coliform bacteria per 100 ml of drinking water was different in different months. In 2005 January the number of fecal coliform (*E. coli*) was 2.9 log cfu, in February 3.1 log cfu, in March 3.2 log cfu, in April 3.3 log cfu, in May 3.4 log cfu, in June 3.4 log cfu, in July 3.5 log cfu, in August 3.6 log cfu, in September 3.5 log cfu, in October 3.0 log cfu, in November 3.1 log cfu and in December the number of fecal coliform (*E. coli*) was 2.8 log cfu/100ml of water, as is evident in Fig 8. The concentration of *E. coli* was significantly higher in summer months than in cold months ($p< 0.004$) In 2006, in January the number of fecal coliform (*E. coli*) was 3.3 log cfu, in February 3.3 log cfu, in March 3.3 log cfu, in April 3.4 log cfu, in May 3.5, log cfu in June 3.5 log cfu, in July 3.6 log cfu, in August 3.6 log cfu, in September 3.6 log cfu, in October 3.7, log cfu in November 3.3 log cfu, and in December the number of fecal coliform (*E. coli*) was 3.3 log cfu/100ml as shown in Fig. 9. The concentration of *E. coli* was significantly higher in summer months than in cold months ($p< 0.037$) In 2007, in January the number of fecal coliform (*E. coli*) was 2.9 log cfu, in February 3.1 log cfu, in March 3.2 log cfu, in April 3.2 log cfu, in May 3. log cfu 3, in June3.4 log cfu, in July3.6 log cfu, in August 3.8, in September 3.5, log cfu in October 3. log cfu 4, in November 3.5 log cfu, and in December the number of fecal coliform (*E. coli*) was 3.0 log cfu/100 ml) as given in Fig.10. The concentration of *E. coli* was significantly higher in summer months than in cold months ($p< 0.042$) Total and fecal coliform bacteria in water

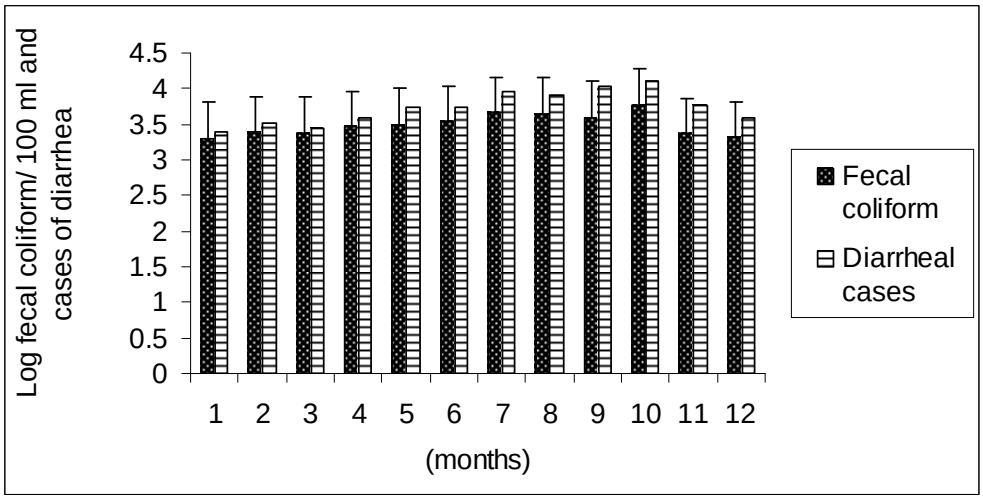
samples collected from randomly selected reservoirs, distribution line, households, hotel and hospitals were determined as shown in table that all samples from all sampling sites were found contaminated with total and fecal coliform (Table 10) .The fecal coliform bacteria per 100 ml in Sukkur municipal water throughout three years of study were lowest in the month of December with the minimum 2.9 log cfu, maximum 3.3 log cfu and the mean 3.0 log cfu and highest in the month of August minimum 3.6, maximum 3.8 log cfu, and the mean 3.7 log cfu/100ml of water were counted (Table 11).

3.1.5 Prevalence of Diarrheal Disease in Sukkur City

In same way as in Khairpur city, for Sukkur city the data of diarrheal diseases were provided by District Health Office and from District Hospital monthly reports. The incidence rate of diarrhoeal disease in 2005, 2006 and 2007 remained 0.07, 0.08 and 0.07 respectively (Fig 11).

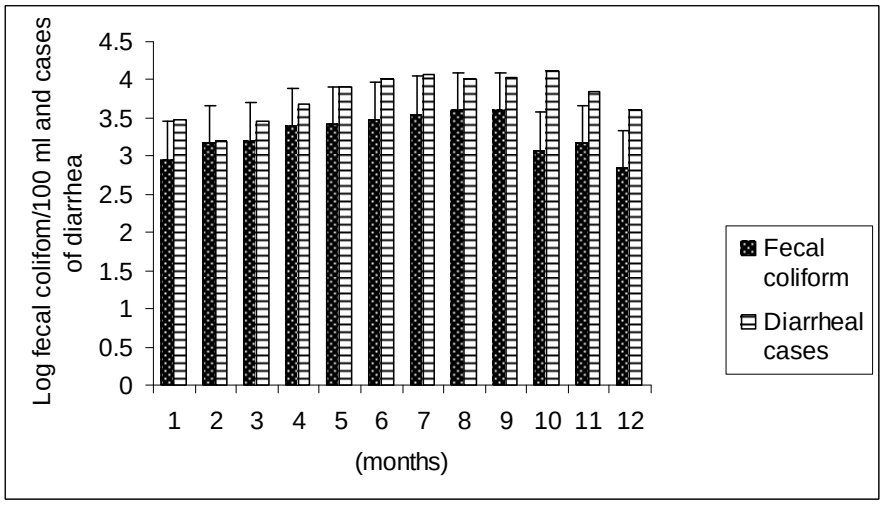
Data of enumeration of fecal coliform (*E. coli*) obtained in this study are Log transformed. According to District Health Authorities the prevalence of diarrheal disease may be due to consumption of contaminated water. According to these data the highest number of diarrheal cases was reported in 2005 throughout the year as shown in Fig 8. In 2006 and 2007 the situation more or less is same (Fig 9 and 10). The number of fecal coliform roughly directly proportional to the diarrheal cases reported by district l hospital Sukkur. A Moderate positive correlation was found between number of fecal coliform per 100 ml of water used for drinking in the city of Khairpur and cases of diarrhea the population using that unsafe water during the study period ($r = 0.60$) (Fig. 12).

Fig. 8
Fecal Coliform per 100 ml Drinking Water of Sukkur City in Comparison with Cases of Diarrhea (2005) (n =24)



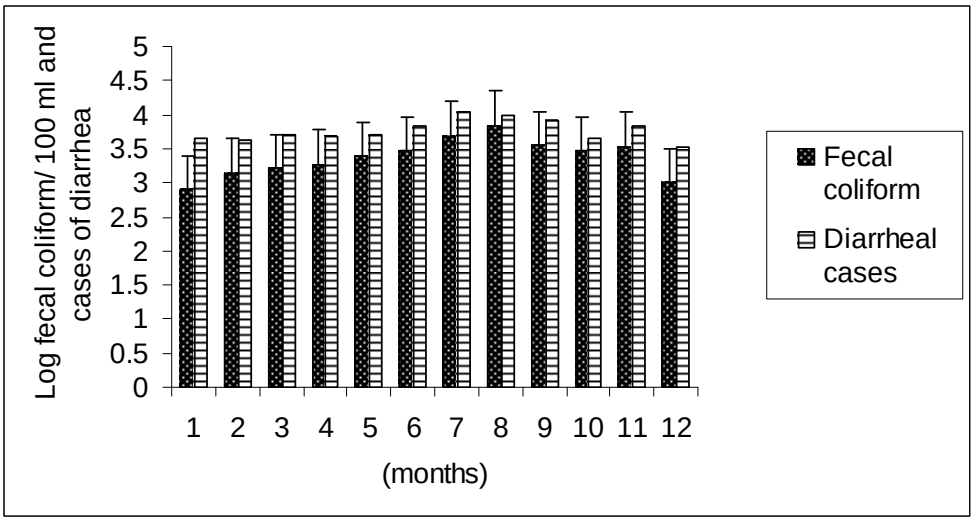
Monthly distribution of fecal coliform (*E. coli*) in 100 ml drinking water sample from municipal water of Sukkur City during 2005. Diarrheal cases are also shown during same time period. Error bar indicate the ± SE Log cfu/100ml

Fig.9
Fecal Coliform per 100 ml Drinking Water of Sukkur City in Comparison with Cases of Diarrhea (2006) (n =24)



Monthly distribution of fecal coliform (*E. coli*) in 100 ml drinking water sample from municipal water of Sukkur City during 2006. Diarrheal cases are also shown during same time period. Error bar indicate the ± SE Log cfu/100ml

Fig. 10
Fecal Coliform per 100 ml Drinking Water of Sukkur City in Comparison with Cases of Diarrhea (2007) (n =24)



Monthly distribution of fecal coliform (*E. coli*) in 100 ml drinking water sample from municipal water of Sukkur city during 2007, Diarrheal cases are also shown during same time period. Error bar indicate the ± SE Log cfu/100ml

Table 10
Bacteriological Results of Total and Fecal coliform Bacteria
(n = 120)

		Total coliform (Log cfu/ 100ml)			*Escherichia coli* (Log cfu/ 100ml)			
Site	n	Max.	Min.	Av.	Max.	Min.	Av.	WHO limits
Main reservoir	24	4.00	4.54	4.36	2.30	3.30	2.90	00/100ml
Distribution line	24	4.23	4.60	4.50	2.60	3.39	3.02	00/100ml
Household	24	4.32	4.65	4.52	2.47	3.36	3.02	00/100ml
Hotel	24	4.14	4.64	4.52	2.69	3.41	3.04	00/100ml
Hospital	24	4.07	4.65	4.49	2.69	3.47	3.03	00/100ml

Table 11
Fecal Coliform Descriptive Statistics of Drinking Water Samples from Sukkur City (2005-2007) (n =72)

MONTH	MINIMUM LOG CFU/100ML	MAXIMUM LOG CFU/100ML	MEAN LOG CFU/100ML	±S.E LOG CFU/100ML
January	2.9	3.3	3.0	2.5
February	3.1	3.3	3.2	2.5
March	3.2	3.3	3.2	2.4
April	3.2	3.4	3.3	2.5
May	3.3	3.5	3.4	2.3
June	3.3	3.4	3.4	2.3
July	3.5	3.6	3.6	2.6
August	3.6	3.8	3.7	2.9
September	3.5	3.6	3.5	2.1
October	3.0	3.7	3.5	2.6
November	3.1	3.5	3.3	2.7
December	2.8	3.3	3.1	2.6

Fig.11
Annual Incidence Rate of Diarrhea in Sukkur City during 2005-2007

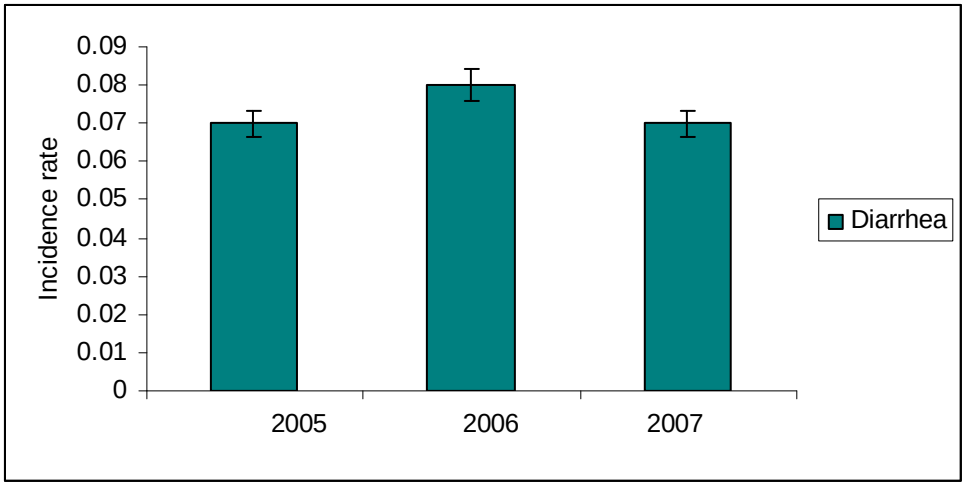
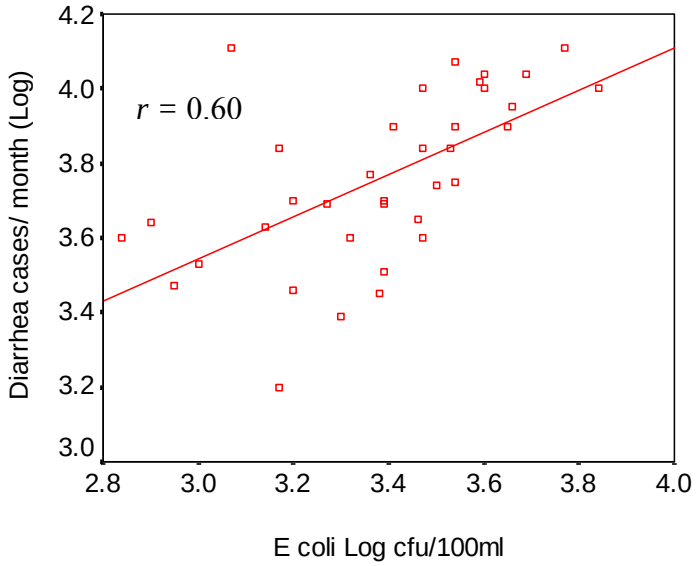


Fig.12



Correlation between *E. coli* counts per100 ml of drinking water and diarrhea cases per month in Sukkur city.

3.1.6 Enumeration of Fecal Coliform (*Escherichia coli)* per 100 ml of Drinking Water of Rohri City

The fecal coliform count per 100 ml drinking water samples collected from the municipal water main reservoirs of Rohri city were higher in the summer months than in winter months throughout study period . In 2005, in January the number of fecal coliform (*E. coli*) was 2.9 log cfu, in February 3.0 log cfu, in March 3.0 log cfu, in April 2.9 log cfu, in May 3.1 log cfu, in June 3.2 log cfu, in July 3.4 log cfu, in August 3.3 log cfu, in September 3.3 log cfu, in October 3.4 log cfu, in November 3.5 log cfu and in December the number was 3.3 log cfu/100 ml as is evident in Fig. 13. The concentration of *E. coli* was significantly higher in summer months than in cold months ($p< 0.022$). In 2006, in January the number of fecal coliform (*E. coli*) was 2.7 log cfu, in February 3.0 log cfu, in March 3.1 log cfu, in April 3.0 log cfu, in May 3.1 log cfu, in June 3.1 log cfu, in July 3.2 log cfu, in August 3. log cfu 2, in September 3.0 log cfu, in October 3.4 log cfu, in November 3.4 log cfu and in December the number of fecal coliform (*E. coli*) was 3.0 log cfu/100 ml) as shown in Fig. 14. The concentration of *E. coli* was significantly higher in summer months than in cold months ($p< 0.013$). In 2007, in January the number of fecal coliform (*E. coli*) was 2.3 log cfu, in February 3.3 log cfu, in March 3.3 log cfu, in April 3.3 log cfu, in May 3.4 log cfu, in June 3.5 log cfu, in July 3.5 log cfu, in August 3.6, log cfu in

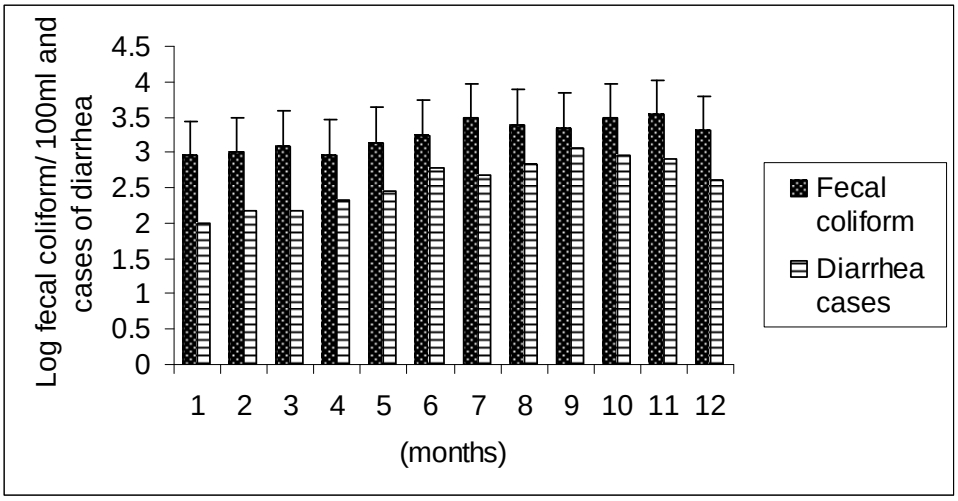
September 3.6 log cfu, in October 3.6 log cfu, in November 3.4 log cfu and in December the number of fecal coliform (*E. coli*) was 3.3 log cfu/100 ml) as given in Fig. 15. The concentration of *E. coli* was significantly higher in summer months than in cold months ($p < 0.003$).The number of fecal coliform bacteria during the three years of study period remained as minimum 2.7 log cfu, maximum 3.1 log cfu, and mean 2.9 log cfu/100 ml in the month of January and highest in October, the minimum number of fecal coliform was 3.4 log cfu, maximum 3.5 log cfu and the mean 3.4 log cfu/100 ml (Table.12).

3.1.7 Prevalence of Diarrheal Disease in Rohri City

Similarly for Rohri city the data of diarrhea diseases were also provided by District Health Office and from District Hospital monthly reports The incidence rate of diarrhoeal disease in 2005, 2006 and 2007 remained 0.06, 0.06 and 0.05 respectively (Fig16)

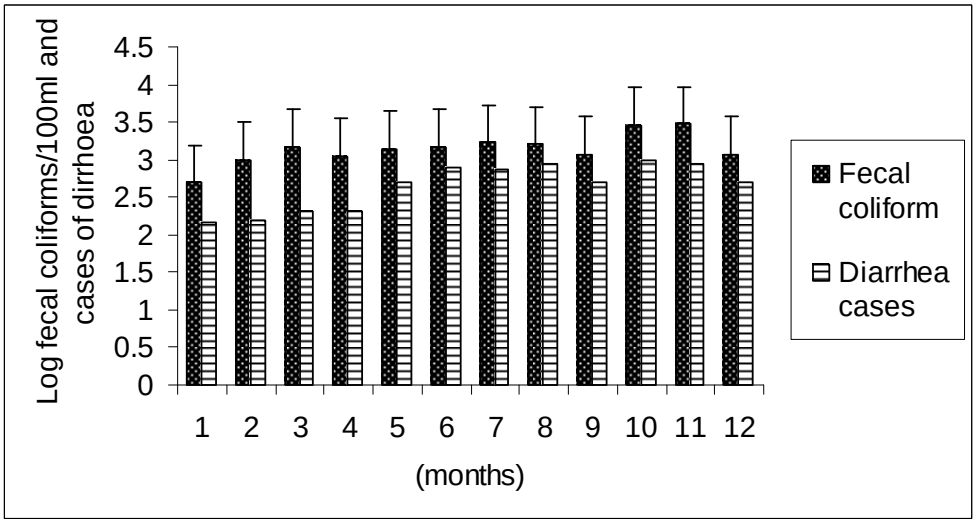
The data of enumerating fecal coliform (*E. coli*) obtained in this study are log transformed. According to District Health Authorities the prevalence of diarrheal disease due to consumption of contaminated water reported through doctors. According to these data the diarrheal case number increased from June- December in 2005 as shown in Fig. 13. The diarrhoeal were observed increasing from May –December and June –October in 2006 and 2007 respectively as shown in Fig 14 and 15. The number of fecal coliform roughly directly proportional to the diarrheal cases by district civil hospital Rohri A moderate positive correlation was found between number of fecal coliform per 100 ml of water used for drinking in the city of Khairpur and cases of diarrhea the population using that unsafe water during the study period ($r = 0.61$) as shown in fig 17.

Fig. 13
Fecal Coliform per 100 ml Drinking Water of Rohri City in Comparison with Cases of Diarrhea (2005) (n =24)



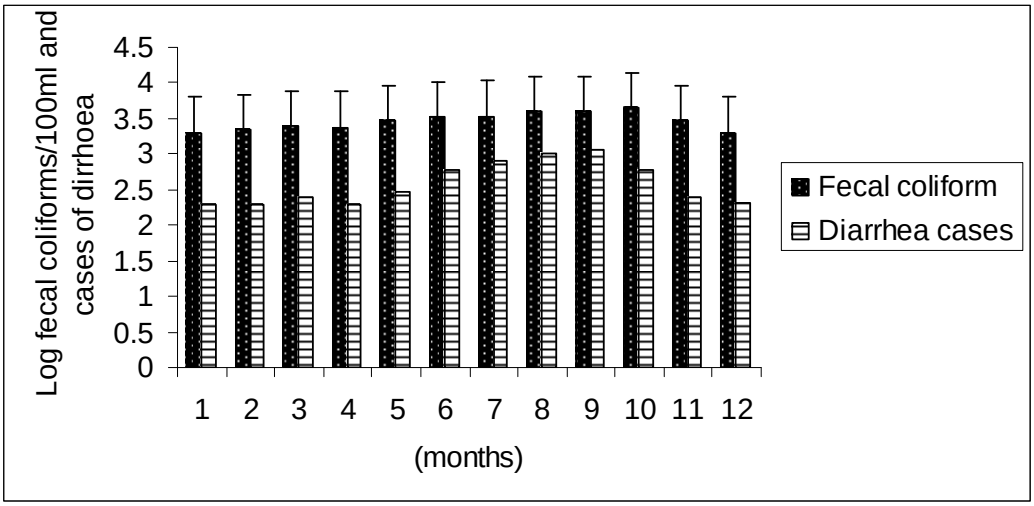
Monthly distribution of fecal coliform (*E. coli*) in 100 ml drinking water sample from municipal water of Rohri city during 2005. Diarrheal cases are also shown during same time period**.** Error bar indicate the ± SE Log cfu/100ml

Fig. 14
Fecal Coliform per 100 ml Drinking Water of Rohri City in Comparison with Cases of Diarrhea (2006) (n =24)



Monthly distribution of fecal coliform (*E. coli*) in 100 ml drinking water sample from municipal water of Rohri city during 2006. Diarrheal cases are also shown during same time period. Error bar indicate the ± SE Log cfu/100ml

Fig. 15
Fecal Coliform per 100 ml Drinking Water of Rohri City in Comparison with Cases of Diarrhea (2007) (n =24)



Monthly distribution of fecal coliform (*E. coli*) in 100 ml drinking water sample from municipal water of Rohri city during 2007. Diarrheal cases are also shown during same time period. Error bar indicate the ± SE Log cfu/100ml

Table 12
Fecal Coliform Descriptive Statistics of Drinking Water Samples from Rohri City (2005-2007) (n =72)

MONTHS	MINIMUM LOG CFU/100ML	MAXIMUM LOG CFU/100ML	MEAN LOG CFU/100ML	± S.E LOG CFU
January	2.6	3.1	2.9	2.4
February	3.0	3.2	3.0	2.3
March	3.0	3.2	3.1	2.3
April	2.9	3.3	3.1	2.6
May	3.1	3.5	3.3	2.8
June	2.9	3.5	3.2	2.8
July	3.2	3.5	3.4	2.8
August	3.2	3.4	3.3	2.6
September	3.3	3.5	3.4	2.6
October	3.4	3.5	3.5	2.2
November	3.4	3.5	3.4	2.3
December	3.0	3.3	3.1	2.4

Fig. 16
Annual Incidence Rate of Diarrhea in Rohri City during 2005-2007

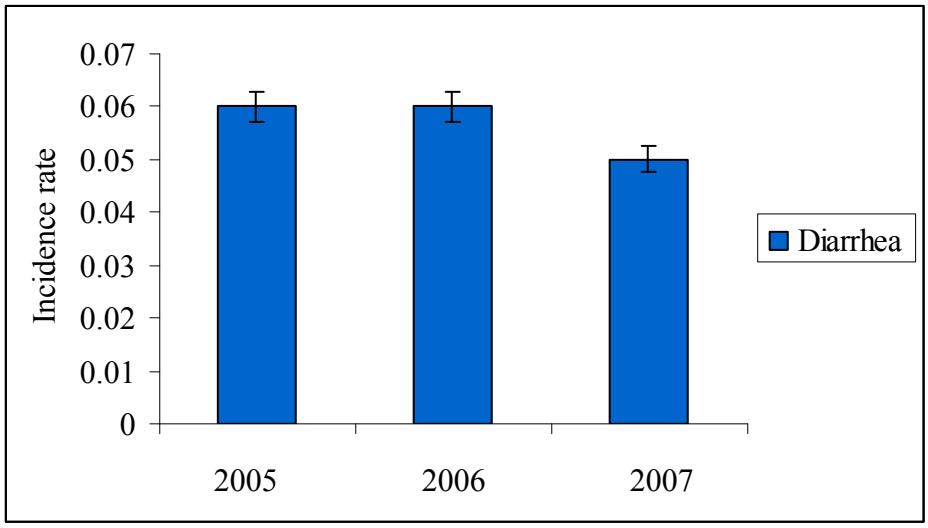
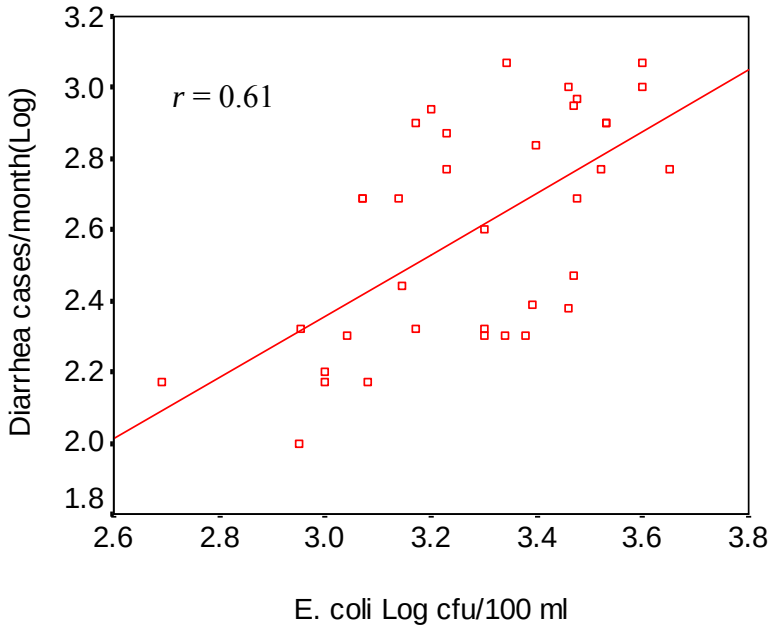


Fig.17



Correlation between *E. coli* counts per100 ml of drinking water and diarrhea cases per month in Rohri city.

3.1.8 Correlation between Number of Fecal Coliform and Diarrheal Cases

Correlation between the number of fecal coliform and diarrhea cases (Data of diarrhea cases were obtained from District health office) were analyzed by using the Special Package for Social Sciences (SPSS version 10). A strong correlation was observed between counts of fecal coliform (*E. coli*) and number of diarrhea cases in Khairpur, Sukkur and Rohri city throughout study period ($r = 0.79$), Fig. 7 ($r = 0.60$) Fig. 12 and ($r = 0.61$) Fig.17 respectively.

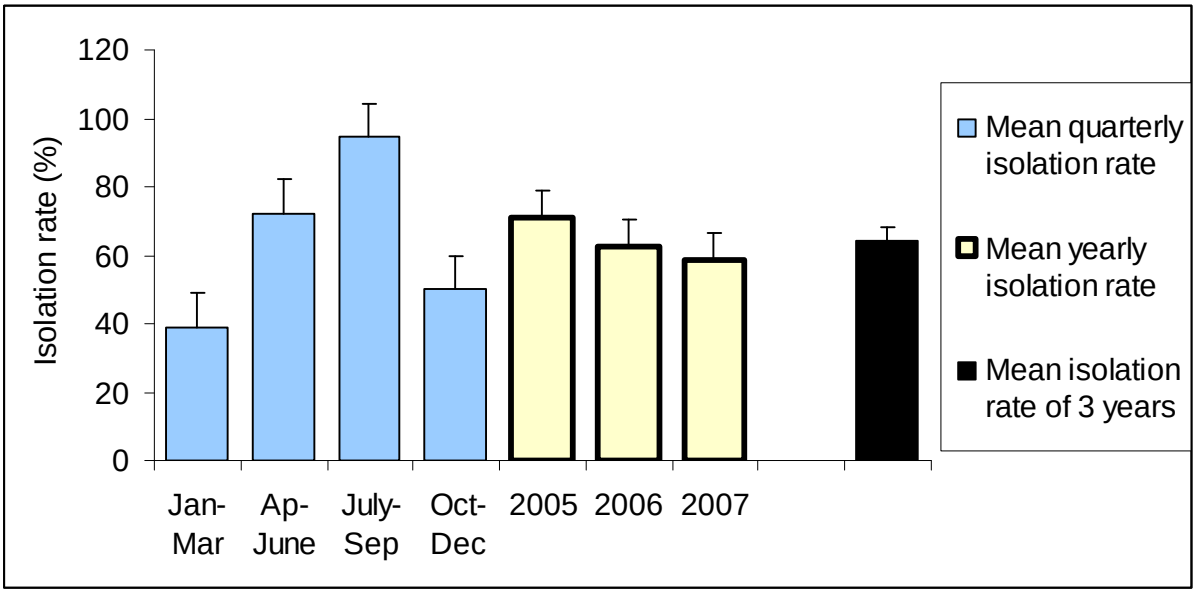
3.2 Isolation and Identification of Waterborne Bacterial Pathogens from Drinking Water (Municipal Water) of Khairpur, Sukkur, and Rohri City (2005-2007).

It has been shown that the presence of pathogens correlates well with presence of fecal contamination (Leclerc *et al.* 2001) for example, an outbreak of *E. coli* O157 and Campylobacteriosis was associated with contamination of drinking water supply reported in 1995 (Jones and Roworth 1996). Keeping in view the public health impact of drinking water quality in the area under study the present study was carried out to isolate bacterial indicator of fecal contamination especially (*E. coli*) and other possible waterborne bacterial pathogens present in the municipal water which was used for drinking, cooking, bathing and other domestic uses by the population of area under study. The comparison of bacteriological quality of drinking water of these cities with the WHO guideline values for drinking water was also intended in present study.

3.2.1 *Escherichia coli* 1 from Drinking Water of Khairpur City.

The *E. coli* 1 was isolated, identified and confirmed by API 20E identification system from drinking water (Municipal water) of Khairpur city during consecutive three years of study (2005-2007) as shown in color plate 4 and Table 13. The isolation rate of *Escherichia coli* 1 was recorded quarterly throughout study period which was varying in different quarter of study period. The mean quarterly isolation rate in January-March was 39%, in April-June it was 72.2%, in July- September it was 94.4% and in October-December it was 50%. The isolation rate was also recorded in every year of the study. In 2005 the mean yearly isolation rate was 71%, in 2006 the mean yearly isolation rate was 62.5% and in 2007 the mean yearly isolation rate was 58.33%. The mean isolation rate of three years was 64% as shown in Fig.18a. The isolation rate of *E. coli* 1was significantly greater in summer months than in winter months ($p<0.01$)

Fig.18a
Yearly, Quarterly and Total Isolation Rate of *Escherichia coli* 1 in Drinking Water of Khairpur City (2005-2007). (n =72)

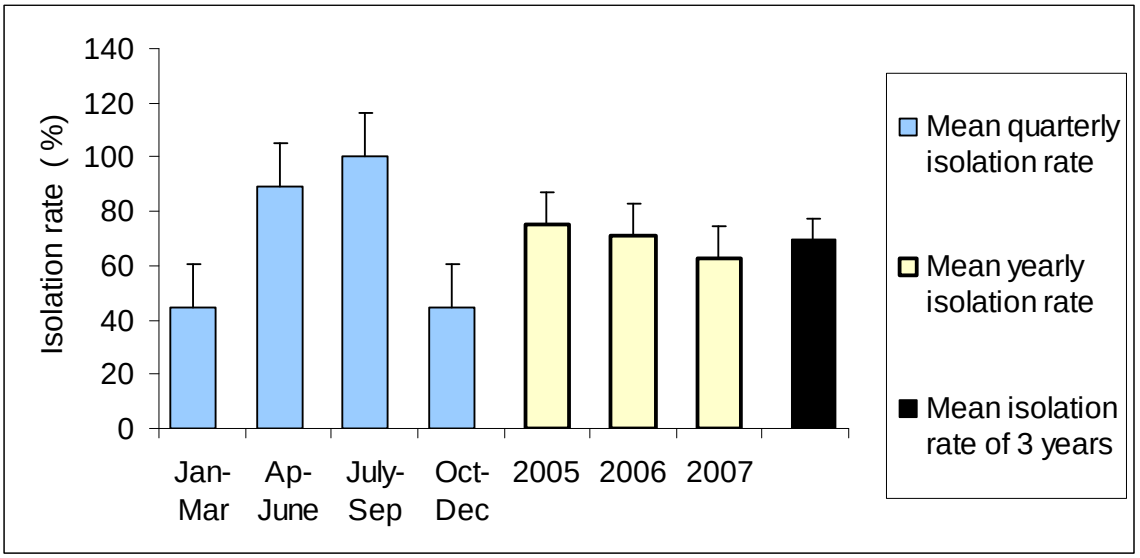


Distribution of *E. coli* 1 in drinking water of Khairpur city. A comparative mean quarterly isolation rate is shown in different quarter of study period. The mean yearly and mean total isolation rate of three years is also shown. Error bar indicate the ± SE of %

3.2.2 *Escherichia coli* 1 from Drinking Water of Sukkur City.

In drinking water (Municipal water) samples collected from Sukkur the *E. coli*1were isolated in present study during 2005-2007. The isolation rate of *Escherichia coli* 1 was recorded quarterly throughout the study period which was varying in different quarters of study period. The mean quarterly isolation rate in January-March was 44.4%, in April-June it was 89%, in July-September it was 100% and in October-December it was 44.4%. In 2005 the mean yearly isolation rate of *E. coli* 1 was 75%, in 2006 the mean yearly isolation rate was 71% and in 2007 the mean yearly isolation rate was 62.5%. The mean isolation rate of three years was 69.4% as given in Fig 18 b. The isolation rate of *E. coli* 1was significantly greater in summer months than in winter months ($p<0.01$)

Fig.18b
Yearly, Quarterly and Total Isolation Rate of *Escherichia coli* 1 in Drinking Water of Sukkur City (2005-2007). (n =72)

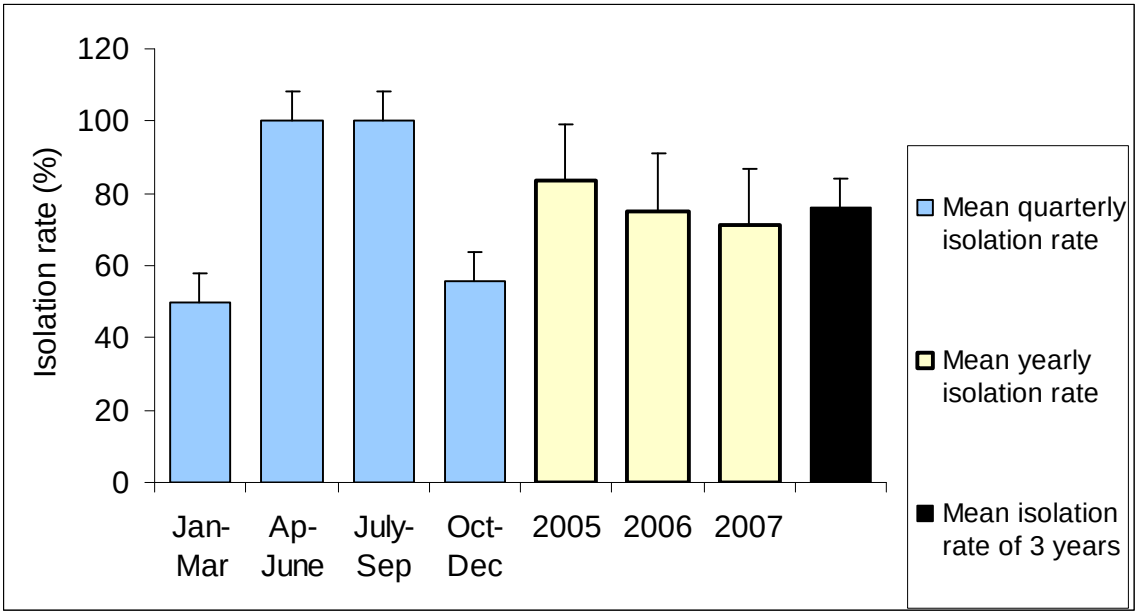


Distribution of *E. coli* 1 in drinking water of Sukkur city. A comparative mean quarterly isolation rate is shown in different quarter of study period. The mean yearly and mean total isolation rate of three years is also shown. Error bar indicate the $\pm$ SEM of %

3.2.3 *Escherichia coli* 1 from Drinking Water of Rohri City.

In Rohri the *E. coli* 1 was isolated from drinking water (Municipal water) samples. The isolation rate of *Escherichia coli* 1 in different quarters throughout the study period was different. The mean quarterly isolation rate in January-March was 50%, in April-June it was 100%, in July-September it was 100% and in October-December the isolation rate was 55.5%. In 2005 the mean yearly isolation rate was 83.3%, in 2006, the mean yearly isolation rate was 75% and in 2007 the mean yearly isolation rate was 71%. The mean isolation rate of three years was 76.3% as shown in Fig.18c. The isolation rate of *E. coli* 1was significantly greater in summer months than in winter months in drinking water sample of Rohri city ($p<0.01$)

Fig.18c
Yearly, Quarterly and Total Isolation Rate of *Escherichia coli* 1 in Drinking Water of Rohri City (2005-2007). (n =72)



Distribution of *E. coli* 1 in drinking water of Rohri city. A comparative mean quarterly isolation rate is shown in different quarter of study period. The mean yearly and mean total isolation rate of three years is also shown. Error bar indicate the ± SEM of %

Color plate 4

The Biochemical Reaction of *Escherichia coli* 1 on API 20 E.

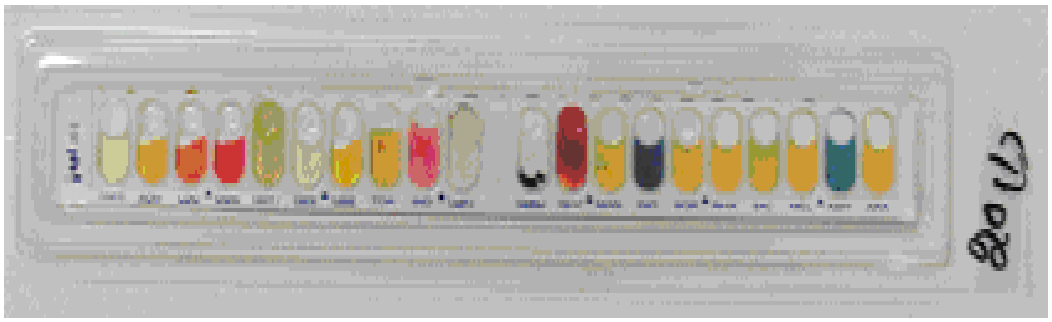


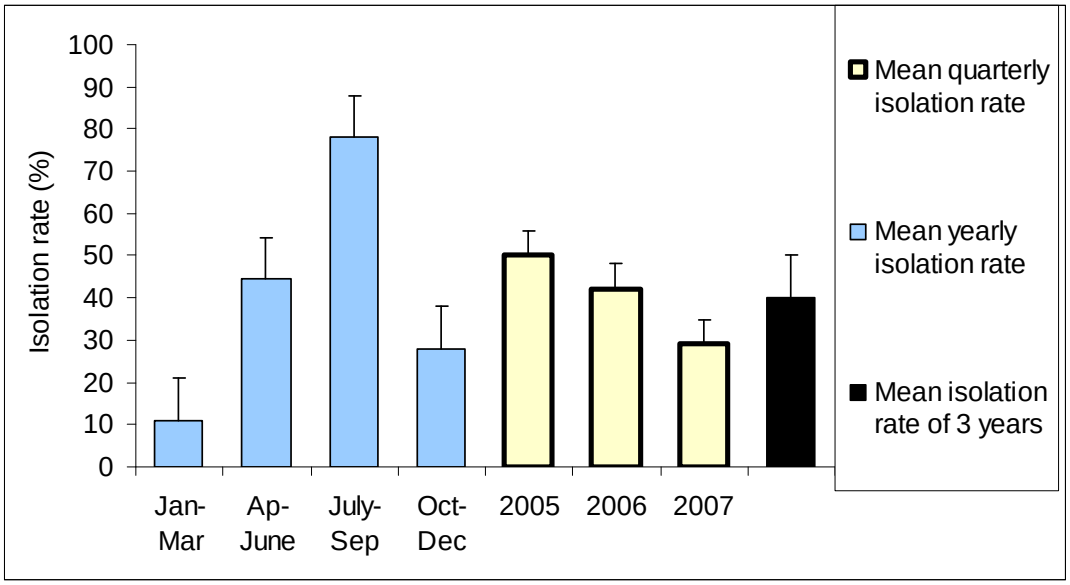
Table 13
Biochemical Reactions of *E. coli* 1 on API 20 E

SUBSTRATE	REACTION	SUBSTRATE	REACTION
ONPG	+	GLU	+
ADH	-	MAN	+
LDC	+	INO	-
ODC	+	SOR	+
CIT	-	RHA	+
H2S	-	SAC	+
URE	-	MEL	+
TDA	-	AMY	-
IND	+	ARA	+
VP	-	OXI	-
GEL	-		

3.2.4 *Escherichia coli* 2 from Drinking Water of Khairpur City

E. coli 2 was isolated, identified and confirmed by API 20E identification system as shown in color plate 5 and Table 14. The isolation rate of *Escherichia coli* 2 in different quarters throughout the study period was different. The mean quarterly isolation rate in January-March was 11.1%, in April-June it was 44.4%, in July- September it was 78% and October-December it was 28%. In 2005 the mean yearly isolation rate was 50%, in 2006 the mean yearly isolation rate was 42%, and in 2007 the mean yearly isolation rate was 29%. The mean isolation rate of three years was 40.2% as shown in Fig. 19a. The difference in isolation rate of *E. coli* 2 in different quarters in drinking water of Khairpur city was not significant.

Fig. 19a
Yearly, Quarterly and Total Isolation Rate of *Escherichia coli* 2 in Drinking Water of Khairpur City (2005-2007). (n =72)

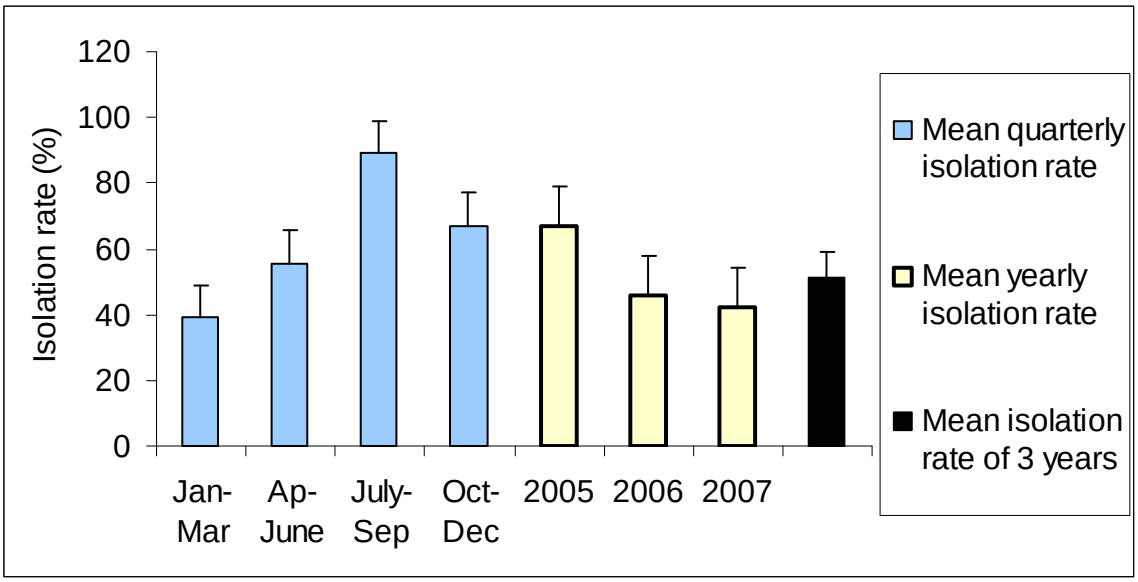


Distribution of *E. coli* 2 in drinking water of Khairpur City. A comparative mean quarterly isolation rate is shown in different quarter of study period. The mean yearly and mean total isolation rate of three years is also shown. Error bar indicate the ± SEM of %

3.2.5 *Escherichia coli* 2 from Drinking Water of Sukkur City

The isolation rate of *Escherichia coli* 2 in different quarters throughout the study period was different. The mean quarterly isolation rate in January-March was 39%, in April-June it was 55.5%, in July- September the isolation rate was 89% and in October-December it was 67%. In 2005 the mean yearly isolation rate was 67%, in 2006 the mean yearly isolation rate was 46% and in 2007 the mean yearly isolation rate was 42%. The mean isolation rate of three years was 51.3% as shown in Fig. 19b. The isolation rate of *E. coli* 2 was significantly greater in summer months than in winter months in drinking water sample of Sukkur city ($p<0.009$)

Fig. 19b
Yearly, Quarterly and Total Isolation Rate of *Escherichia coli* 2 in Drinking Water of Sukkur City (2005-2007). (n =72)

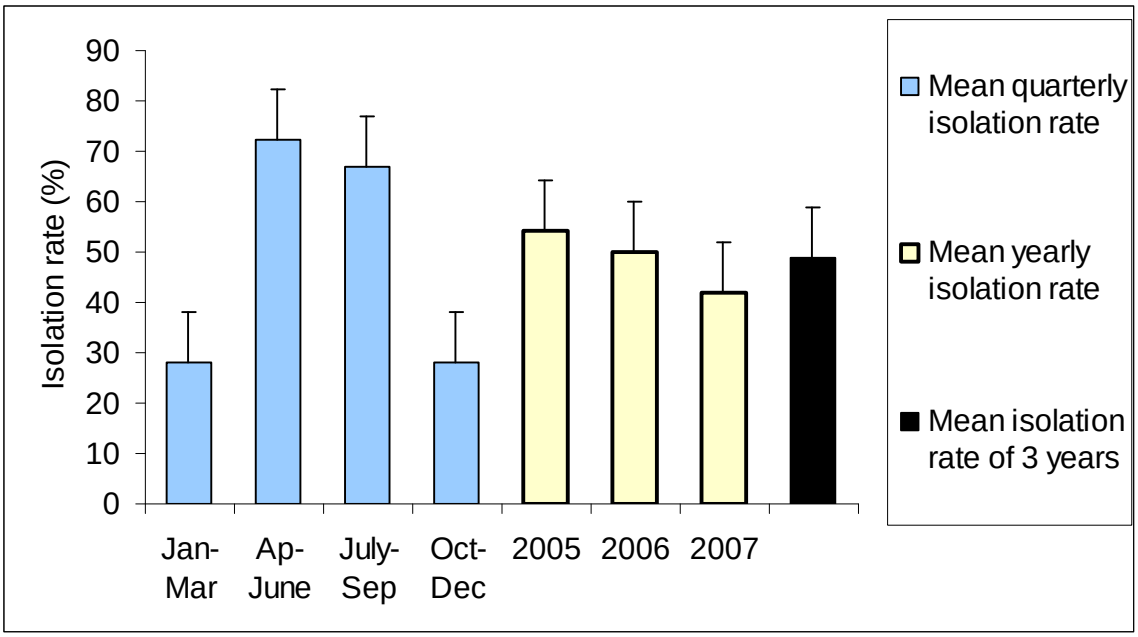


Distribution of *E. coli* 2 in drinking water of Sukkur city. A comparative mean quarterly isolation rate is shown in different quarter of study period. The mean yearly and mean total isolation rate of three years is also shown. Error bar indicate the $\pm$ SEM of %

3.2.6 *Escherichia coli* 2 from Drinking Water Rohri City

The isolation rate of *E. coli* 2 was different in different seasons in drinking water sample of Rohri city. The isolation rate in different quarters of three years i.e. in January-March the isolation rate was 28%, in April-June it was 72.2%, in July-September it was 67% and in October-December the isolation rate was 28%. In 2005 mean yearly isolation rate 54.1%, in 2006 mean yearly isolation rate was 50% and in 2007 mean yearly isolation rate was 42%. The mean isolation rate of three years was 49% as shown in Fig.19c The isolation rate of *E. coli* 1was significantly greater in summer months than in winter months in drinking water of Rohri city ($p<0.027$)

Fig.19c
Yearly, Quarterly and Total Isolation Rate of *Escherichia coli 2* in Drinking Water of Rohri City(2005-2007). (n =72)



Distribution of *E. coli* 2 in drinking water of Rohri city. A comparative mean quarterly isolation rate is shown in different quarter of study period. The mean yearly and mean total isolation rate of three years is also shown. Error bar indicate the ± SEM of %

Color plate 5 Biochemical Reaction of *E. coli* 2 on API 20E

Table 14
Biochemical Features of *E. coli* 2 on API 20E

SUBSTRATE	REACTION	SUBSTRATE	REACTION
ONPG	+	GLU	+
ADH	-	MAN	-
LDC	-	INO	-
ODC	-	SOR	+
CIT	-	RHA	+
H2S	-	SAC	-
URE	-	MEL	-
TDA	-	AMY	-
IND	-	ARA	+
VP	-	OXI	-
GEL	-		

3.3 Isolation and Identification of *Proteus spp.* from Drinking Water (Municipal Water) of Khairpur, Sukkur, and Rohri City (2005-2007)

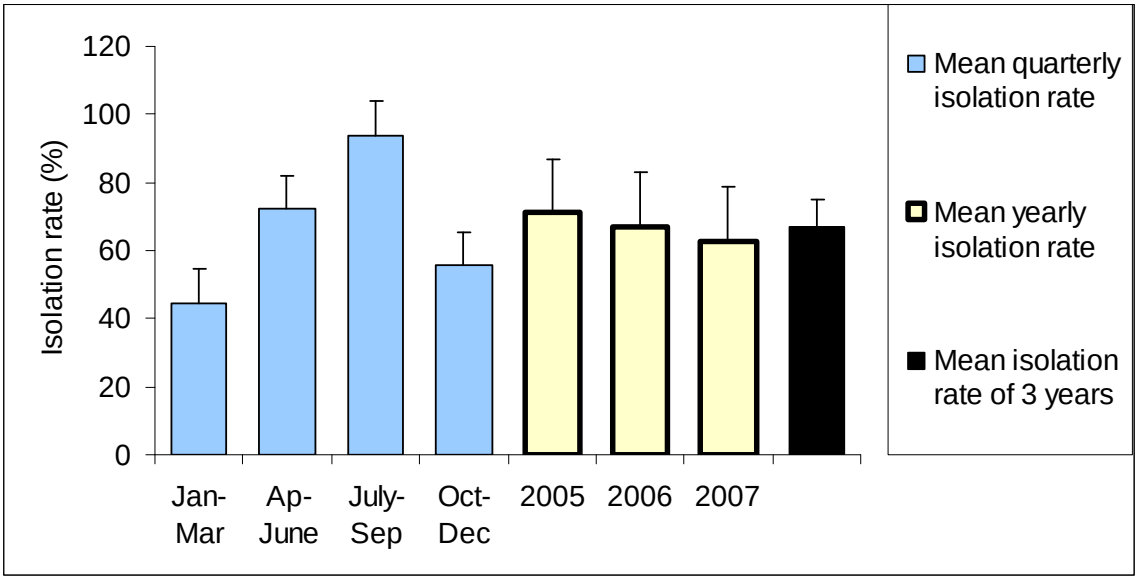
After *Escherichia coli, Proteus mirabilis* is on of the most frequent etiological agents associated with urinary tract infections (UTIs). *P. mirabilis* is a member of the normal microbiota of the mammalian intestinal tract and has been isolated from humans, dogs, monkeys, pigs, sheep, cattle, raccoons, cats, rats, and other mammals (Guentzel 1991). Additionally *P. mirabilis* is widely distributed in the environment, occurring in polluted water, manure, and soil. Besides UTI, this pathogen has been described as an opportunistic etiological agent in infection of respiratory tract and of the wounds burns, skin, ears, nose and throat as well gastroenteritis resulting from the contaminated food (Manos and Belas 2005., Muller 1989., Rozalski *et al*. 1997).

3.3.1 *Proteus mirabilis* from Drinking Water of Khairpur City

P. mirabilis was isolated, identified and confirmed by API 20E identification system from drinking water of Khairpur city during study period as shown in color plate 6 and table 15. The mean quarterly isolation rate in different quarters was different throughout study period. In

January-March the isolation rate was 44.4%, in April-June it was 72.2%, in July-September it was 94.4% and in October- December isolation rate was 55.5%. In 2005 the mean yearly isolation rate was 71%, in 2006 the mean yearly isolation rate was 67% and in 2007 the mean yearly isolation rate was 62.5%. The mean isolation rate of three years was 67% as is evident from Fig. 20a. The isolation rate of *P. mirabilis* was significantly greater in summer months than in winter months in drinking water of Sukkur city ($p<0.009$)

Fig. 20a
Yearly, Quarterly and Total Isolation Rate of *P. mirabilis* in Drinking Water of Khairpur City (2005-2007). (n =72)



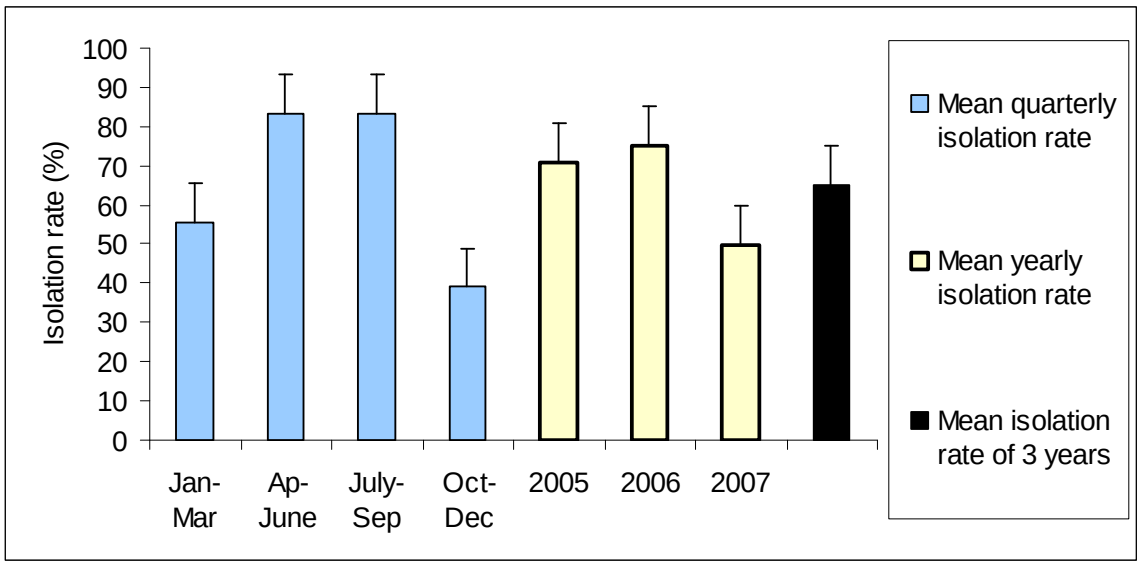
Distribution of *P. mirabilis* in drinking water of Khairpur city. A comparative mean quarterly isolation rate is shown in different quarter of study period. The mean yearly and mean total isolation rate of three years is also shown. Error bar indicate the ± SEM of %

3.3.2 *Proteus mirabilis* from Drinking Water of Sukkur City

In drinking water of Sukkur city the *P. mirabilis* was isolated during 2005-2007. The quarterly isolation rate in different quarters was different. The mean quarterly isolation rate in January-March was 55.5%, in April-June it was 83.3%, in July-September it was 83.3% and in October-December isolation was 39%. In 2005 the mean yearly isolation rate was 71%, in 2006 the mean yearly isolation rate was 75% and in 2007 the mean yearly isolation rate was 50%. The mean

isolation rate of three years was 65.2% as is shown in Fig. 20b. The isolation rate of *P. mirabilis* was significantly greater in summer months than in winter months in drinking water of Sukkur city ($p<0.009$)

Fig. 20b
Yearly, Quarterly and Total Isolation Rate of *P. mirabilis* in Drinking Water of Sukkur City (2005-2007). (n =72)

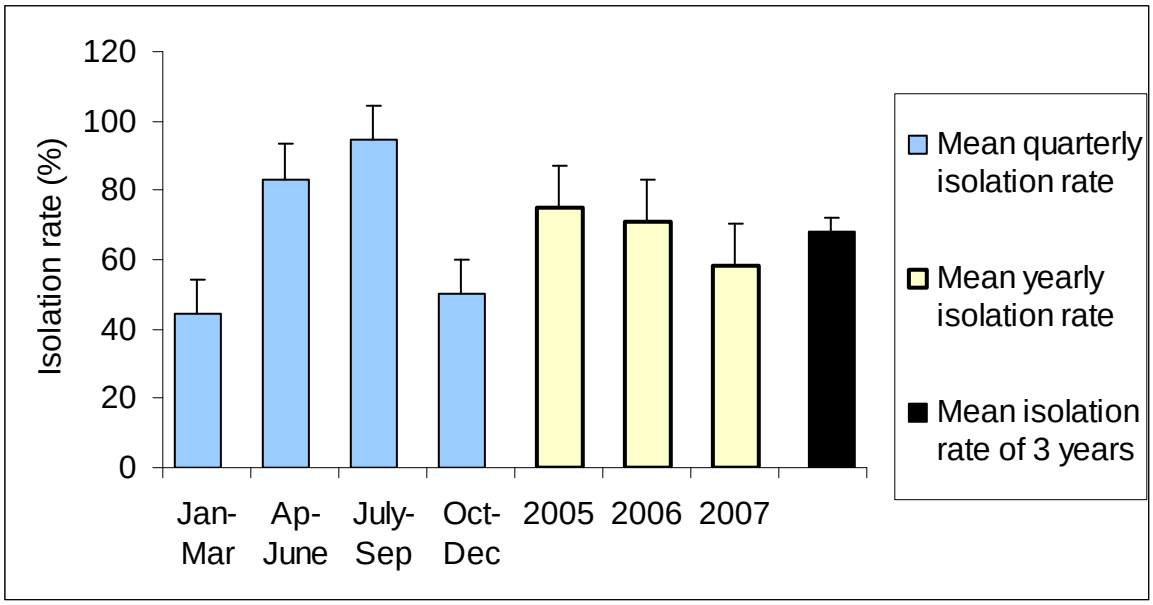


Distribution of *P. mirabilis* in drinking water of Sukkur city. A comparative mean quarterly isolation rate is shown in different quarter of study period. The mean yearly and mean total isolation rate of three years is also shown. Error bar indicate the $\pm$ SEM of %

3.3.3 *Proteus mirabilis* from Drinking Water of Rohri City

In drinking water of Rohri city the *P. mirabilis* was isolated during 2005-2007. The quarterly isolation rate in different quarters was different. The mean quarterly isolation rate in January-March it was 44.4%, in April-June it was 83.3%, in July-September it was 94.4% and in October- December the isolation rate was 50%. In 2005 the mean yearly isolation rate was 75%, in 2006 the mean yearly isolation rate was 71% and in 2007 the mean yearly isolation rate was 58.3%. The mean isolation rate of three years was 68% as is shown in Fig. 20c. The isolation rate of *P. mirabilis* was significantly greater in summer months than in winter months in drinking water of Rohri city ($p<0.012$)

Fig. 20c
Yearly, Quarterly and Total Isolation Rate of *P. mirabilis* in Drinking Water of Rohri City (2005-2007). (n =72)



Distribution of *P. mirabilis* in drinking water of Rohri city. A comparative mean quarterly isolation rate is shown in different quarter of study period. The mean yearly and mean total isolation rate of three years is also shown. Error bar indicate the ± SEM of %

Color plate 6 Biochemical Reaction of *P. mirabilis* on API 20 E

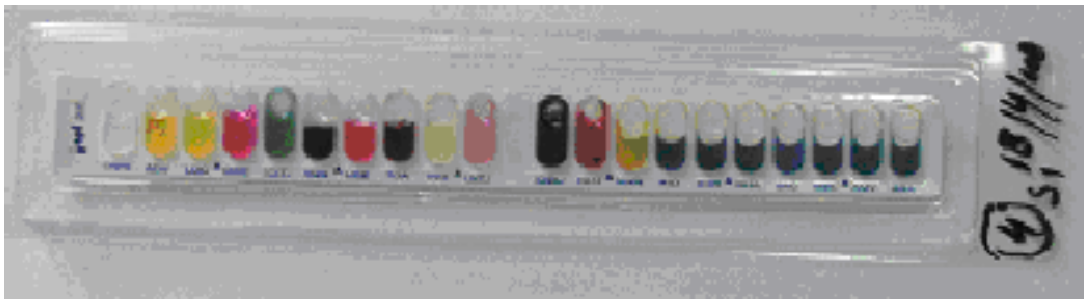


Table 15
Biochemical Features of *P. mirabilis* on API 20 E

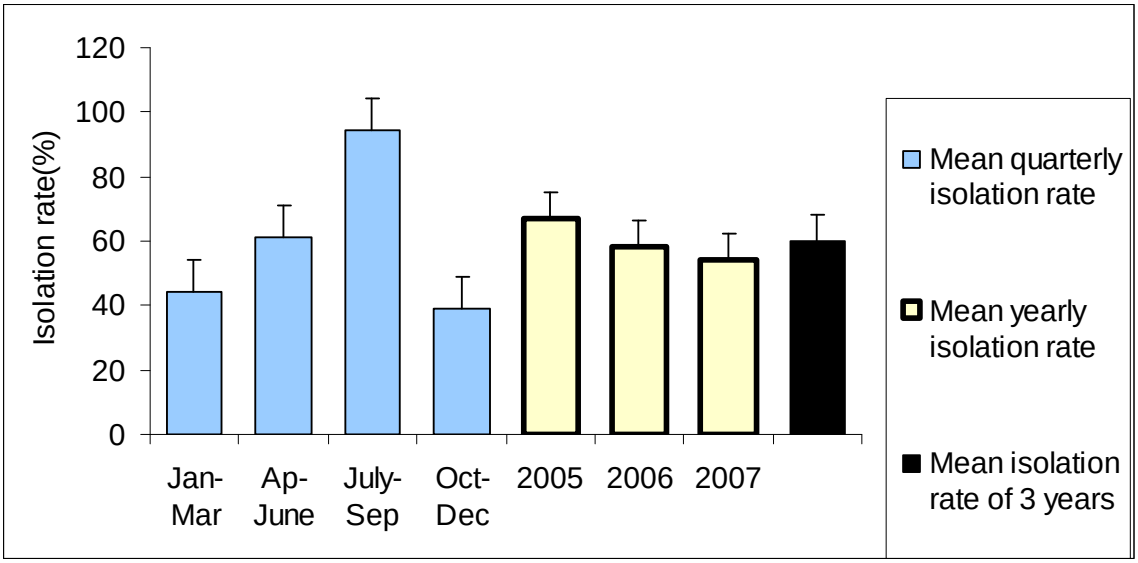
SUBSTRATE	REACTION	SUBSTRATE	REACTION
ONPG	-	GLU	+
ADH	-	MAN	+
LDC	-	INO	-
ODC	+	SOR	-
CIT	+	RHA	-
H2S	+	SAC	-
URE	+	MEL	-
TDA	+	AMY	-
IND	-	ARA	-
VP	+	OXI	-
GEL	+		

3.4. Isolation and Identification of *Providencia spp.* from Drinking Water (Municipal Water) of Khairpur, Sukkur and Rohri City (2005-2007)

3.4.1 *Providencia rettgeri* from Drinking Water Khairpur City

The *P. rettgeri* was isolated, identified and confirmed by API 20E identification system from drinking water of Khairpur city as shown in color plate 7 and Table 16. The quarterly isolation rate in different quarters was different throughout study period. The mean quarterly isolation rate in January-March was 44.4%, in April-June it was 61.1%, in July-September it was 94.4% and in October- December the isolation rate was 39%. In 2005 the mean yearly isolation rate was 67%, in 2006 the mean yearly isolation rate was 58.3% and in 2007 the mean yearly isolation rate was 62.5%. The mean isolation rate of three years was 60% as is shown in Fig 21a. The isolation rate of *P. rettgeri* was significantly greater in summer months than in winter months in drinking water of Khairpur city ($p<0.017$)

Fig. 21a
Yearly Quarterly and Total Isolation Rate of *P. rettgeri* in Drinking Water of Khairpur City (2005-2007). (n =72)

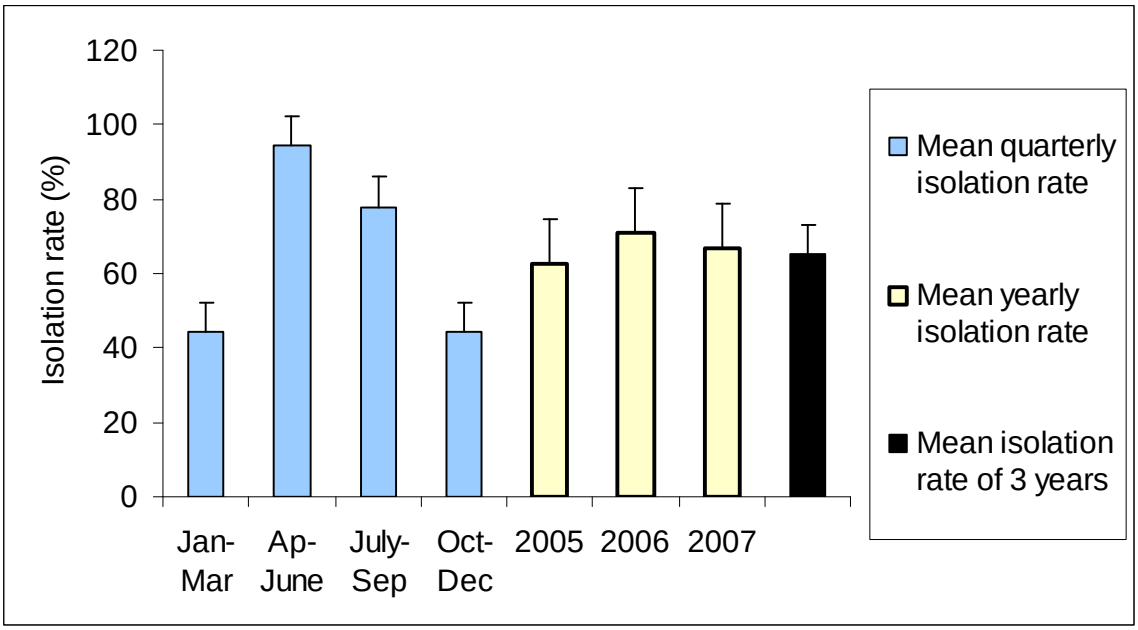


Distribution of *P. rettgeri* in drinking water of Khairpur City. A comparative mean quarterly isolation rate is shown in different quarter of study period. The mean yearly and mean total isolation rate of three years is also shown. Error bar indicate the ± SEM of %

3.4.2 *Providencia rettgeri* from Drinking Water of Sukkur City

In drinking water of Sukkur city the *P. rettgeri* was isolated during 2005-2007. The quarterly isolation rate in different quarters throughout study period was different. In January-March the mean quarterly isolation rate was 44.4%, in April-June it was 94.4%, in July-September it was 78%, and in October- December the isolation rate was 44.4%. In 2005 the mean yearly isolation rate was 62.5%, in 2006 the mean yearly isolation rate was (71%). and in 2007 the mean yearly isolation rate was 67%. The mean isolation rate of three years was 65.2% as is given in Fig 21b. The isolation rate of *P. rettgeri* was significantly greater in summer months than in winter months in drinking water of Sukkur city ($p<0.014$)

Fig.21b
Yearly, Quarterly and Total Isolation Rate of *P. rettgeri* in Drinking Water of Sukkur City (2005-2007). (n =72)

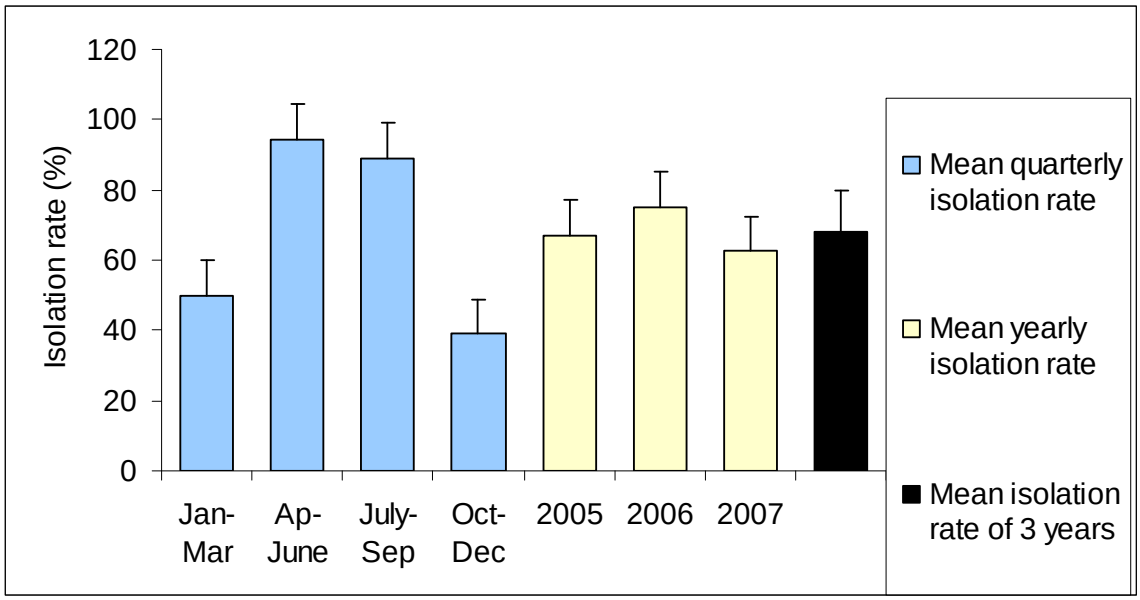


Distribution of *P. rettgeri* in drinking water of Sukkur city. A comparative mean quarterly isolation rate is shown in different quarter of study period. The mean yearly and mean total isolation rate of three years is also shown. Error bar indicate the $\pm$ SEM of %

3.4.3 *Providencia rettgeri* from Drinking Water of Rohri City

In the drinking water (Municipal Water) of Rohri city the isolation rate of *P. rettgeri* was varying during three years of study (2005-2007). In January-March the mean quarterly isolation rate was 50%, and in April-June it was 94.4%, in July-September it was 89%, and in October- December it was 39%. In 2005 the mean yearly isolation rate was 67% in 2006 the mean yearly isolation rate was 75% and in 2007 the mean yearly isolation rate was 62.5%. The mean isolation rate of three years was 65.2% as is shown in Fig 21c. The isolation rate of *P. rettgeri* was significantly greater in summer months than in winter months in drinking water of Rohri city ($p<0.016$)

Fig.21c
Yearly, Quarterly and Total Isolation Rate of *P. rettgeri* in Drinking Water of Rohri City (2005-2007). (n =72)



Distribution of *P. rettgeri* in drinking water of Rohri city. A comparative mean quarterly isolation rate is shown in different quarter of study period. The mean yearly and mean total isolation rate of three years is also shown. Error bar indicate the ± SEM of %

Color plate 7 Biochemical Reaction of *P. rettgeri* on API 20E Kit

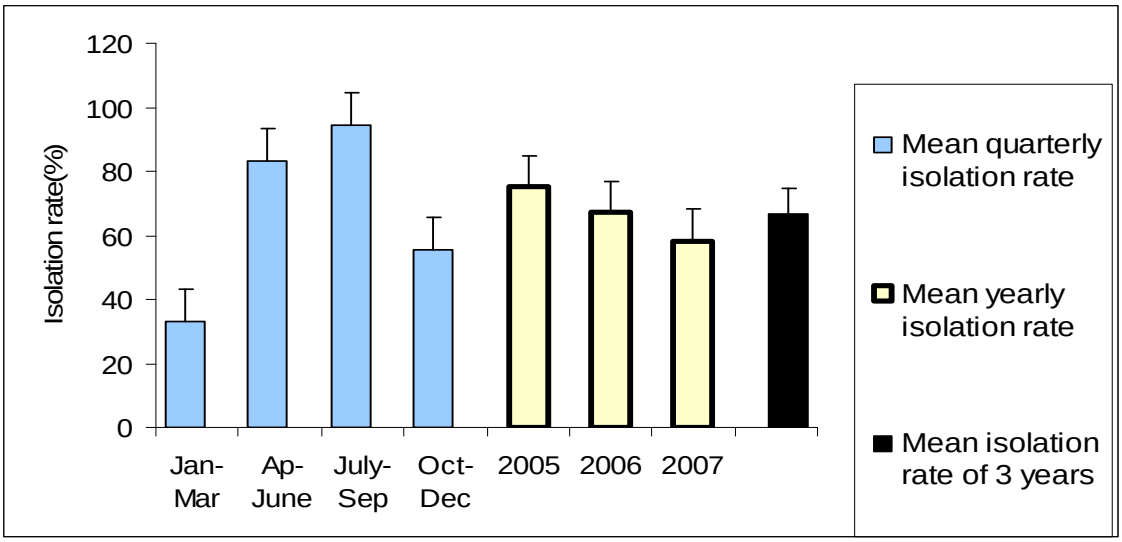
Table 16
Biochemical Features of *P. rettgeri* on API 20E

SUBSTRATE	REACTION	SUBSTRATE	REACTION
ONPG	-	GLU	+
ADH	-	MAN	+
LDC	-	INO	+
ODC	-	SOR	-
CIT	+	RHA	+
H2S	-	SAC	-
URE	+	MEL	-
TDA	+	AMY	+
IND	+	ARA	-
VP	-	OXI	-
GEL	-		

3.4.4 *Providencia stuarti* from Drinking Water of Khairpur City

P. stuarti was isolated, identified and confirmed by API 20E identification system from drinking water (Municipal water) of Khairpur city as shown in color plate 8 and Table 17. The isolation rate of these bacteria in drinking water was also varying. The mean quarterly isolation rate in different quarters of three years i.e. in January-March was 33.3%, in April-June it was 83.3%, in July-September it was 94.4%, and in October- December the isolation rate was 55.5.4%). The mean yearly isolation rate in 2005 was 75%, in 2006 it was 67% and in 2007 the mean yearly isolation rate was 58.3%. The mean isolation rate of three years was 66.5%, as is shown in Fig. 22a. The isolation rate of *P. stuarti* was significantly greater in summer months than in winter months in drinking water of Khairpur city ($p<0.017$)

Fig. 22a
Yearly, Quarterly and Total Isolation Rate of *P. stuarti* in Drinking Water of Khairpur City (2005-2007). (n =72)

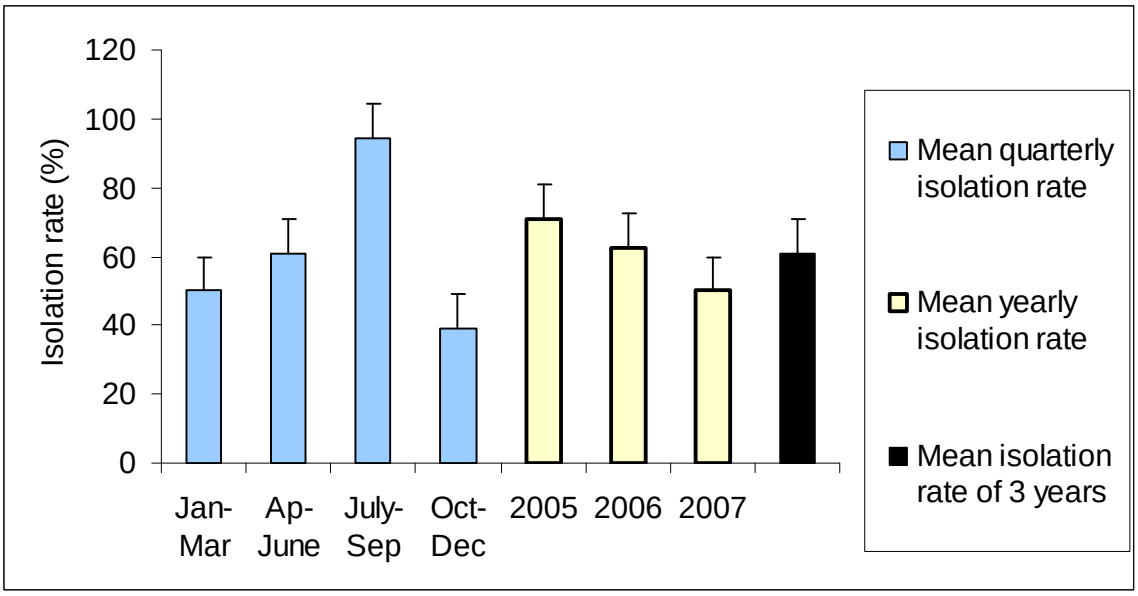


Distribution of *P. stuarti* in drinking water of Khairpur city. A comparative mean quarterly isolation rate is shown in different quarter of study period. The mean yearly and mean total isolation rate of three years is also shown. Error bar indicate the ± SEM of %

3.4.5 *Providencia stuarti* from Drinking Water of Sukkur City

P. stuarti was isolated during 2005-2007 from drinking water (Municipal water) of Sukkur city. The isolation rate of these bacteria in drinking water was also varying in different quarters. The mean quarterly isolation rate in different quarters of three years i.e. in January-March was 50%,in April-June it was 61%, in July-September it was 94.4%, and in October- December the isolation rate was 39%. The mean yearly isolation rate in 2005 was 71%. In 2006 the mean yearly isolation rate was 62.4% nd in 2007 the mean yearly isolation rate was 62.49%. The mean isolation rate of three years was 61% as is shown in Fig. 22b. The isolation rate of *P. stuarti* was significantly greater in summer months than in winter months in drinking water of Sukkur city ($p<0.015$)

Fig. 22b
Yearly, Quarterly and Total Isolation Rate of *P. stuarti* in Drinking Water of Sukkur City (2005-2007). (n =72)

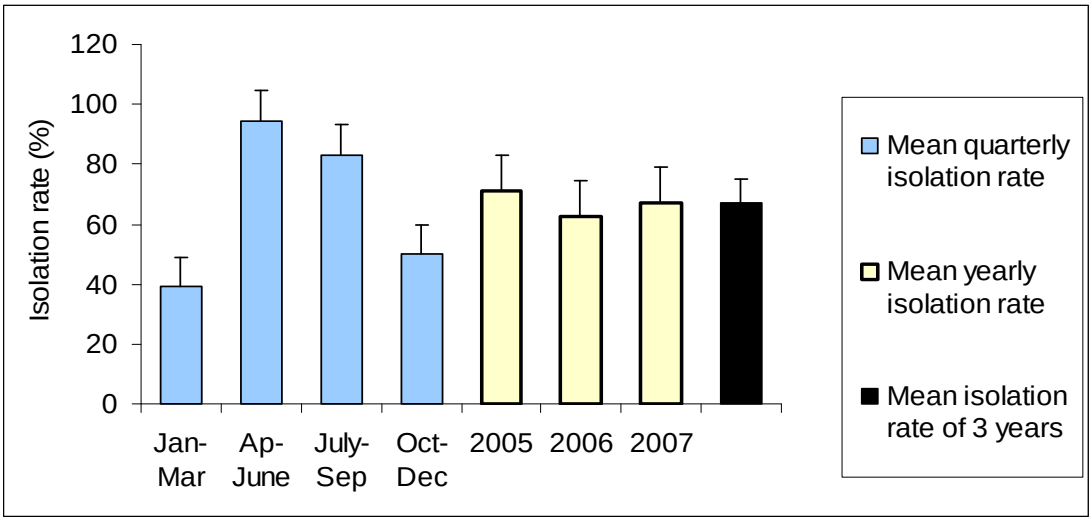


Distribution of *P. stuarti* in drinking water of Sukkur city. A comparative mean quarterly isolation rate is shown in different quarter of study period. The mean yearly and mean total isolation rate of three years is also shown. Error bar indicate the ± SEM of %

3.4.6 *Providencia stuarti* from Drinking Water Rohri City

P. stuarti was isolated from drinking water of Rohri city during the three year of study 2005-2007. The isolation rate of *P. stuarti* in drinking water samples was different in drinking water (Municipal water) of Sukkur city. The isolation rate of these bacteria in drinking water was also varying in different quarters during study period. The mean quarterly isolation rate in different quarters of three years i.e. in January-March was 39%, in April-June it was 94.4%, in July-September it was 83.3% and in October- December the isolation rate was 50%. The mean yearly isolation rate in 2005 was 71%, in 2006 it was 62.49% and in 2007 the mean yearly isolation rate was 67% . The mean isolation rate of three years was 66.5% as is shown in Fig. 22c. The isolation rate of *P. stuarti* was significantly greater in summer months than in winter months in drinking water of Rohri city ($p<0.015$)

Fig.22c
Yearly, Quarterly and Total Isolation Rate of *P. stuarti* in Drinking Water of Rohri City (2005-2007). (n =72)



Distribution of *P. stuarti* in drinking water of Rohri city. A comparative mean quarterly isolation rate is shown in different quarter of study period. The mean yearly and mean total isolation rate of three years is also shown. Error bar indicate the ± SEM of %

Color plate 8 Biochemical Reaction of *P. stuarti* on API 20 E

Table 17
Biochemical Features of *P. stuarti* on API 20 E

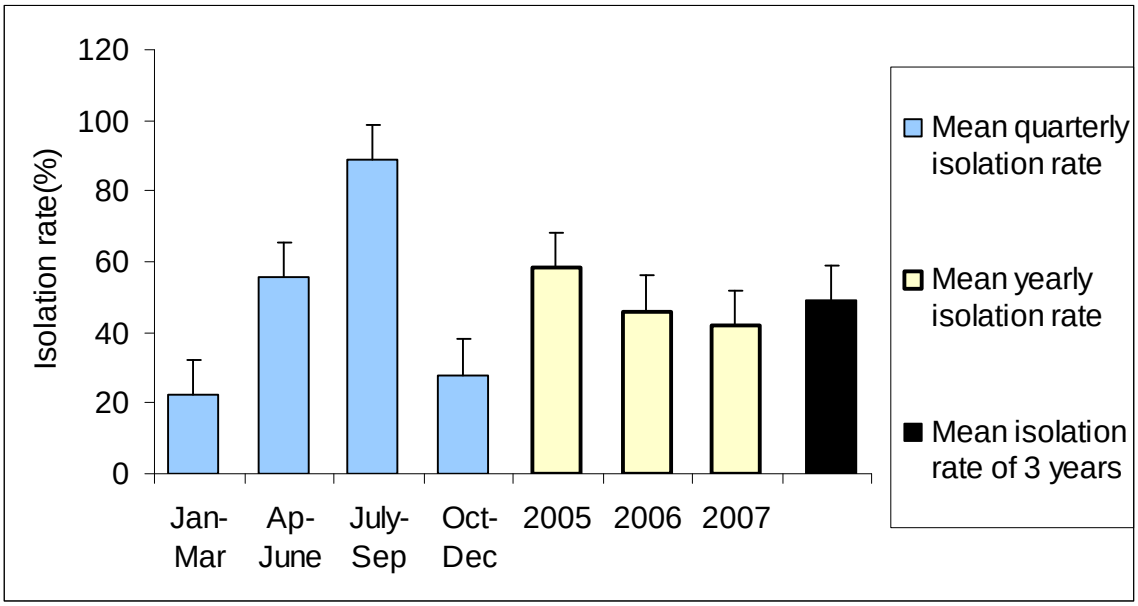
SUBSTRATE	REACTION	SUBSTRATE	REACTION
ONPG	-	GLU	+
ADH	-	MAN	+
LDC	-	INO	-
ODC	-	SOR	-
CIT	+	RHA	+
H2S	-	SAC	-
URE	-	MEL	+
TDA	+	AMY	-
IND	+	ARA	-
VP	-	OXI	-
GEL	-		

3.5 Isolation and Identification of *Klebsiella spp.* from Drinking Water (Municipal Water) of Khairpur, Sukkur and Rohri City (2005-2007)

3.5.1 *Klebsiella oxytoca* from Drinking Water of Khairpur City

The *K. oxytoca* was isolated, identified and confirmed by API 20E identification system as shown in color plate 9 and Table 18. The quarterly isolation rate of *K. oxytoca* was different in different quarter throughout study period. The mean quarterly isolation rate in different quarters i.e. in January-March was 22.2%, in April-June it was 55.5%, in July-September it was 89% and in October- December the mean quarterly isolation rate was 28%. The mean yearly isolation rate in 2005 was 58.3%, in 2006 the mean yearly isolation rate was 46% and in 2007 the mean yearly isolation rate was 42%. The mean isolation rate of three years was 49% as is shown in 23a. The difference in isolation rate of *K. oxytoca* in different quarters in drinking water of Khairpur city was not significant.

Fig. 23a
Yearly, Quarterly and Total Isolation Rate of *K. oxytoca* in Drinking Water of Khairpur City (2005-2007). (n =72)

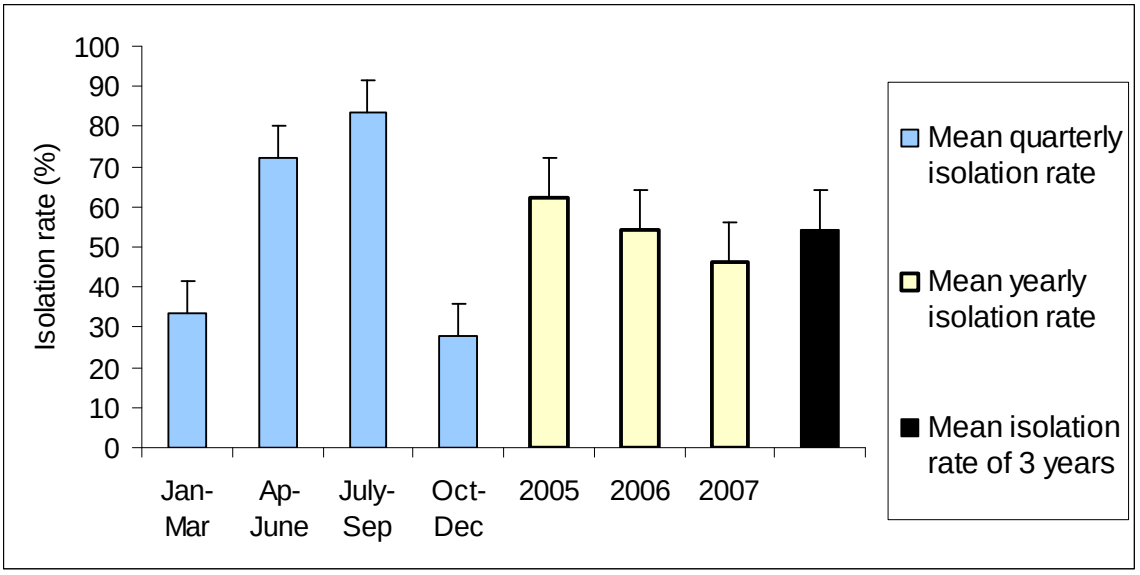


Distribution of *K. oxytoca* in drinking water of Khairpur City. A comparative mean quarterly isolation rate is shown in different quarter of study period. The mean yearly and mean total isolation rate of three years is also shown. Error bar indicate the ± SEM of %

3.5.2 *Klebsiella oxytoca* from Drinking Water of Sukkur City

The *K. oxytoca* was isolated from drinking water (Municipal water) of Sukkur city during 2005-2007. The isolation rate was different in different seasons. The mean quarterly isolation rate in different quarters i.e. in January-March was 33.3%, in April-June it was 72.2%, in July-September it was 83.3%, in October-December the mean quarterly isolation rate was 28%. In 2005 mean yearly isolation rate was 62.4%, in 2006 it was 54.1% and in 2007 the mean yearly isolation rate was 46%. The mean isolation rate of three years was 54.1% as is shown in Fig.23b. The isolation rate of *K. oxytoca* was significantly greater in summer months than in winter months in drinking water of Sukkur city ($p<0.029$)

Fig. 23b
Yearly, Quarterly and Total Isolation rate of *K. oxytoca* in Drinking Water of Sukkur City (2005-2007) (n =72)

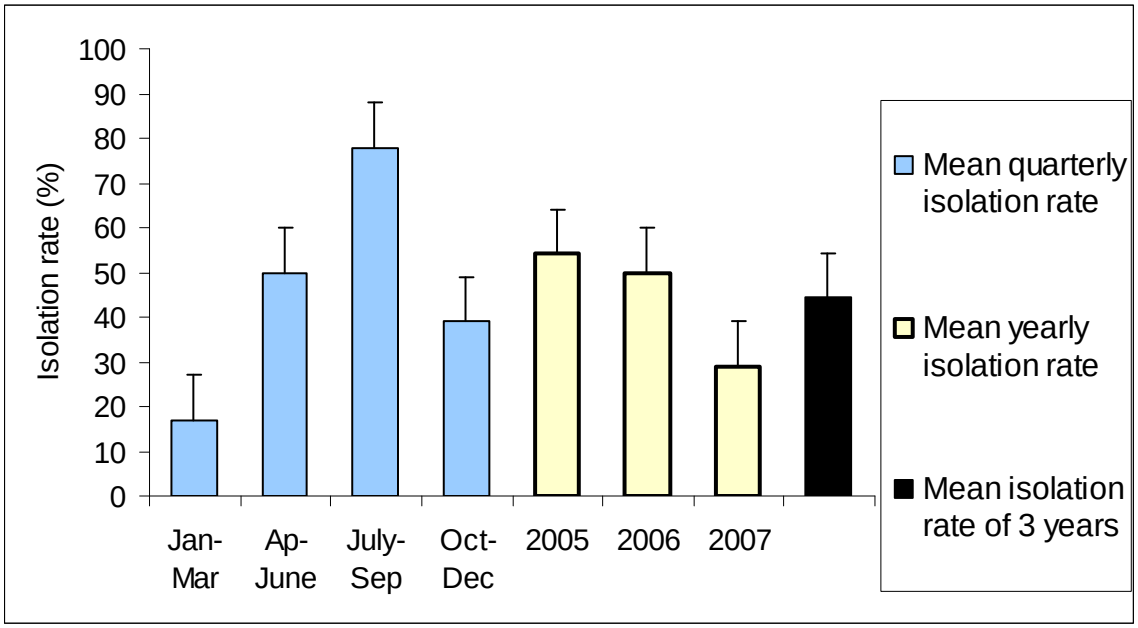


Distribution of *K. oxytoca* in drinking water of Sukkur city. A comparative mean quarterly isolation rate is shown in different quarter of study period. The mean yearly and mean total isolation rate of three years is also shown. Error bar indicate the ± SEM of %

3.5.3 *Klebsiella oxytoca* from Drinking Water of Rohri City

The *K. oxytoca* was isolated from drinking water (Municipal water) of Rohri city during 2005-2007. The isolation rate was different in different seasons. The mean quarterly isolation rate in different quarters of three years i.e. in January-March was 17%, in April-June it was 50%, in July-September it was 78% and in October- December the isolation rate was 39%. In 2005 the mean yearly isolation rate was 54.1%, in 2006 it was 50% and in 2007 the mean yearly isolation rate was 29.1%. The mean isolation rate in three years was 44.4% as shown in Fig 23c. The isolation rate of *K. oxytoca* was significantly greater in summer months than in winter months in drinking water of Rohri city ($p<0.036$)

Fig. 23c
Yearly, Quarterly and Total Isolation Rate of *K. oxytoca* in Drinking Water of Rohri City (2005-2007). (n =72)



Distribution of *K. oxytoca* in drinking water of Rohri city. A comparative mean quarterly isolation rate is shown in different quarter of study period. The mean yearly and mean total isolation rate of three years is also shown. Error bar indicate the ± SEM of %

Color plate 9 Biochemical Reaction of *K. oxytoca* on API 20 E

Table 18
Biochemical Features of *K. oxytoca* on API 20 E

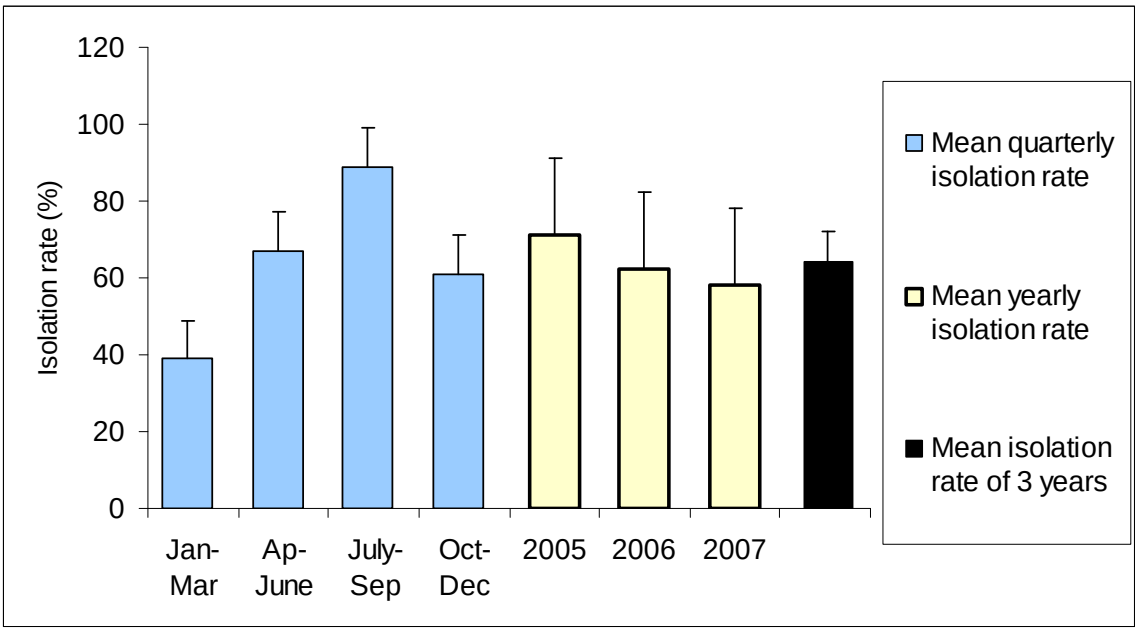
SUBSTRATE	REACTION	SUBSTRATE	REACTION
ONPG	+	GLU	+
ADH	-	MAN	+
LDC	-	INO	+
ODC	-	SOR	+
CIT	+	RHA	+
H2S	-	SAC	+
URE	-	MEL	+
TDA	-	AMY	+
IND	+	ARA	+
VP	+	OXI	-
GEL	-		

3.6 Isolation and Identification of *Citrobacter spp.* from Drinking (Municipal) Water of Khairpur, Sukkur and Rohri City (2005-2007)

3.6.1 *Citrobacter youngae* from Drinking Water of Khairpur City.

The *C. youngae* was isolated, identified and confirmed by API 20E identification system as shown in color plate 10 and Table 19. The isolation rate of *C. youngae* was different in different seasons. The mean quarterly isolation rate in different quarters i.e. in January-March was 39% in April-June it was 67%, in July-September it was 89%, in October-December the mean quarterly isolation rate was 61%. The mean yearly isolation of in 2005 was 71%, in 2006 isolation rate was 62.4% and in 2007. The mean yearly isolation rate was 58.3%. The mean isolation rate of three years was 64% as shown in Fig.24a. The isolation rate of *C. youngae* was significantly greater in summer months than in winter months in drinking water of Khairpur city ($p<0.008$)

Fig. 24a
Yearly, Quarterly and Total Isolation Rate of *C. youngae* in Drinking Water of Khairpur City (2005-2007).

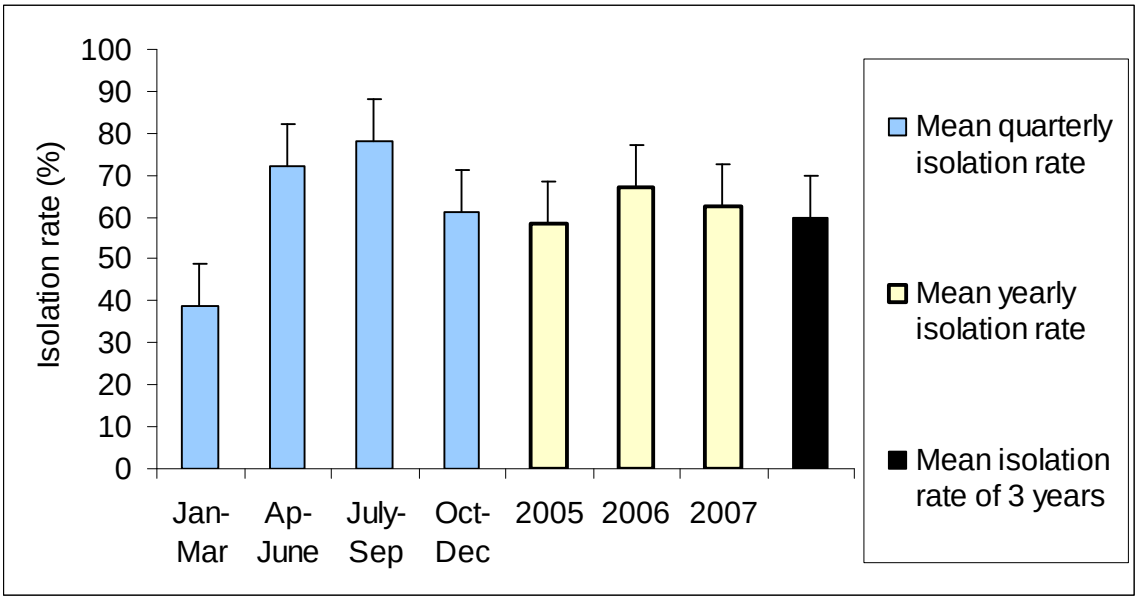


Distribution of *C. youngae* in drinking water of Khairpur City. A comparative mean quarterly isolation rate is shown in different quarter of study period. The mean yearly and mean total isolation rate of three years is also shown. Error bar indicate the ± SEM of %

3.6.2 *C. youngae* from Drinking Water of Sukkur City.

The isolation rate of *C. youngae* was different in different seasons. The mean quarterly isolation rate in different quarters of three years i.e. in January-March was 39%, in April-June it was 72.2%, in July-September it was 78% and in October- December the mean quarterly isolation rate was 61.1%. The mean yearly isolation rate in 2005 was 58.3%, in 2006 it was 67% and in 2007 it was 62.4%. The mean isolation rate of three years was 60% as shown in Fig. 24b. The isolation rate of *C. youngae* was significantly greater in summer months than in winter months in drinking water of Khairpur city ($p<0.005$)

Fig. 24b
Yearly, Quarterly and Total Isolation Rate of *C. youngae* in Drinking Water of Sukkur City (2005-2007).

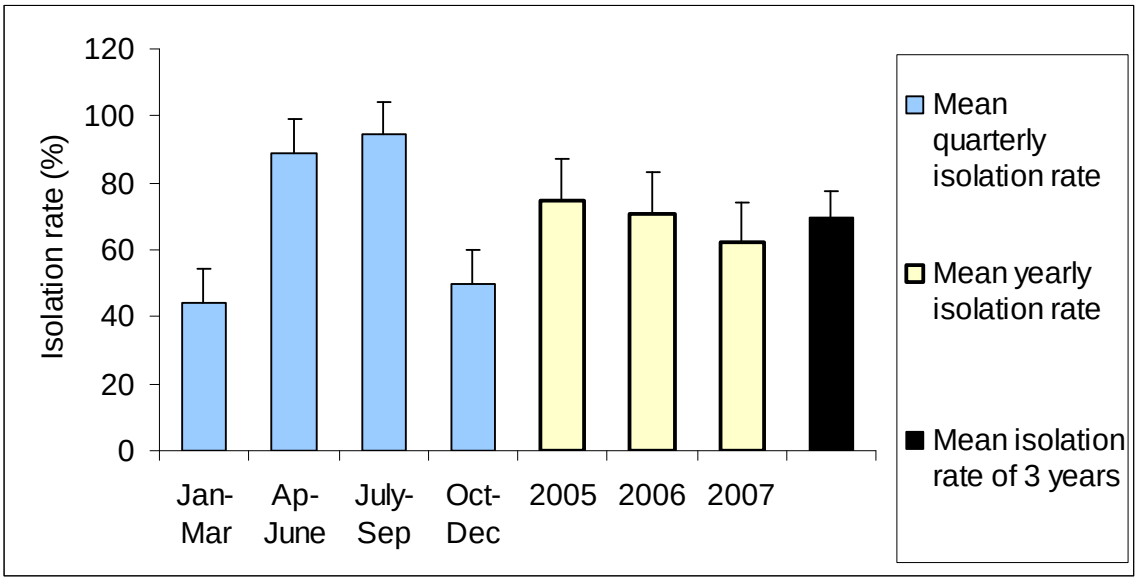


Distribution of *C. youngae* in drinking water of Sukkur city. A comparative mean quarterly isolation rate is shown in different quarter of study period. The mean yearly and mean total isolation rate of three years is also shown. Error bar indicate the $\pm$ SEM of %

3.6.3 *C. youngae* from Drinking Water of Rohri City.

The isolation rate of *C. youngae* was different in different seasons. The mean quarterly isolation rate in different quarters throughout study period was observed as: in January-March the mean quarterly isolation rate was 44.4%, in April-June the mean isolation rate was 89%, in July-September mean isolation rate was 94.4% and in October-December the mean isolation rate was 50%. The mean yearly isolation rate was also recorded. In 2005, the mean yearly isolation rate was 75%, in 2006, the mean yearly isolation rate was 71% and in 2007 the mean yearly isolation rate was 62.4%. The mean total isolation rate of three years was 69.4% as shown in Fig. 24c. The isolation rate of *C. youngae* was significantly greater in summer months than in winter months in drinking water of Rohri city ($p<0.013$)

Fig. 24c
Yearly, Quarterly and Total isolation Rate of *C. youngae* in Drinking Water of Rohri City (2005-2007).



Distribution of *C. youngae* in drinking water of Rohri city. A comparative mean quarterly isolation rate is shown in different quarter of study period. The mean yearly and mean total isolation rate of three years is also shown. Error bar indicate the ± SEM of %

Color plate 10 Biochemical Reaction of *C. youngae* on API 20 E

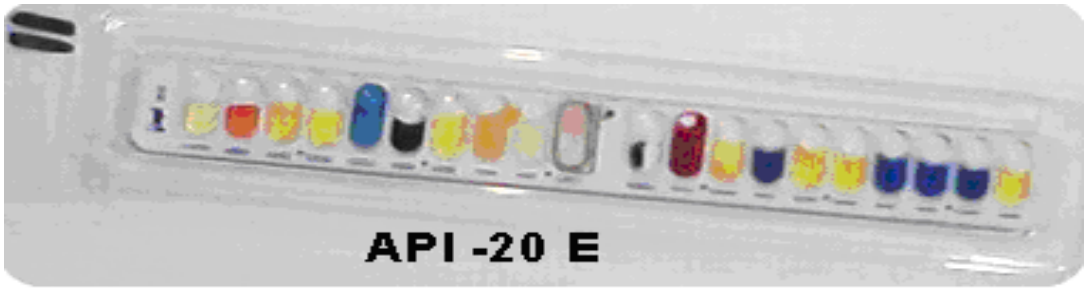


Table 19
Biochemical Features of *C. youngae* on API 20 E

SUBSTRATE	REACTION	SUBSTRATE	REACTION
ONPG	+	GLU	+
ADH	+	MAN	+
LDC	-	INO	-
ODC	-	SOR	+
CIT	+	RHA	+
H2S	+	SAC	-
URE	-	MEL	-
TDA	-	AMY	-
IND	-	ARA	+
VP	-	OXI	-
GEL	-		

3.7. Isolation and Identification of *Non-fermenter* Species from Drinking (Municipal Water) of Khairpur, Sukkur, and Rohri City 2005-2007

Non-fermenter species

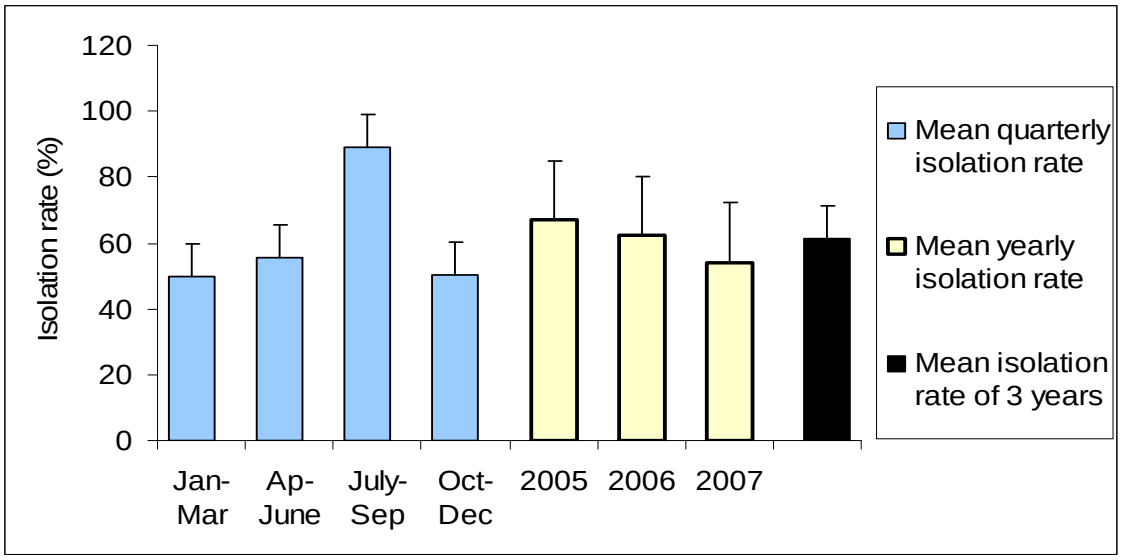
These are a heterogeneous bacteria and capable of growing aerobically, non-fermenting gram-negative bacilli. Utilize carbohydrates as a source of energy or break them down via aerobic rather than anaerobic pathway.(Koneman *et. al.* 1988). *Pseudomonas, Acinetobacter, Alcaligenes, Flavobacter, Oligella, Flavimonas, Agrobacter* and *Weeksiella,* are listed as non-fermenters. In order to detect the non-fermenter species from drinking water of study area this study was carried out.

3.7.1 *Non-fermenter* Species, from Drinking Water of Khairpur City

The *Non- Fermenter spp*. was isolated, identified and confirmed by API 20E identification system as shown in color plate11 and Table 20. The mean quarterly isolation rate in different quarters throughout study period was observed as: in January-March the mean quarterly isolation rate was 50%, in April-June it was 55.5% in July- September it was 89% and in October-December the mean quarterly isolation rate was 50%. The mean yearly isolation rate

was also recorded. In 2005, the mean yearly isolation rate was 67%, in 2006, the mean yearly isolation rate was 62.4%and in 2007 the mean yearly isolation rate was 54.16%. The mean isolation rate of three years was 61.1% as shown in Fig 25a. The isolation rate of *Non-fermenters* was significantly greater in summer months than in winter months in drinking water of Khairpur city ($p<0.007$)

Fig. 25a
Yearly, Quarterly and Total Isolation Rate of *Non-fermenter species* in Drinking Water of Khairpur City (2005-2007)



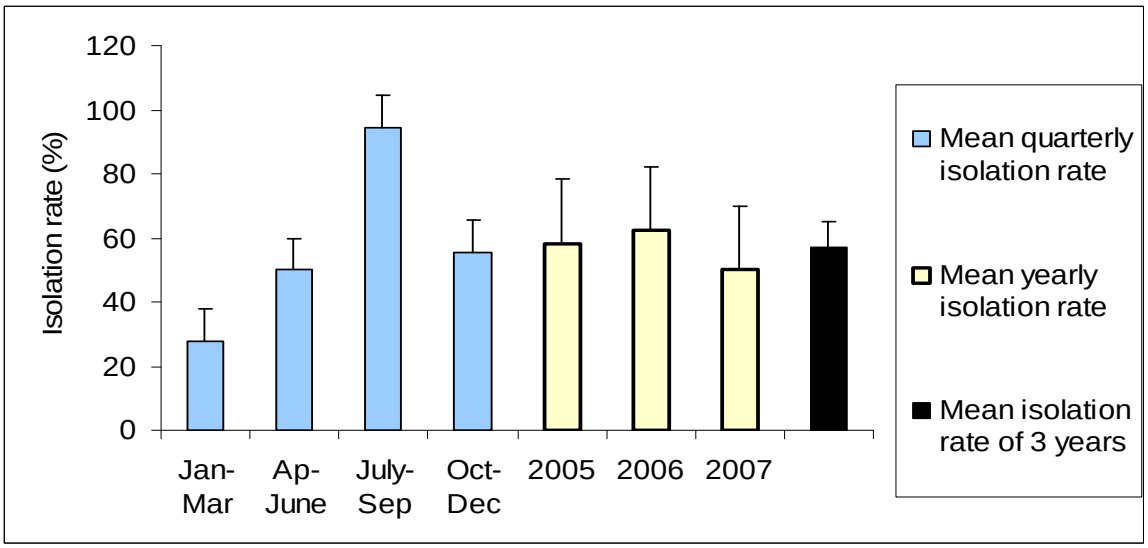
Distribution of *Non-fermenter species* in drinking water of Khairpur City. A comparative mean quarterly isolation rate is shown in different quarter of study period. The mean yearly and mean total isolation rate of three years is also shown. Error bar indicate the ± SEM of %

3.7.2 *Non-fermenter* Species from Drinking Water of Sukkur City.

The drinking water of Sukkur city was also investigated for waterborne bacterial pathogens. The *Non-fermenter* species was isolated during study period. The mean quarterly isolation rate in different quarters throughout study period was observed as: in January-March the mean isolation rate was 28%, in April- June it was 50%, in July-September it was 94.4% and in October-December the mean quarterly isolation rate was 55.5%. The mean yearly isolation rate was also recorded. In 2005 the mean yearly isolation rate was 58.3%, in 2006. The mean yearly

isolation rate was 62.4% and in 2007 the mean yearly isolation rate was 50%. The mean isolation rate in three years was 57% as shown in Fig. 25b. The isolation rate of *Non-fermenters* was significantly greater in summer months than in winter months in drinking water of Sukkur city ($p<0.028$)

Fig. 25b
Yearly, Quarterly and Total Isolation Rate of *Non-fermenter species* in Drinking Water of Sukkur City (2005-2007)



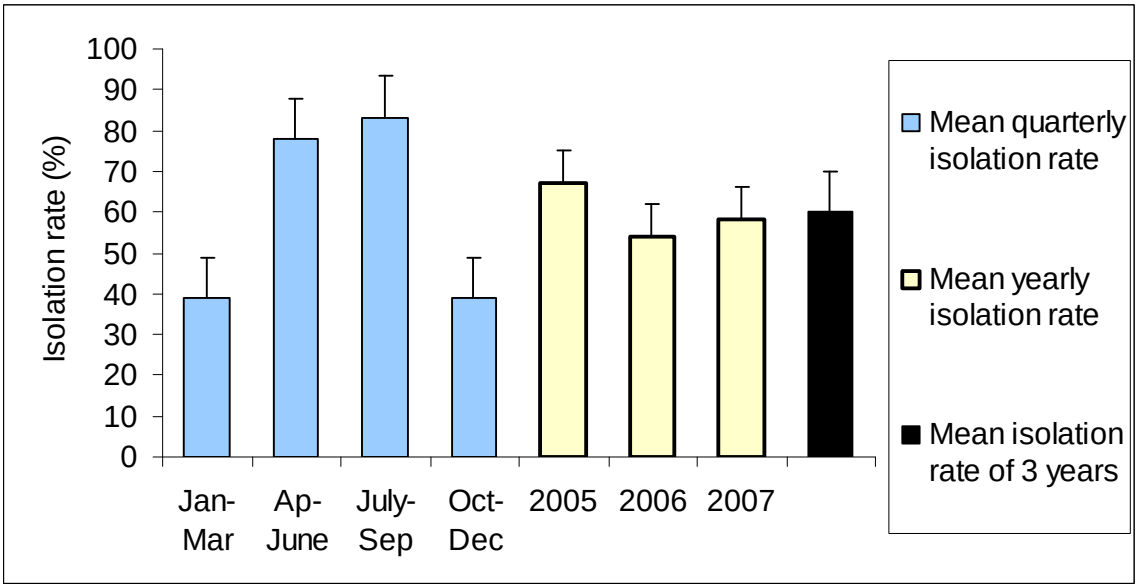
Distribution of *Non-fermenter species* in drinking water of Sukkur city. A comparative mean quarterly isolation rate is shown in different quarter of study period. The mean yearly and mean total isolation rate of three years is also shown. Error bar indicate the ± SEM of %

3.7.3 *Non-fermenter* Species from Drinking Water of Rohri City

In Rohri drinking water, the isolation rate of *Non-fermenter spp*. was varying in different quarter during study period. The mean quarterly isolation rate in different quarters was observed as: in January-March the mean quarterly isolation rate was 39%, in April-June the mean quarterly isolation rate was 78%, in July-September the mean quarterly isolation rate was 83.3%, and in October- December the mean quarterly isolation rate was 39%.The mean yearly isolation rate was also recorded throughout study period. In 2005 the mean yearly isolation rate was 67%, in 2006 the mean yearly isolation rate was 54.1% and in 2007 the mean yearly isolation rate was 58.3%. The mean isolation rate of three year was 60% as shown in Fig.25c. The isolation rate of

Non-fermenters was significantly greater in summer months than in winter months in drinking water of Rohri city ($p<0.016$)

Fig. 25c
Yearly, Quarterly and Total Isolation Rate of *Non-fermenter species* in Drinking Water of Rohri City (2005-2007)



Distribution of *Non-fermenter species* in drinking water of Rohri city. A comparative mean quarterly isolation rate is shown in different quarter of study period. The mean yearly and mean total isolation rate of three years is also shown. Error bar indicate the ± SEM of %

Color Plate 11. The Biochemical Reaction of *Non-fermenter species* on API 20 E

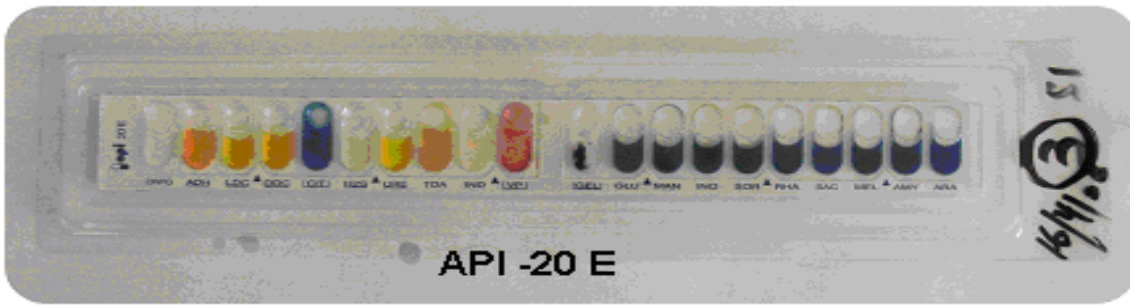


Table 20
Biochemical Features of *Non-fermenter on* API 20 E

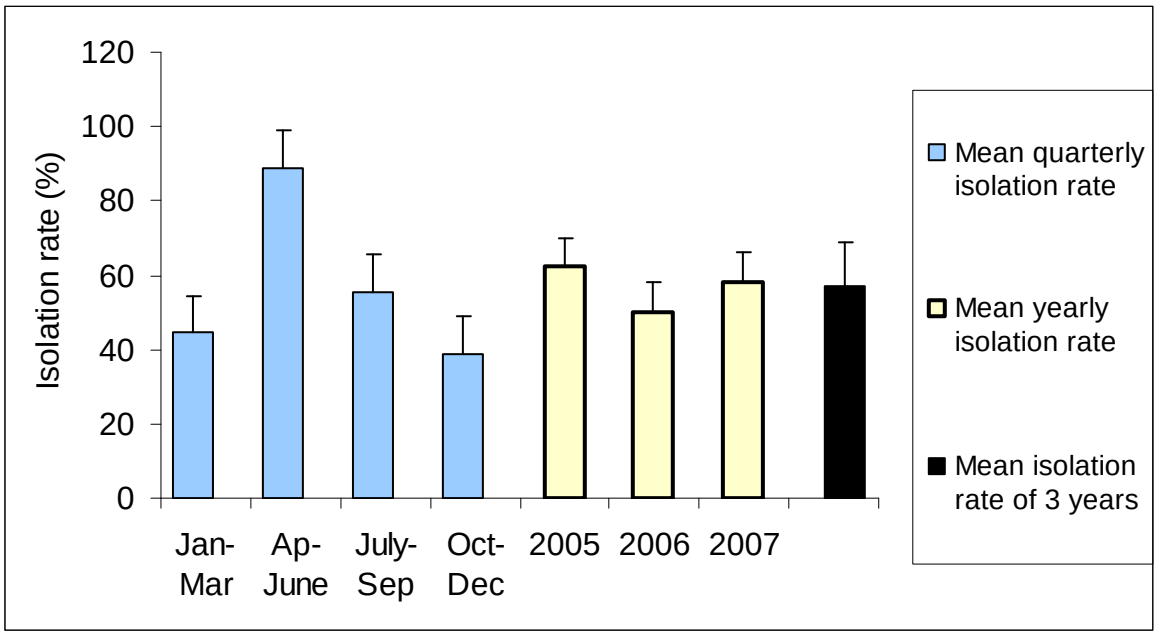
SUBSTRATE	REACTION	SUBSTRATE	REACTION
ONPG	-	GLU	-
ADH	+	MAN	-
LDC	-	INO	-
ODC	-	SOR	-
CIT	+	RHA	-
H2S	-	SAC	-
URE	-	MEL	-
TDA	-	AMY	-
IND	-	ARA	-
VP	+	OXI	-
GEL	-		

3.8 Isolation and Identification of *Chryseobacterium spp.* from Drinking Water (Municipal Water) of Khairpur, Sukkur, and Khairpur City (2005-2007).

3.8.1 *C. meningosepticum* from Drinking Water of Khairpur City

C. meningosepticum was isolated, identified and confirmed by API 20E identification system as shown in color plate 12 and Table 21. The isolation rate of *C. meningosepticum* was different in different seasons. The mean quarterly isolation rate in different quarters throughout study period was observed as: in January-March the mean quarterly isolation rate was 44.4%, in April-June it was 89%, in July-September it was 55.5% and in October- December the mean quarterly isolation was 39%. The mean yearly isolation rate was also recorded throughout study period. In 2005, the mean yearly isolation rate was 62.2%, in 2006 the mean yearly isolation rate was 50% and in 2007 the mean yearly isolation rate was 58.3%. The mean isolation rate of three year was 57% as shown in Fig. 26a.The isolation rate of *C. meningosepticum* was significantly greater in summer months than in winter months in drinking water of Khairpur city ($p<0.015$)

Fig. 26a
Yearly, Quarterly and Total Isolation Rate of *C. meningosepticum* in Drinking Water (Municipal Water) of Khairpur City (2005-2007)

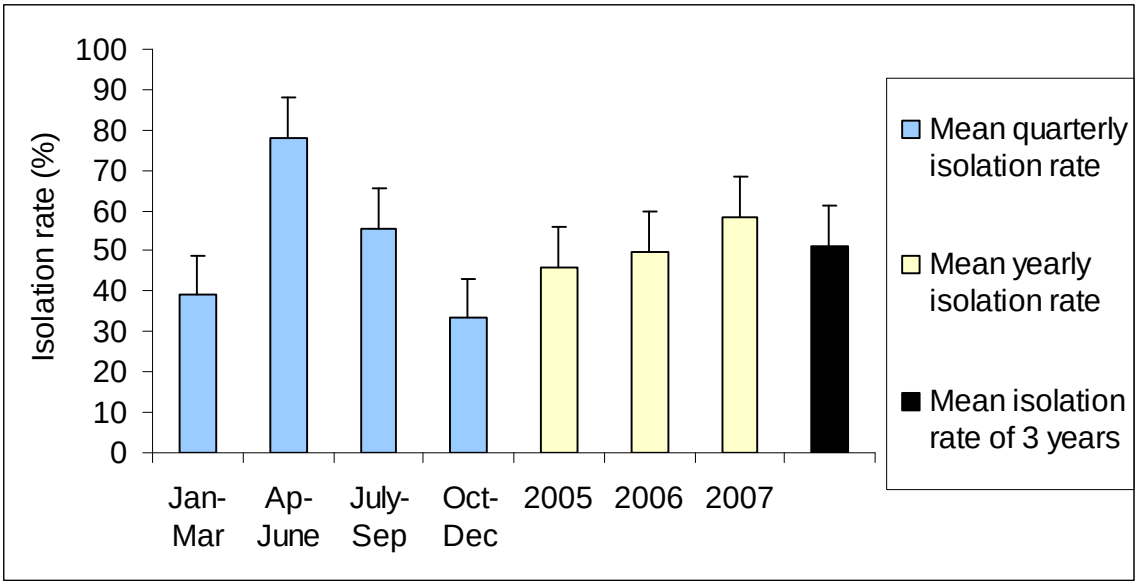


Distribution of *C. meningosepticum* in drinking water of Khairpur city. A comparative mean quarterly isolation rate is shown in different quarter of study period. The mean yearly and mean total isolation rate of three years is also shown. Error bar indicate the ± SEM of %

3.8.2 *C. meningosepticum* from Drinking Water of Sukkur City

The isolation rate of *C. meningosepticum* was different in different seasons in drinking water of Sukkur city. The mean quarterly isolation rate in different quarters throughout study period was observed as: in January-March, April-June, July-September, October- December, the mean isolation rate was 39%, 78%, 55.5% and 33.3% respectively. The mean yearly isolation rate was also recorded throughout study period. In 2005, in 2006 and in 2007 the mean yearly isolation rate was 46%, 50% and 58.3%) respectively. The mean isolation rate of three years was observed according to these results the mean total isolation rate was 51.38% as shown in Fig. 26b. The isolation rate of *C. meningosepticum* was significantly greater in summer months than in winter months in drinking water of Sukkur city ($p<0.014$)

Fig. 26b
Yearly, Quarterly and Total Isolation Rate of *C. meningosepticum* in Drinking Water of Sukkur City (2005-2007).



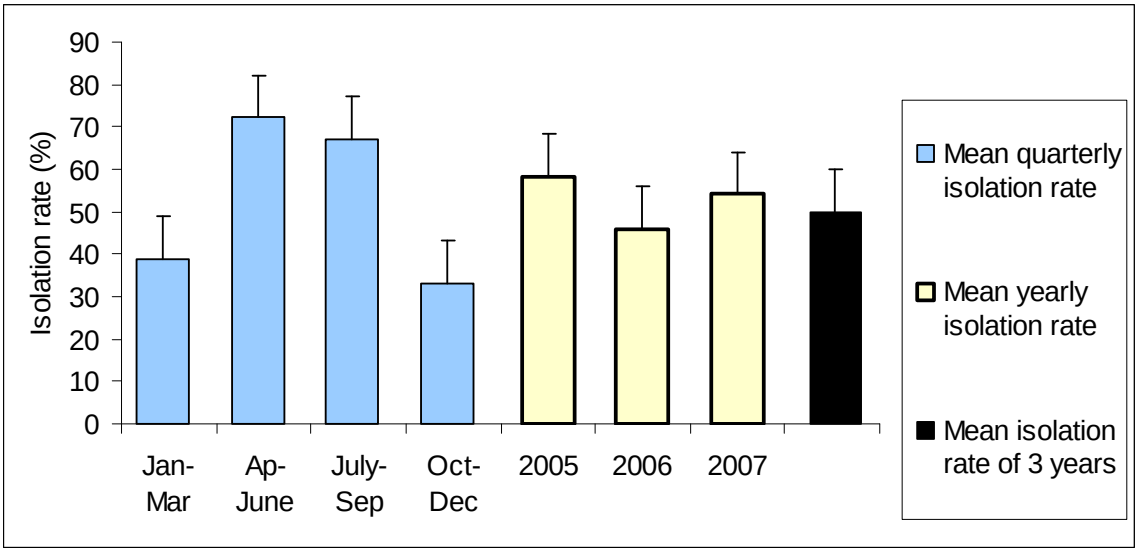
Distribution of *C.meningosepticum* in drinking water of Sukkur city. A comparative mean quarterly isolation rate is shown in different quarter of study period. The mean yearly and mean total isolation rate of three years is also shown. Error bar indicate the ± SEM of %

3.8.3 *C. meningosepticum* from Drinking Water of Rohri City

The isolation rate of *C. meningosepticum* was different in different seasons The mean quarterly isolation rate in different quarters throughout study period was observed as: in January-March, April-June, July-September, October- December, the mean isolation rate was 39%, 72.2%, 67% and 33.3% respectively. The mean yearly isolation rate was also recorded throughout study period. In 2005, in 2006 and in 2007 the mean yearly isolation rate was 58.3%, 46% and 54.1% respectively. The mean total isolation rate of three years was observed according to these results the mean isolation rate was 50% as shown in Fig.26c The isolation rate of *C. meningosepticum* was significantly greater in summer months than in winter months in drinking water of Rohri city ($p<0.012$)

Fig. 26c

Yearly, Quarterly and Total Isolation Rate of *C. meningosepticum* in Drinking Water of Rohri City (2005-2007)



Distribution of *C.meningosepticum* in drinking water of Rohri city. A comparative mean quarterly isolation rate is shown in different quarter of study period. The mean yearly and mean total isolation rate of three years is also shown. Error bar indicate the ± SEM of %

Color plate 12. Biochemical Reaction of *C.meningosepticum* on API 20E

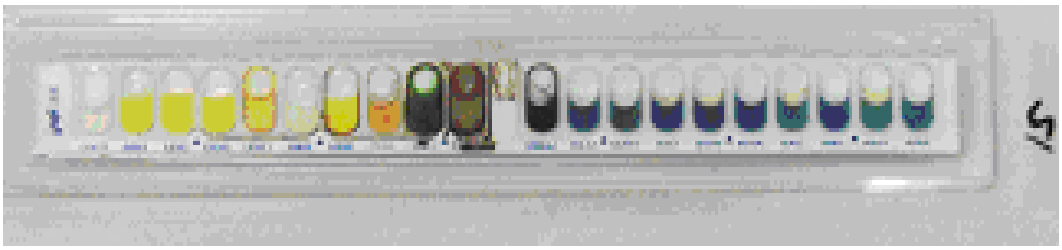


Table 21
Biochemical Features of *C. meningosepticum* on API 20 E

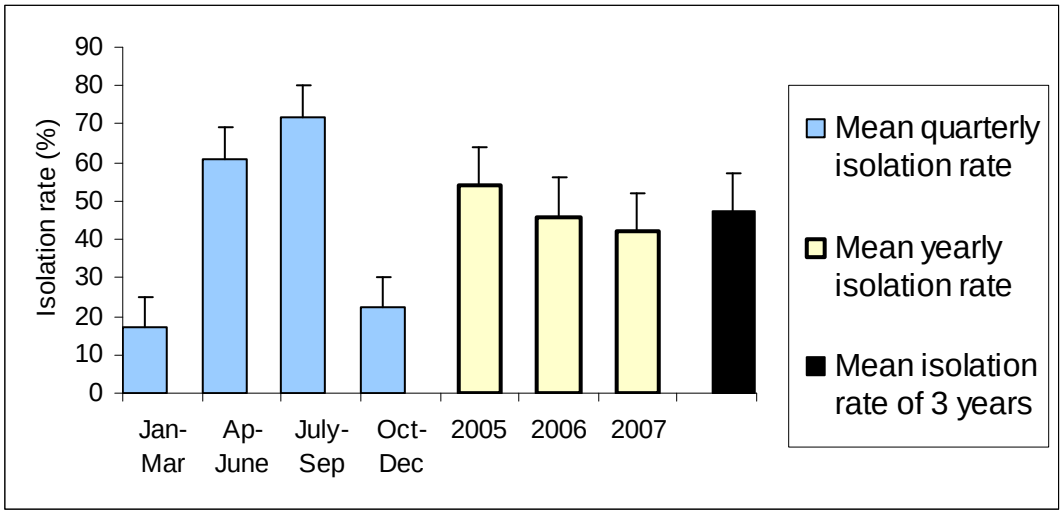
SUBSTRATE	REACTION	SUBSTRATE	REACTION
ONPG	-	GLU	-
ADH	-	MAN	-
LDC	-	INO	-
ODC	-	SOR	-
CIT	-	RHA	-
H2S	-	SAC	-
URE	-	MEL	-
TDA	+	AMY	-
IND	-	ARA	-
VP	-	OXI	+
GEL	+		

3.9 Isolation and Identification of *Vibrio spp.* from Drinking Water (Municipal water) of Khairpur, Sukkur, and Rohri (2005-2007).

3.9.1 *Vibrio mimicus* from Drinking Water of Khairpur City.

V. mimicus was isolated, identified and confirmed by API 20E identification system as shown in color plate 13 and Table 22. The isolation rate of *V. mimicus* was different in different seasons in drinking water. The mean quarterly isolation rate in different quarters throughout study period was observed as: in January-March, April-June, July-September, October- December, the mean isolation rate was 17%, 61%, 72% and 22.3% respectively. The mean yearly isolation rate was also recorded throughout study period. In 2005, in 2006 and in 2007 the mean yearly isolation rate was 54.1%, 46% and 42% respectively. The mean total isolation rate of three years was observed according to these results the mean total isolation rate was 47.2% as shown in Fig. 27a. The difference in isolation rate of *V. mimicus* in different quarters in drinking water of Khairpur city was not significant

Fig. 27a
Yearly, Quarterly and total Isolation Rate of *V. mimicus* in Drinking Water of Khairpur City (2005-2007).

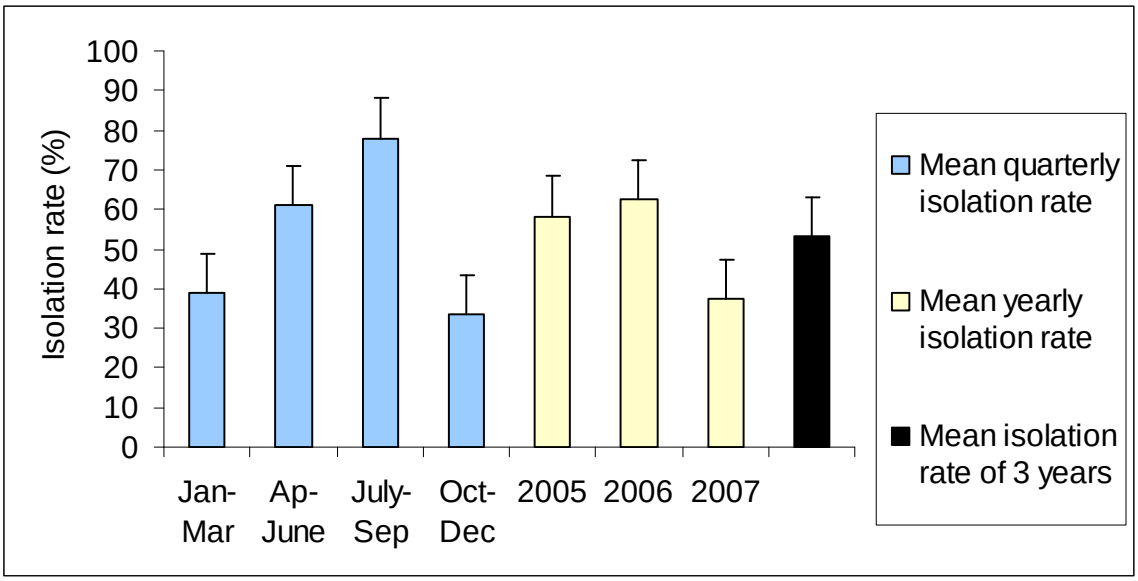


Distribution of *V. mimicus* in drinking water of Khairpur city. A comparative mean quarterly isolation rate is shown in different quarter of study period. The mean yearly and mean total isolation rate of three years is also shown. Error bar indicate the ± SEM of %

3.9.2 *V. mimicus* from Drinking Water of Sukkur City.

The isolation rate of *V. mimicus* was different in different seasons in drinking water samples The mean quarterly isolation rate in different quarters throughout study period was observed as: in January-March, April-June, July-September, October- December, the mean isolation rate was 39%, 61%, 78% and 33.4% respectively. The mean yearly isolation rate was also recorded throughout study period. In 2005, in 2006 and in 2007 the mean yearly isolation rate was 58.3%, 62.5% and 39%, respectively. The mean total isolation rate of three years was observed according to these results the mean total isolation rate was 53% as shown in Fig. 27b. The isolation rate of *V. mimicus* was significantly greater in summer months than in winter months in drinking water of Sukkur city ($p<0.014$)

Fig. 27b
Yearly, Quarterly and Total Isolation Rate of *V. mimicus* in Drinking Water of Sukkur City (2005-2007).

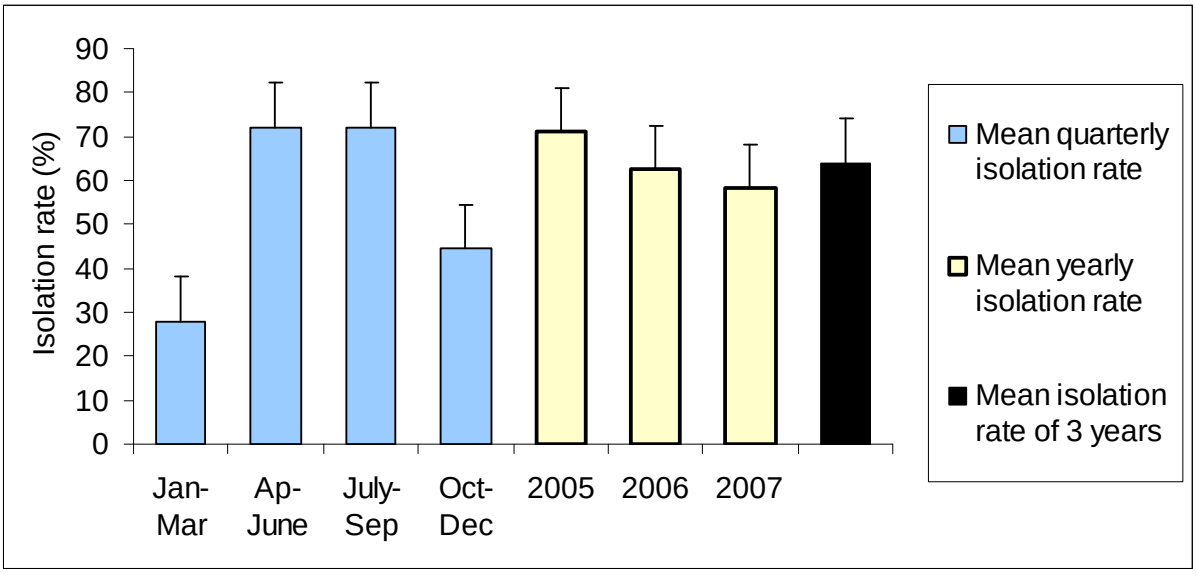


Distribution of *V. mimicus* in drinking water of Sukkur city. A comparative mean quarterly isolation rate is shown in different quarter of study period. The mean yearly and mean total isolation rate of three years is also shown. Error bar indicate the ± SEM of %

3.9.3 *V. mimicus* from Drinking Water of Rohri City.

The isolation rate of *V. mimicus* was different in different seasons in drinking water samples. The mean quarterly isolation rate in different quarters throughout study period was observed as: in January-March, April-June, July-September, October- December, the mean isolation rate was 28%, 72.2%, 72.2% and 44.4% respectively. The mean yearly isolation rate was also recorded throughout study period. In 2005, in 2006 and in 2007 the mean yearly isolation rate was 71%, 62.5% and 58.3% respectively. The mean total isolation rate of three years was observed according to these results the mean total isolation rate was 64% as shown in Fig 27c. The isolation rate of *V. mimicus* was significantly greater in summer months than in winter months in drinking water of Rohri city ($p<0.016$)

Fig. 27c
Yearly, Quarterly and Total Isolation Rate of *Vibrio mimicus* in Drinking Water of Rohri City (2005-2007)



Distribution of *V. mimicus* in drinking water of Rohri city. A comparative mean quarterly isolation rate is shown in different quarter of study period. The mean yearly and mean total isolation rate of three years is also shown. Error bar indicate the ± SEM of %

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Color Plate 13 Biochemical Reaction of *V. mimicus* on API 20 E

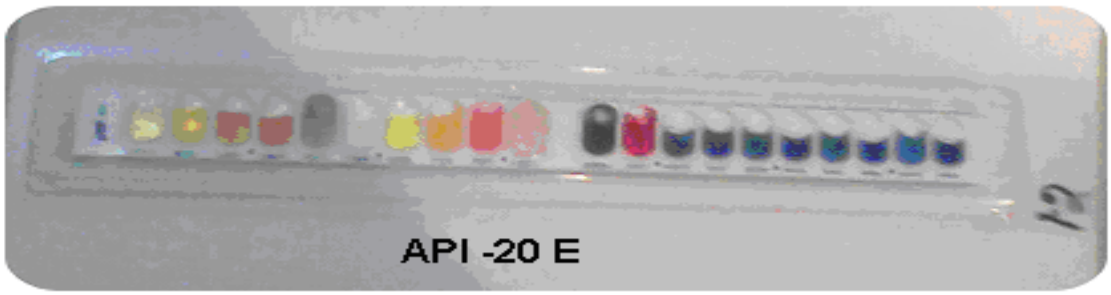


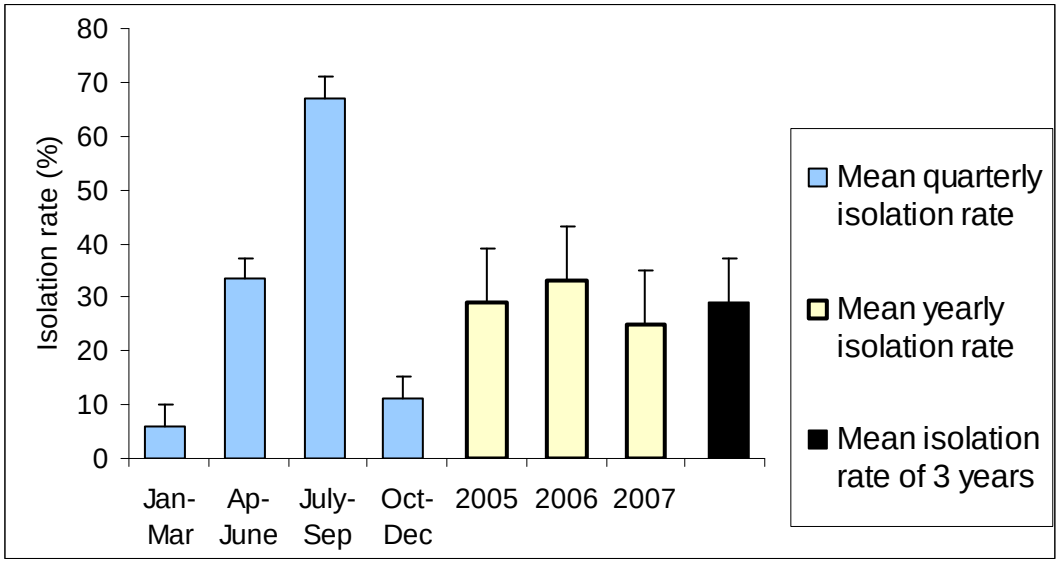
Table 22
Biochemical Features of *V. mimicus* on API 20 E

SUBSTRATE	REACTION	SUBSTRATE	REACTION
ONPG	+	GLU	+
ADH	-	MAN	-
LDC	+	INO	-
ODC	+	SOR	-
CIT	-	RHA	-
H2S	-	SAC	-
URE	-	MEL	-
TDA	-	AMY	-
IND	+	ARA	-
VP	-	OXI	+
GEL	+		

3.9.4 *V. cholerae* from Drinking Water of Khairpur City.

V. cholerae was isolated, identified and confirmed by API 20E identification system from drinking water samples as shown in color plate 14 and Table 23. The isolation rate of *V. cholerae* was varying in different seasons. The mean quarterly isolation rate in different quarters throughout study period was observed as: in January-March, April-June, July-September, October- December, the mean isolation rate was 6%, 33.3%, 67% and 11.1% respectively. The mean yearly isolation rate was also recorded throughout study period. In 2005, in 2006 and in 2007 the mean yearly isolation rate was 29%, 33.3% and 25%, respectively. The mean isolation rate of three years was observed according to these results the mean total isolation rate was 29.1% as shown in Fig. 28a. The difference in isolation rate of *V. cholerae* in different quarters in drinking water of Khairpur city was not significant

Fig. 28a
Yearly, Quarterly and Total Isolation rate of *V. cholerae* in Drinking Water of Khairpur City (2005-2007)

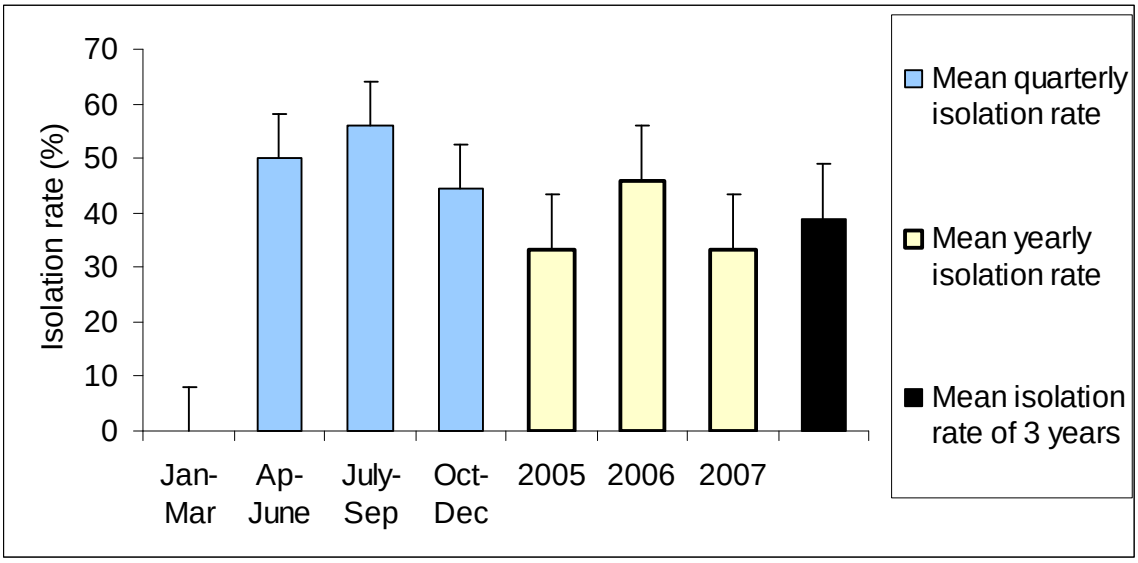


Distribution of *V. cholerae* in drinking water of Khairpur city. A comparative mean quarterly isolation rate is shown in different quarter of study period. The mean yearly and mean total isolation rate of three years is also shown. Error bar indicate the $\pm$ SEM of %

3.9.5 *Vibrio cholerae* from Drinking Water of Sukkur City

The isolation rate of *V. cholerae* was different in different seasons in drinking water samples. The mean quarterly isolation rate in different quarters throughout study period was observed as: in January-March, April-June, July-September, October- December, the mean isolation rate was 0%, 50%, 56% and 44.4% respectively. The mean yearly isolation rate was also recorded throughout study period. In 2005, in 2006 and in 2007 the mean yearly isolation rate was 33.3%, 46% and 33.3% respectively. The mean isolation rate of three years was observed according to these results the mean total isolation rate was 39% as shown in Fig. 28b. The difference in isolation rate of *V. cholerae* in different quarters in drinking water of Sukkur city was not significant

Fig. 28b
Yearly, Quarterly and Total Isolation Rate of *V. cholerae* in Drinking Water of Sukkur City (2005-2007).

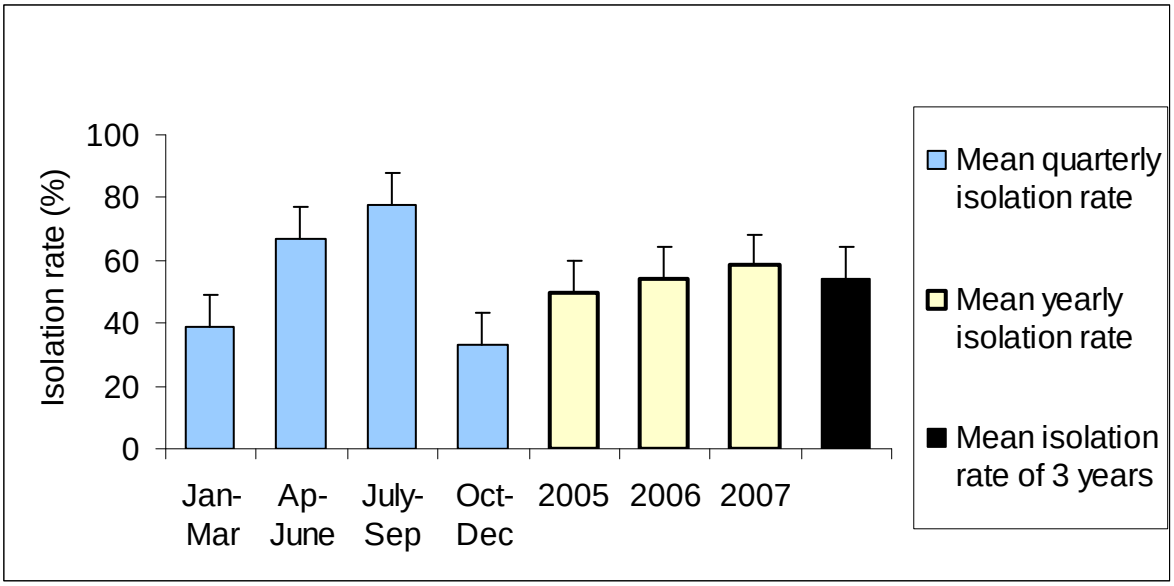


Distribution of *V. cholerae* in drinking water of Sukkur city. A comparative mean quarterly isolation rate is shown in different quarter of study period. The mean yearly and mean total isolation rate of three years is also shown. Error bar indicate the $\pm$ SEM of %

3.9.6 *Vibrio cholerae* from Drinking Water of Rohri city.

The isolation rate of *V. cholerae* was different in different seasons in drinking water samples. The mean quarterly isolation rate in different quarters throughout study period was observed as: in January-March, April-June, July-September, October- December, the mean isolation rate was 39%, 67%, 78% and 33.3% respectively. The mean yearly isolation rate was also recorded throughout study period. In 2005, in 2006 and in 2007 the mean yearly isolation rate was 50%, 54.1% and 58.4% respectively. The mean total isolation rate of three years was observed according to these results the mean total isolation rate was 54.3% as shown in Fig.28c. The isolation rate of *V. cholerae* was significantly greater in summer months than in winter months in drinking water of Rohri city ($p<0.015$)

Fig. 28c
Yearly, Quarterly and Total Isolation Rate of *V. cholerae* in Drinking Water of Rohri City (2005-2007).



Distribution of *V. cholerae* in drinking water of Rohri city. A comparative mean quarterly isolation rate is shown in different quarter of study period. The mean yearly and mean total isolation rate of three years is also shown. Error bar indicate the ± SEM of %

Color plate 14 **Biochemical Reaction of *V. cholerae* on API 20E**

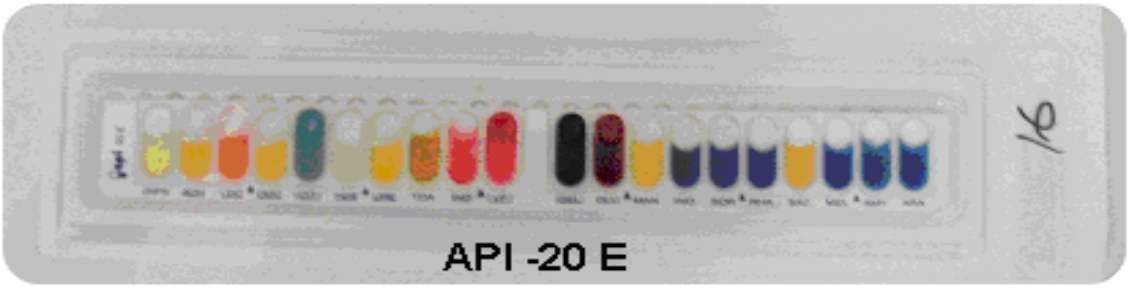


Table 23
Biochemical Features of *V. cholerae* on API 20 E

SUBSTRATE	REACTION	SUBSTRATE	REACTION
ONPG	+	GLU	+
ADH	-	MAN	+
LDC	+	INO	-
ODC	-	SOR	-
CIT	+	RHA	-
H2S	-	SAC	+
URE	-	MEL	-
TDA	-	AMY	-
IND	+	ARA	-
VP	+	OXI	+
GEL	+		

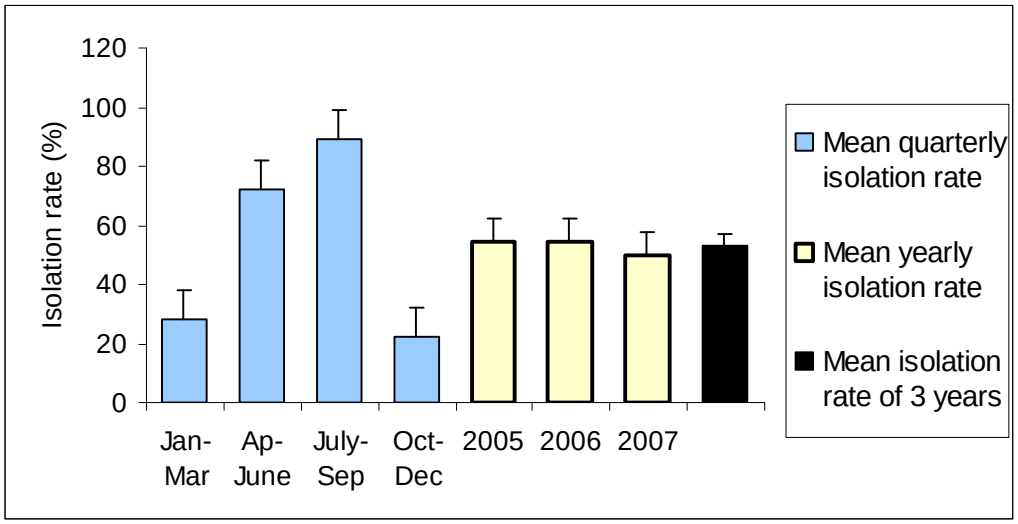
3.10 Isolation and Identification of *Aeromonas spp.* from Drinking Water (Municipal Water) of Khairpur, Sukkur and Rohri 2005-2007

In present study *Aeromonas hydrophila* was isolated from the drinking water of Khairpur, Sukkur and Rohri during three years study (2005-2007).

3.10.1 *A. hydrophila* from Drinking Water of Khairpur City

A. hydrophila was isolated, identified and confirmed by API 20E identification system from drinking water samples as shown in color plate 14 and Table 24 The mean quarterly isolation rate of *A. hydrophila* in different quarters throughout study period was observed as: in January-March, April-June, July-September, October- December, the mean isolation rate was 28%, 72.2%, 89% and 22.2%, respectively. The mean yearly isolation rate was also recorded throughout study period. In 2005, in 2006 and in 2007 the mean yearly isolation rate was 54.2%, 54.2% and 50% respectively. The mean total isolation rate of three years was observed according to these results the mean total isolation rate was 53% as is given in Fig.29a. The isolation rate of *A. hydrophila* was significantly greater in summer months than in winter months in drinking water of Khairpur city ($p<0.014$)

Fig. 29a
Yearly, Quarterly and Total Isolation Rate of *A. hydrophila* in Drinking Water of Khairpur City 2005-2007.

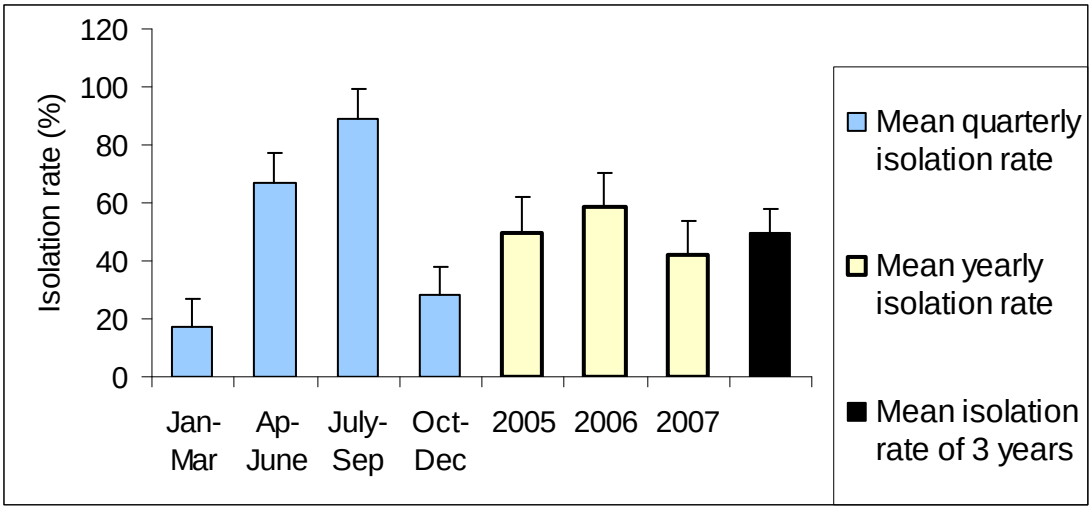


Distribution of *A. hydrophila* in drinking water of Khairpur City. A comparative mean quarterly isolation rate is shown in different quarter of study period. The mean yearly and mean total isolation rate of three years is also shown. Error bar indicate the ± SEM of %

3.10.2 *A. hydrophila* from Drinking Water of Sukkur City.

In drinking water samples from Sukkur city the *A. hydrophila* was isolated. The mean quarterly isolation rate in different quarters throughout study period was observed as: in January-March, April-June, July-September, October- December, the mean isolation rate was 17%, 67%, 89 and 28% respectively. The mean yearly isolation rate was also recorded throughout study period. In 2005, in 2006 and in 2007 the mean yearly isolation rate was 50%, 58.3 and 42% respectively. The mean total isolation rate of three years was observed according to these results the mean total isolation rate was 50% as shown in Fig. 29b. The isolation rate of *A. hydrophila* was significantly greater in summer months than in winter months in drinking water of Sukkur city ($p<0.012$)

Fig. 29b
Yearly, Quarterly, and Total Isolation Rate of *Aeromonas hydrophila* in Drinking Water of Sukkur City (2005-2007).

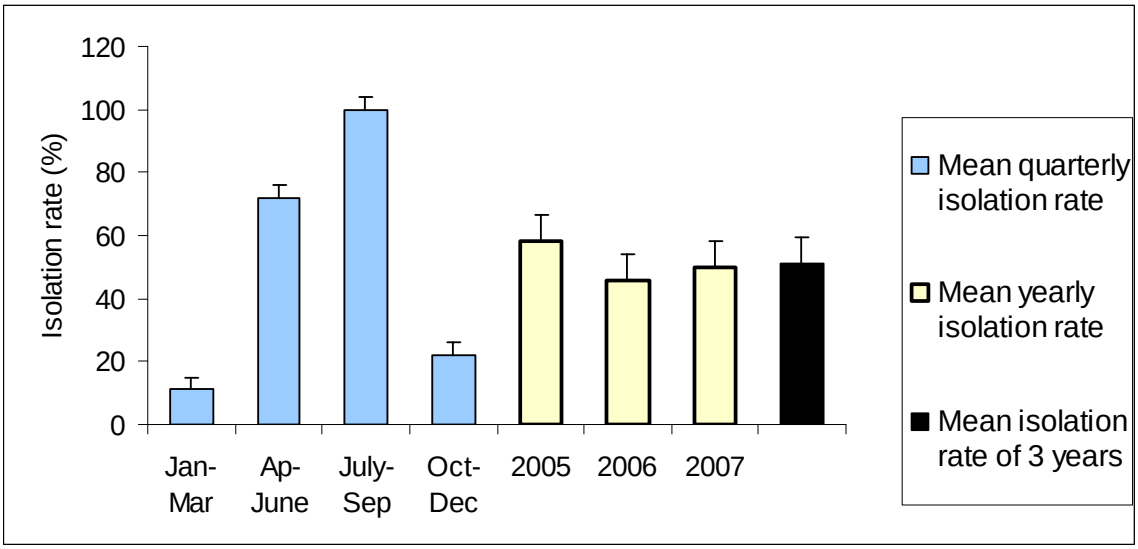


Distribution of *A. hydrophila* in drinking water of Sukkur city. A comparative mean quarterly isolation rate is shown in different quarter of study period. The mean yearly and mean total isolation rate of three years is also shown. Error bar indicate the ± SEM of %

3.10.3 *A. hydrophila* from Drinking Water of Rohri City.

The *A. hydrophila* was isolated from drinking water of Rohri during present study. The mean quarterly isolation rate in different quarters throughout study period was observed as: in January-March, April-June, July-September, October- December, the mean isolation rate was 11%, 72%, 100% and 22.2% respectively. The mean yearly isolation rate was also recorded throughout study period. In 2005, in 2006 and in 2007 the mean yearly isolation rate was 58.3%, 46% and 50%, respectively. The mean total isolation rate of three years was observed according to these results the mean total isolation rate was 51.3% as shown in Fig.29c. The difference in isolation rate of *A. hydrophila* in different quarters in drinking water of Rohri city was not significant

Fig.29c
Yearly, Quarterly and Total Isolation Rate of *A. hydrophila* in Drinking Water of Rohri City (2005-2007).



Distribution of *A. hydrophila* in drinking water of Rohri city. A comparative mean quarterly isolation rate is shown in different quarter of study period. The mean yearly and mean total isolation rate of three years is also shown. Error bar indicate the ± SEM of %

Color plate 15 Biochemical Reaction of *A. hydrophila* on API 20E

Table 24
Biochemical Features of *A. hydrophila* on API 20 E

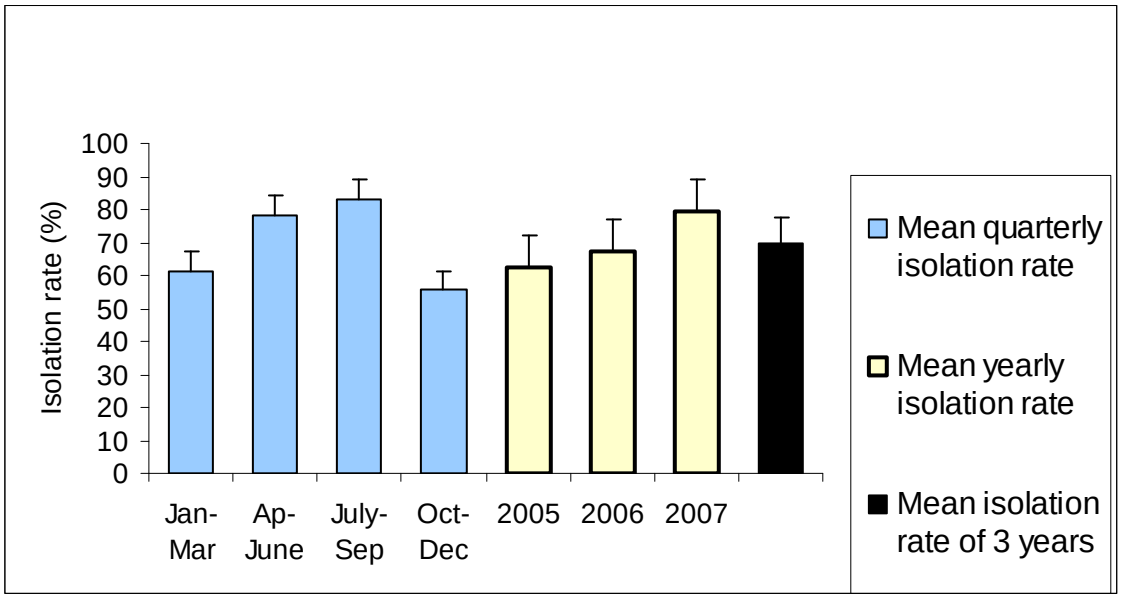
SUBSTRATE	REACTION	SUBSTRATE	REACTION
ONPG	+	GLU	+
ADH	+	MAN	+
LDC	+	INO	-
ODC	-	SOR	-
CIT	+	RHA	-
H2S	-	SAC	+
URE	-	MEL	-
TDA	-	AMY	+
IND	+	ARA	-
VP	+	OXI	+
GEL	+		

3.11 Isolation and Identification of *Pseudomonas spp.* from Drinking Water (Municipal water) of Khairpur, Sukkur, and Rohri city (2005-2007)

3.11.1 *P. aeruginosa* from Drinking Water of Khairpur city

The *P. aeruginosa* was isolated, identified and confirmed by API 20E identification system as shown in color plate 16 and Table 25.The isolation rate of *P. aeruginosa* was different in different seasons in drinking water samples tested in present study. The mean quarterly isolation rate in different quarters throughout study period was observed as: in January-March, April-June, July-September, October- December, the mean isolation rate was 61.1%, 78%, 83.3% and 55.5% respectively. The mean yearly isolation rate was also recorded throughout study period. In 2005, in 2006 and in 2007 the mean yearly isolation rate was 79.1%, 67% and 62.4% respectively. The mean total isolation rate of three years was observed according to these results the mean total isolation rate was 69.4% as shown in Fig. 30a. The isolation rate of *P. aeroginosa* was significantly greater in summer months than in winter months in drinking water of Khairpur city ($p<0.002$)

Fig. 30a
Yearly, Quarterly and Total Isolation Rate of *P. aeruginosa* in Drinking Water of Khairpur City (2005-2007).

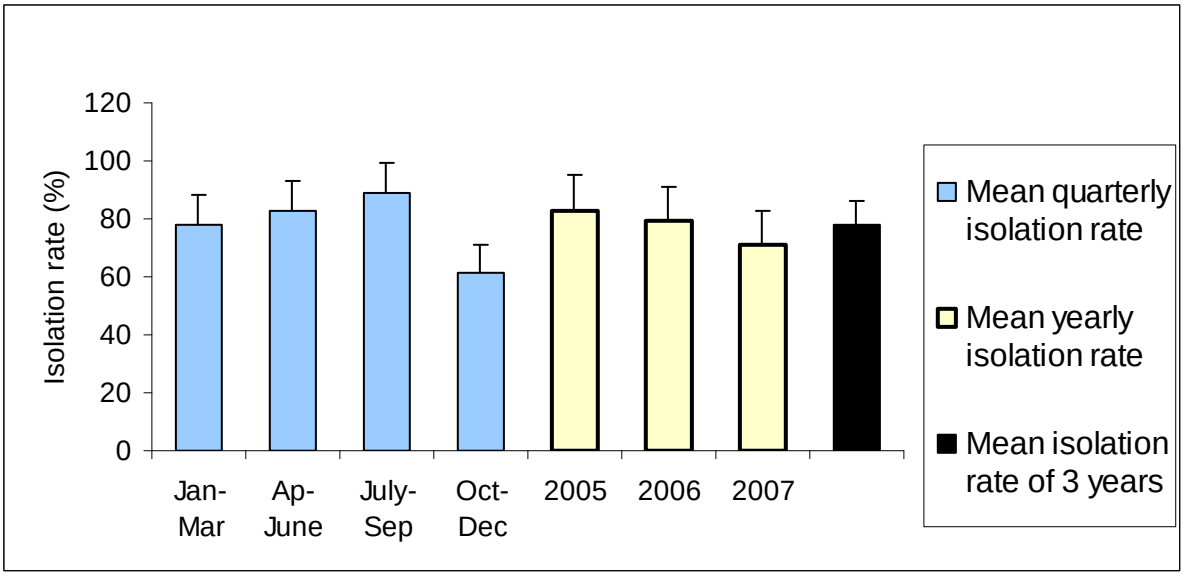


Distribution of *P. aeruginosa* in drinking water of Khairpur City. A comparative mean quarterly isolation rate is shown in different quarter of study period. The mean yearly and mean total isolation rate of three years is also shown. Error bar indicate the $\pm$ SEM of %

3.11.2 *P. aeruginosa* from Drinking Water of Sukkur City

The isolation rate of *P. aeruginosa* was different in different seasons in drinking water samples from Sukkur city. The mean quarterly isolation rate in different quarters throughout study period was observed as: in January-March, April-June, July-September, October- December, the mean isolation rate was 78%, 83.3%, 89% and 61.1% respectively. The mean yearly isolation rate was also recorded throughout study period. In 2005, in 2006 and in 2007 the mean yearly isolation rate was 83.3%, 79.1% and 71% respectively. The mean total isolation rate of three years was observed according to these results the mean total isolation rate was 78% as shown in Fig. 30b. The isolation rate of *P. aeroginosa* was significantly greater in summer months than in winter months in drinking water of Sukkur city ($p<0.001$)

Fig. 30b
Yearly, Quarterly and Total Isolation rate of *P. aeruginosa* in Drinking Water of Sukkur City (2005-2007).

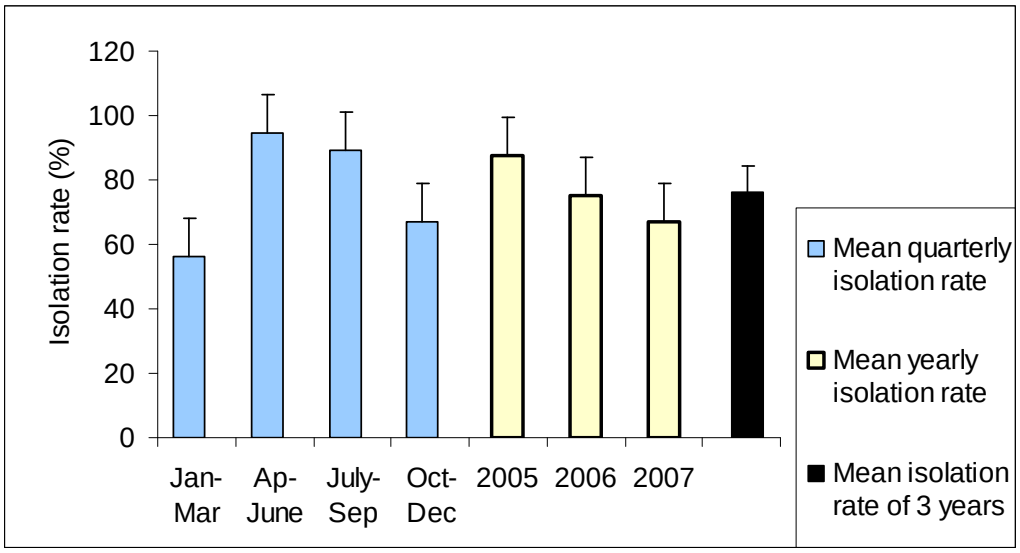


Distribution of *P. aeruginosa* in drinking water of Sukkur city. A comparative mean quarterly isolation rate is shown in different quarter of study period. The mean yearly and mean total isolation rate of three years is also shown. Error bar indicate the ± SEM of %

3.11.3 *Pseudomonas aeruginosa* from Drinking Water of Rohri city

The isolation rate of *P. aeruginosa* was different in different seasons. The mean quarterly isolation rate in different quarters throughout study period was observed as: in January-March, April-June, July-September, October- December, the mean isolation rate was 56%, 94.4%, 89% and 67% respectively. The mean yearly isolation rate was also recorded throughout study period. In 2005, in 2006 and in 2007 the mean yearly isolation rate was 87.5 %, 75% and 67% respectively. The mean total isolation rate of three years was observed according to these results the mean total isolation rate was 76.3% as shown in Fig, 30c. The isolation rate of *P. aeroginosa* was significantly greater in summer months than in winter months in drinking water of Rohri city ($p<0.003$)

Fig. 30c
Yearly, Quarterly and Total Isolation Rate of *P. aeruginosa* in Drinking Water of Rohri City (2005-2007).



Distribution of *P. aeruginosa* in drinking water of Rohri City. A comparative mean quarterly isolation rate is shown in different quarter of study period. The mean yearly and mean total isolation rate of three years is also shown. Error bar indicate the ± SEM of %

Color plate 16 Biochemical reaction of *P. aeruginosa* on API 20E

Table 25
Biochemical Reaction of *P. aeruginosa* on API 20E

SUBSTRATE	REACTION	SUBSTRATE	REACTION
ONPG	-	GLU	+
ADH	+	MAN	-
LDC	-	INO	-
ODC	-	SOR	-
CIT	+	RHA	-
H2S	-	SAC	-
URE	-	MEL	-
TDA	-	AMY	-
IND	-	ARA	-
VP	-	OXI	+
GEL	+		

3.12. Isolation and Identification of *Salmonella spp.* from Drinking Water (Municipal water) of Khairpur, Sukkur, and Rohri city (2005-2007)

3.12.1 *Salmonella spp.*

The *salmonella spp*. was not isolated from drinking water tested in this study; reasons for their absence from drinking water samples are discussed in the discussion section (see page no. 157).

Correlation among Isolated Waterborne Bacterial Pathogens

The correlation matrices among waterborne bacterial pathogens are given in Table 29 All isolated bacteria were strongly correlated to each other except *C. meningosepticum* which showed a strong correlation with only three species i.e. *A. hydrophila* (r = 0.70), *P. stuarti* (r = 0.67) and *P. aeruginosa* (r = 0.66) However the *C . meningosepticum* showed a moderate correlation with all remaining isolated waterborne bacteria *V. mimicus* (r = 0.56), *E. coli* 1 (r =0.47), *Nonfermenter spp.* (r = 0.53), *P. mirabilis*(r =0.42), *K. oxytoca*(r = 0.42) and showed a weak correlation with *P. rettgeri* (r =0.33) *C. youngae* (r =0.30), *V. cholerae* (r = 0.37), *and E. coli* 2 (r = 0.35).

3.13 Temperature Measurement of Drinking Water (Municipal Water) Samples 2005-2007

Temperature is widely recognized as an important controlling factor in influencing bacterial growth. In climate where water temperatures are warm, bacterial growth may be very rapid. However the minimum temperature at which microbial activity was observed varied from system

to system (WHO 2003). In the present study the temperature of drinking water samples collected from main reservoirs of municipal water of Khairpur, Sukkur and Rohri cities was analyzed to observe the effect of temperature on the prevalence of waterborne bacterial pathogens in different seasons during study period.

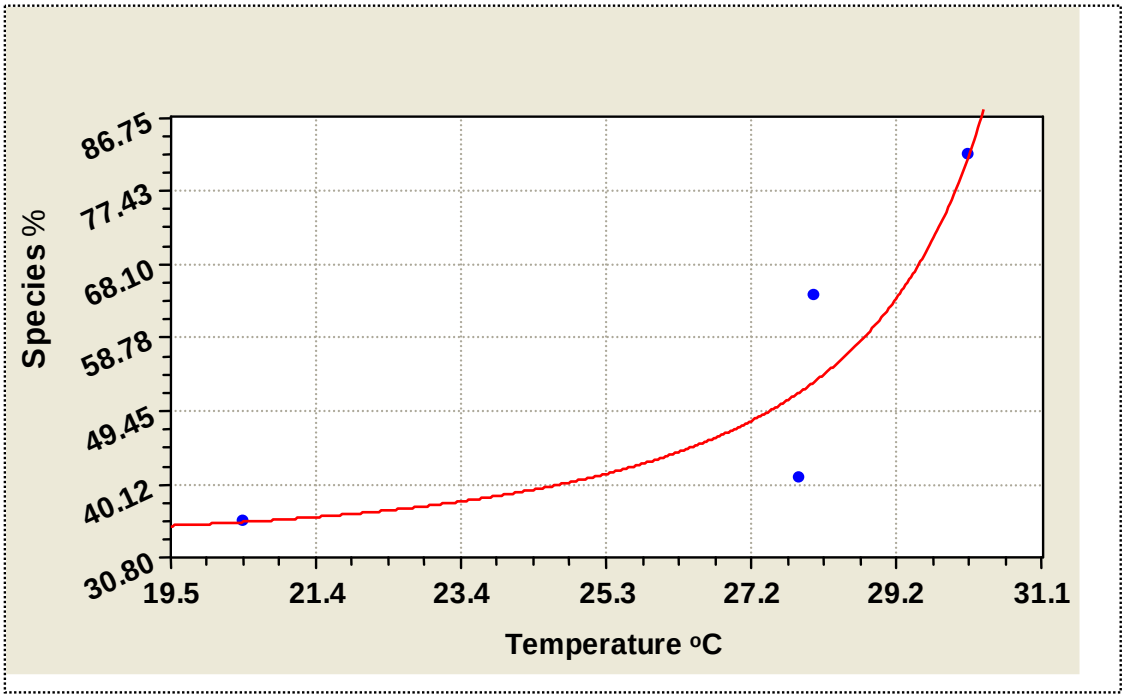
3.13.1 Temperature Measurement of Drinking Water of Khairpur City

The temperature of drinking water (municipal water n=72) was analyzed. Samples were collected from main reservoirs of Khairpur in different months during 2005-2007. In January-March the minimum temperature 17.0^{o}C, maximum temperature 23^{o}C and mean temperature was 20.4^{o}C, in April-June, minimum temperature 26.0^{o}C, maximum temperature 29.8^{o}C and mean temperature was 28.0^{o}C, in July-September minimum temperature 29.0^{o}C, maximum temperature 31.2^{o}C, and mean temperature 30.1^{o}C and in October-December minimum temperature 21.0^{o}C, maximum temperature 29.5^{o}C, mean temperature was 26.3^{o}C (Table 26. The relation of temperature with frequency of waterborne bacterial pathogens in fresh water environment is illustrated in Fig.31a

Table 26
Temperature of Drinking Water Samples of Khairpur City (2005-2007)
(n =72)

MONTH	MINIMUM ^{O}C	MAXIMUM ^{O}C	MEAN ^{O}C	STD. DEVIATION
Jan-Mar	17.0	23.0	20.4	1.6
Apr-Jun	26.0	29.8	28.0	1.2
Jul.-Sep.	29.0	31.2	30.1	0.5
Oct-Dec.	21.0	29.5	26.3	2.6

Fig. 31a
The Relation of Temperature with Prevalence of Waterborne Bacterial Pathogen in Drinking Water Collected from Main Storage Reservoir of Khairpur City.(n = 72)



The relation of temperature of water samples colleted from municipal water of Khairpur city with the isolation rate of waterborne bacterial pathogens during study period. The points represent the % of species isolated at various temperatures of water samples. The mean quarterly data was used to show the relation ship between temperature taken in those quarters and the bacterial species.

3.13.2 Temperature Measurement of Drinking Water of Sukkur City

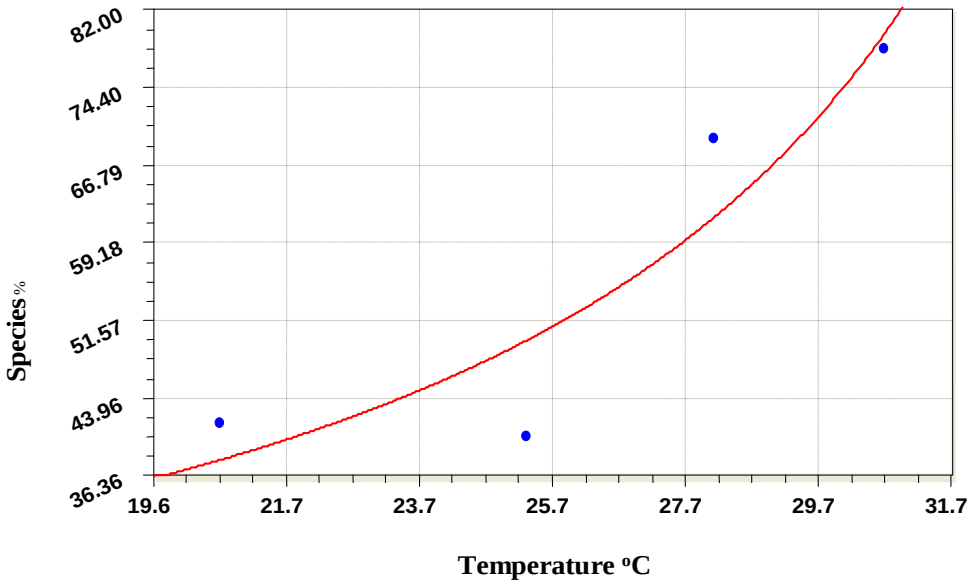
Temperature of 72 drinking water samples (municipal water n=72) 18 samples in each quarter was measured during 2005-2007. January-March minimum temperature was 16.0^{o}C, the maximum temperature was 23.3^{o}C, and mean temperature was 20.6^{o}C, April-June minimum temperature was 26.0^{o}C, maximum temperature was 30.2^{o}C mean temperature was 28.1^{o}C, July-September the minimum temperature was 30.0^{o}C, the maximum temperature was 31.0^{o}C and the

mean temperature was 30.7^{o}C and in October-December the minimum temperature was 20.0^{o}C, maximum temperature was 29.5^{o}C and mean temperature was 25.70^{o}C Table 27 The relation of temperature with frequency of waterborne bacterial pathogens in freshwater environment was observed in present study is illustrated in Fig.31b

Table 27
Temperature of Drinking Water Sample of Sukkur City (2005-2007)
(*n* =72)

MONTH	MINIMUM ^{O}C	MAXIMUM ^{O}C	MEAN ^{O}C	STD. DEVIATION
Jan-Mar	16.0	23.3	20.6	1.9
Apr-June	26.0	30.2	28.1	1.3
Jul-Sep.	30.0	31.6	30.7	0.5
Oct-Dec.	20.0	29.5	25.2	2.9

Fig. 31b
The Relation of Water Temperature with the Prevalence of Waterborne Bacterial Pathogens in Drinking Water of Sukkur City. (n =72)



The relation of temperature of water samples colleted from municipal water of Sukkur city with isolation rate of waterborne bacterial pathogens during study period. The points represent the % of species isolated at various temperatures of water samples. The mean quarterly data was used to show the relationship between temperature taken in those quarters and the bacterial species.

3.13.3 Temperature Measurement of Drinking Water of Rohri city

Temperature of 72 drinking water samples (municipal water n=72) 18 samples in each quarter was measured during 2005-2007. In January-March minimum temperature 17.6°C, maximum temperature 22.5°C and mean temperature was 20.7°C, April-June minimum temperature 23.6°C, maximum temperature 30.2°C and the mean temperature was 27.0°C, July-September minimum temperature 29.0°C, maximum temperature 32.0°C and mean temperature was 30.4°C and in October-December minimum temperature 18.0°C maximum temperature 29.0°C mean

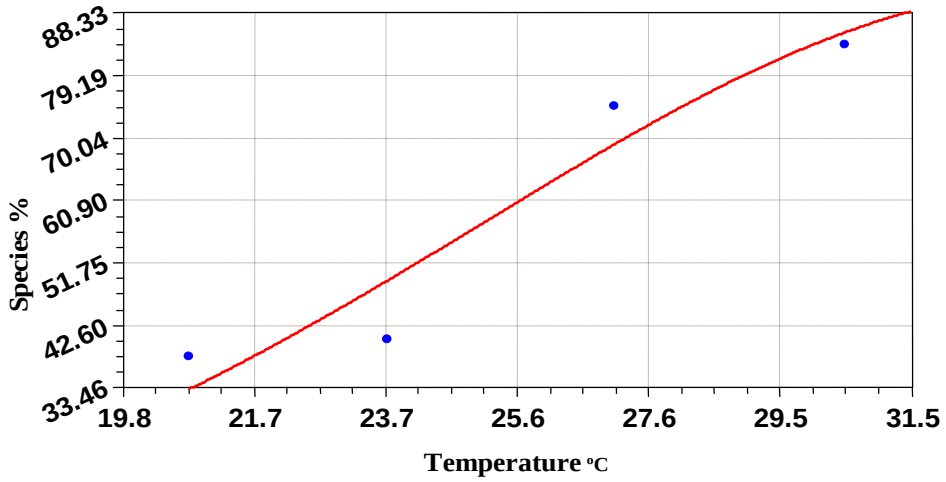
temperature was 23.7^{o}C Table 28 The relation of temperature with frequency of waterborne bacterial pathogens in fresh environment was observed in present study is illustrated in Fig.31c

Table 28
Temperature of Drinking Water Samples of Rohri City (2005-007)
(*n* =72)

MONTH	MINIMUM ^{O}C	MAXIMUM ^{O}C	MEAN ^{O}C	STD. DEVIATION
Jan-Mar	17.6	22.5	20.7	1.3
Apr-June	23.6	30.2	27.0	2.0
Jul-Sep.	29.0	32.0	30.4	0.8
Oct-Dec.	18.0	29.0	23.7	3.2

Fig 31c

The Relation of Water Temperature to the Prevalence of Waterborne Bacterial Pathogens in Drinking Water from Rohri City. (n =72)

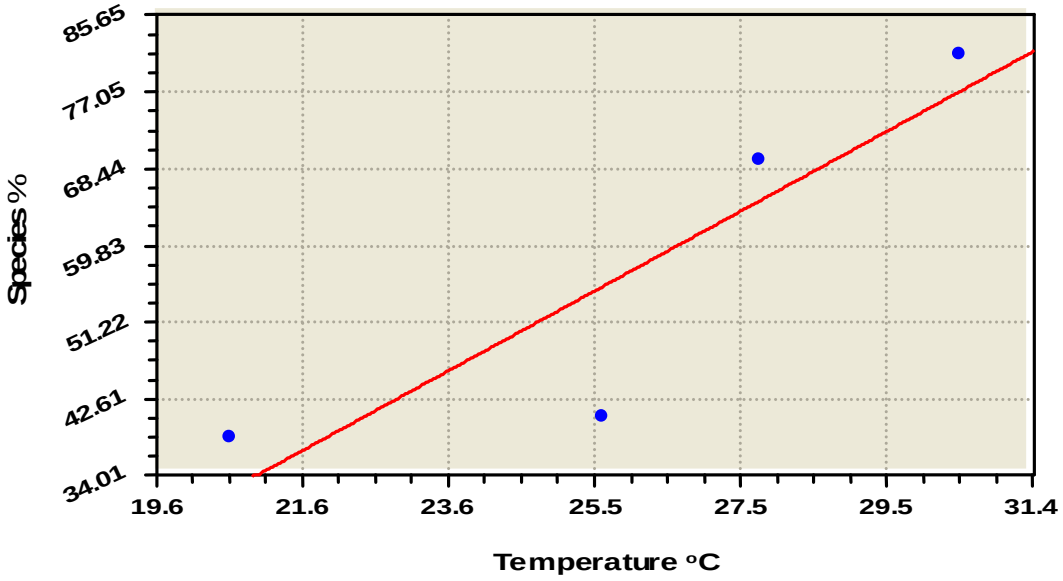


The relation of temperature of water samples colleted from municipal water of Rohri city with the isolation rate of waterborne bacterial pathogens during study period. The points represent the % of species isolated at various temperatures of water samples. The mean quarterly data was used to show the relationship between temperature taken in those quarters and the bacterial species

3.13.4 Temperature of Drinking Water of Khairpur, Sukkur and Rohri (2005-2007)

The relation of water temperature to isolation rate of waterborne bacterial pathogen has been observed during three years of study of drinking water of Khairpur Sukkur and Rohri cities during 2005-2007. It is evident in Fig. 31d that as temperature increases, the isolation rate of waterborne bacterial pathogens also increases. When temperature was 19°C the isolation rate was 34%, when the temperature was 25°C then the isolation rate was 40%, when temperature was 27°C the overall isolation rate was 70% and when temperature of water was 30°C the over all isolation rate was 80% In the present study it was observed that very high isolation rate was recorded during warmer months.

Fig. 31d
The Relation of Water Temperature to the Prevalence of Waterborne Bacterial Pathogens in Drinking Water of area under Study during Study Period (n = 216)



The relation of water temperature with the isolation rate of waterborne bacterial pathogens during study period. The points represent the % of species isolated at various temperatures of water samples. The mean quarterly data was used to show the relationship between temperature taken in those quarters and the bacterial species

3.14 Correlation between Isolated Waterborne Bacterial Pathogens and Water Temperature

The correlation matrices between water temperature and isolated waterborne bacterial pathogen are shown in Table 29.The isolated waterborne bacterial pathogens showed strong correlation with water temperature throughout the study period (*r* ranging from 0.60-0.98). *E. coli* 1 (*r* = 0.81) *Nonfermeter spp*,(*r* = 0.60), *P. mirabilis* (*r* = 0.82) *A. hydrophila* (*r* = 0.98), *P. rettgeri* (*r* = 0.60), *P. stuarti* (*r* = 0.69), *K. oxytoca* (*r* = 0.74), *C. youngae* (*r* = 0.92), *V. mimicus* (*r* = 0.91), *V. cholerae* (*r* = 0.73) *and E. coli* 2 (*r* = 0.83). However *C. meningosepticum* showed weak (r = 0.33) *and P. aeruginosa* showed moderate (*r* = 0.56) correlation with water temperature.

3.15 pH analysis of Drinking Water (Municipal Water) of Khairpur, Sukkur and Rohri Cities (2005-2007).

The pH of water entering the distribution system must be controlled to minimize the corrosion of water mains and pipes in household system. Failure to do so can result in the contamination of drinking water and in adverse effects on its taste, odor and appearance (WHO 2007). The pH of water was analyzed in order to observe the condition of drinking water of Khairpur, Sukkur and Rohri. It was observed that the pH of drinking water was in the range (6.5-8.5) fixed by World Health Organization (WHO) standard (WSC 2004).

3.15.1 pH Analysis of Drinking Water of Khairpur City.

The pH of drinking water of Khairpur was measured in different months during 2005-2007. In January-March the minimum pH was 7.2, the maximum pH was 8.3 and the mean pH was 7.8. In April-June the minimum pH was 7.5, maximum pH 8.6 and the mean pH was 7.9, in July-September minimum pH was 7.0, maximum pH was 8.4 and mean pH was 7.6. In October-December minimum pH was 7.2, maximum pH was 8.5 and mean pH 7.8 was recorded (Table 30)The pH of water samples from distribution line and consumers tape was also determined. The data is presented in table in

Table 30
pH of Drinking Water, Samples of Khairpur City (2005-2007)
(*n* =72)

Month	Minimum	Maximum	Mean	Std. Deviation
January-March	7.2	8.3	7.8	0.2
April-June	7.5	8.6	7.9	0.2
July-September	7.0	8.4	7.6	0.4
October-December	7.2	8.5	7.8	0.3

Table 31
pH of Water Samples Collected from Different Sites

Site	n	Maximum	Minimum	Average	WHO limits
Main reservoir	30	6.31	7.41	7.37	6.5-6.8
Distribution line	30	6.78	7.81	7.09	6.5-6.8
Consumer tap	30	6.87	7.5	7.01	6.5-6.8

3.15.2 pH Analysis of Drinking Water of Sukkur City.

The pH of drinking water (municipal water) of Sukkur city was analyzed during 2005-2007. In January-March the minimum pH was 7.2, maximum pH was 8.3, and the mean pH was 7.8. In April-June minimum pH was 7.0, maximum pH was 8.2 and mean pH was 7.81. In July-September minimum pH was 7.1, the maximum pH was 8.5, and the mean pH 7.7. In October-December minimum pH was 7.0, maximum pH was 8.5 and the mean pH was 7.8 as is given in Table 32

Table 32
pH of Drinking Water Samples of Sukkur city(2005-2007)
(*n* =72)

Month	Minimum	Maximum	Mean	Std. Deviation
January-March	7.2	8.3	7.8	0.3
April-June.	7.0	8.2	7.81	0.2
July-September.	7.1	8.5	7.7	0.3
October-December.	7.0	8.5	7.8	0.4

3.15.3 pH Analysis of Drinking Water of Rohri City.

pH of drinking water (municipal water) of Rohri city was analyzed during 2005-2007. In January-March minimum pH was 7.0, maximum pH was 8.0 and the mean pH was 7.7. In April-June minimum pH was 7.3, maximum pH was 8.5 and the mean pH was 7.9. In July- September minimum pH was 7.2, maximum pH was 8.30 and the mean pH was 7.8. In October- December the minimum pH was 7.0, maximum pH was 8.2 and the mean pH 7.5 was recorded (Table 33

Table 33
pH of Drinking Water Samples of Rohri City (2005-2007)
(*n*=72)

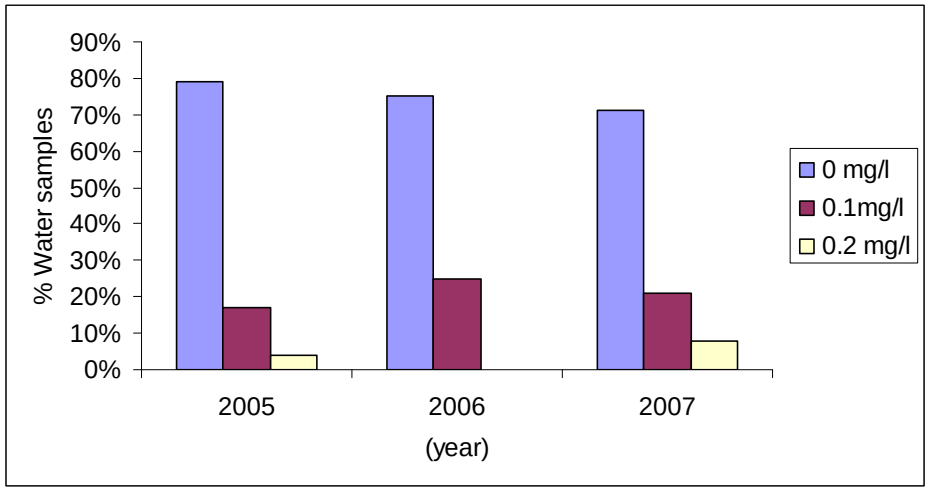
Month	Minimum	Maximum	Mean	Std. Deviation
January-March	7.0	8.0	7.7	0.2
April-June	7.3	8.5	7.9	0.2
July-September	7.2	8.3	7.8	0.2
October-December	7.0	8.2	7.5	0.3

3.16 Analysis of Free Residual Chlorine in Drinking Water (Municipal Water) of Khairpur, Sukkur and Rohri Cities (2005-2007)

3.16.1 Free Residual Chlorine in Drinking Water of Khairpur City

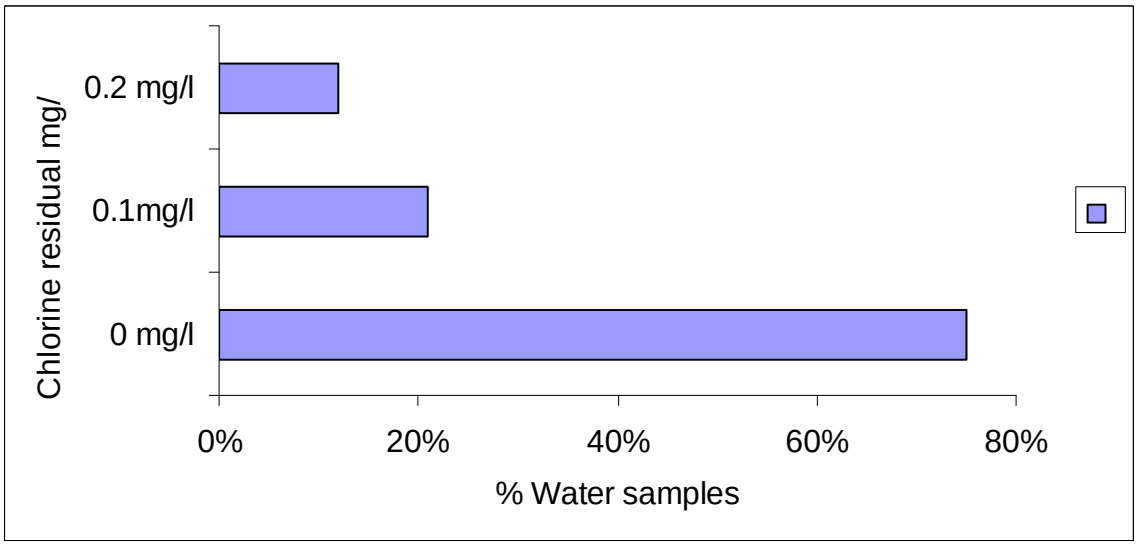
Free residual chlorine was measured in drinking water samples collected from municipal water of Khairpur city during 2005-2007. In 2005 out of 24 samples, 19 (79.1%) samples of drinking samples were without chlorine, only 4 (17%) samples with 0.1 mg/l, and 1 (4%) samples were containing 0.2mg/l. In 2006 18 (75%) samples were observed without chlorine, and 6 (25%) containing 0.1 mg/l chlorine residual whereas in 2007 17(71%) samples were found without chlorine, 5 (21%) samples with 0.1mg/l, and 2(8%) were containing 0.2 mg/l residual chlorine, Fig. 32a. The percentage of total drinking water samples (n =72) containing residual chlorine were 25% out of which 21% contained 0.1 mg/L and 4% contained 0.2 mg/L. Where as 75% did not contain any residual chlorine as shown in Fig. 27b. Residual chlorine was also determined in water sample collected from randomly selected distribution line and consumers tap it was also observed that the residual chlorine was not detected in any sample tested (Table 34)

Fig. 32a
Percentage of Drinking Water Samples Observed with and without Free Residual Chlorine in Drinking Water of Khairpur City during Study Period(n =72)



The comparative percentage of drinking water samples containing varying amount of free residual chlorine during study period.

Fig. 32b
Total Percentage of Drinking Water Samples Found with and without Free Residual Chlorine in Drinking Water of Khairpur City (n =72)



The mean percentage of drinking water samples containing varying amount of free residual chlorine during study period.

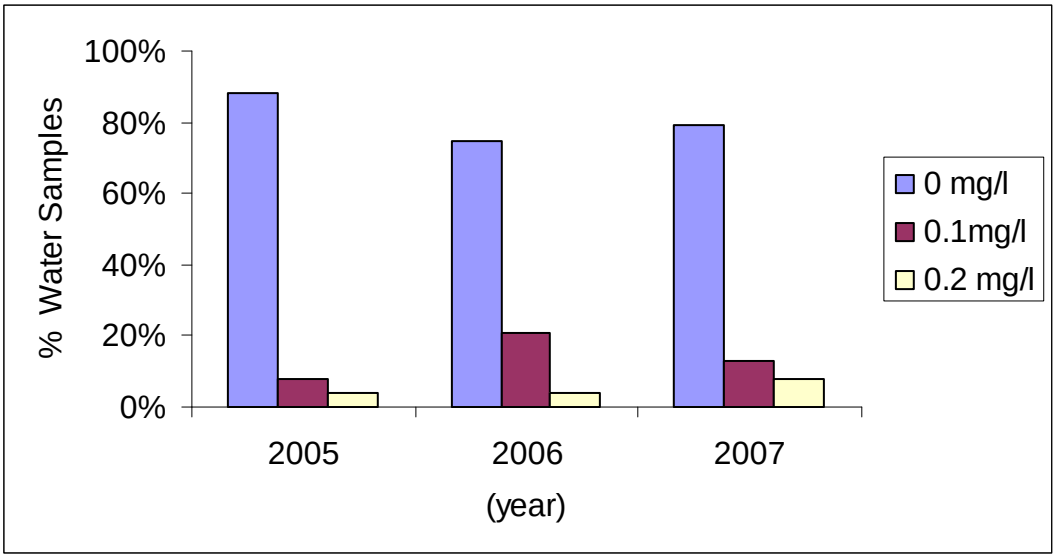
Table 34
Residual Chlorine Content in Water Sample Collected from Different Sites in Khairpur city

Site	n	Maximum	Minimum	Average	WHO limits
Main reservoir	30	00	00	00	0.2-0.5 mg/L
Distribution line	30	00	00	00	0.2-0.5 mg/L
Consumer tap	30	00	00	00	0.2-0.5 mg/L

3.16.2 Free Residual Chlorine in Drinking Water of Sukkur City

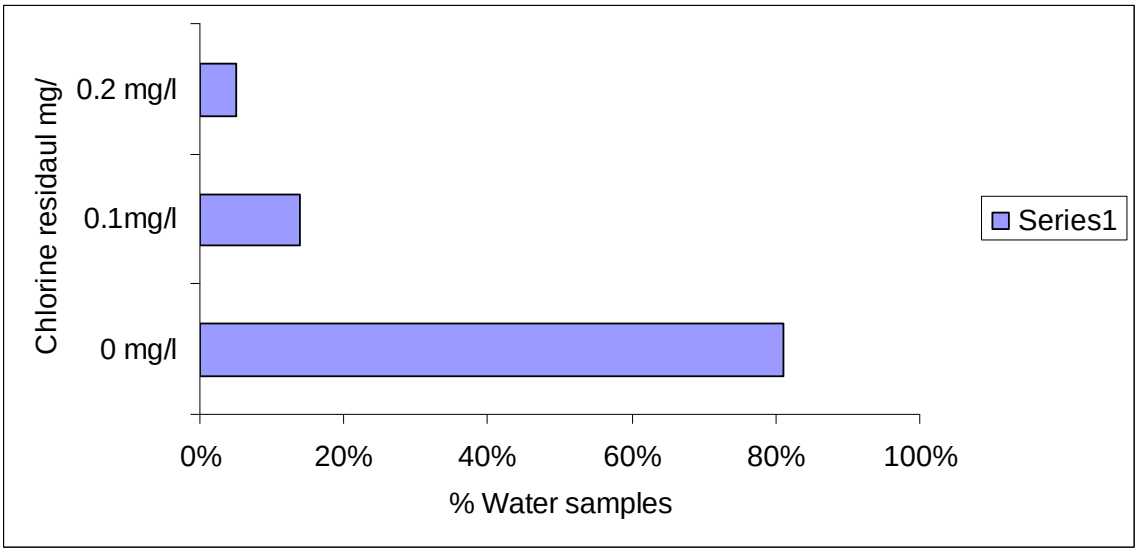
Free residual chlorine was analyzed in drinking water (municipal water) of Sukkur city in 2005-2007. It was observed that 21(88%) samples were found without residual chlorine, 3 (8%) and 1(4%) samples were found with 0.1mg/l and 0.2mg/l respectively in 2005. In 2006, 18(75 %) samples were found without residual chlorine, 5(21%) and 1(4%) samples were found with 0.1mg/l and 0.2mg/l respectively. In 2007, 19(79 %) samples were found without residual chlorine, 3(13%) and 2(8%) samples were found with 0.1mg/l and 0.2mg/l respectively. Fig 33a. The mean percentage of drinking water samples containing free residual chlorine throughout study period from Sukkur city was, out of 72 (100%) drinking water samples, 58(81%) were found without chlorine, 10(14%) contained 0.1 mg/l, and only 4(5.5%) contained .02 mg/l as shown in Fig.33b. the residual chlorine was also determine in distribution line.

Fig. 33a
Percentage of Drinking Water Samples Observed with and without Free Residual Chlorine in Drinking Water of Sukkur City (n =72)



The comparative percentage of drinking water samples containing varying amount of free residual chlorine during study period collected from drinking water of Sukkur city.

Fig. 33b
Total Percentage of Drinking Water Samples Found with and without Free Residual Chlorine in Drinking Water of Sukkur City (n =72)



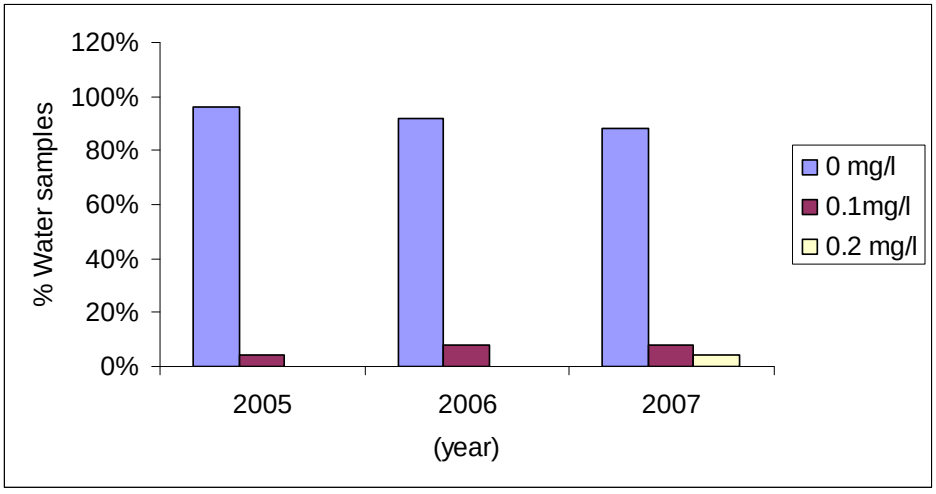
The mean percentage of drinking water samples containing varying amount of free residual chlorine during study period.

3.16.3 Free Residual Chlorine in Drinking Water of Rohri City

Free residual Chlorine was analyzed in drinking water (municipal water) of Rohri city, in 2005. 23(96%) samples were found without chlorine and only 1(4%) samples were found with 0.1mg/l residual chlorine which was also below the standards of WHO for drinking water. In 2006, 22 (92%) samples were found without chlorine, 2(8%) samples found with 0.1mg/l residual chlorine. In 2007, 21(88%) samples were found without residual chlorine, and only 2 (8%) samples were found with 0.1mg/l residual chlorine, and 1(4%) samples were containing .2 mg/l residual chlorine (Fig 34a). The percentage of total drinking water samples (n =72) containing residual chlorine were 8% out of which 7% contained 0.1 mg/L and 1.38% contained 0.2 mg/L. Where as 92% did not contain any residual chlorine as shown in Fig. 34b

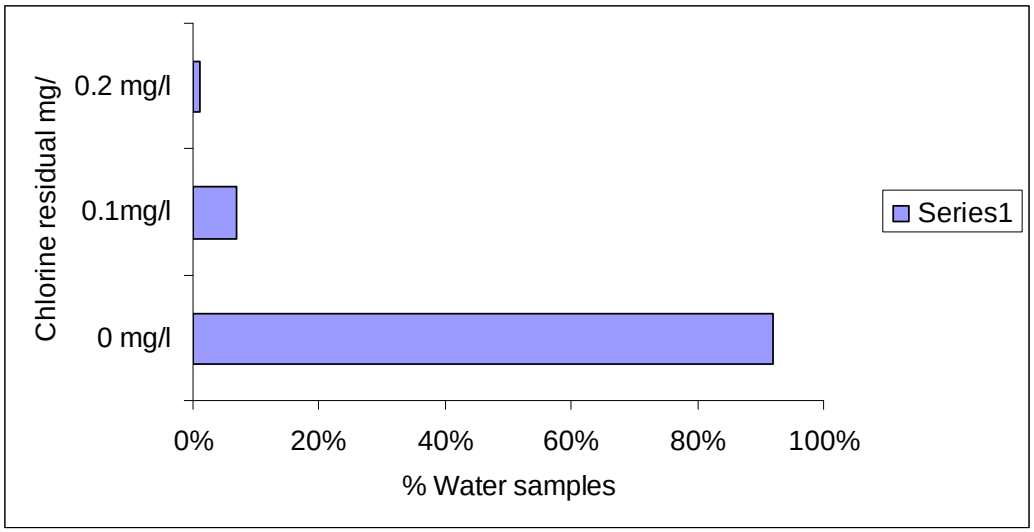
Total percentage of drinking water sample containing free and residual chlorine from area under study i.e. Khairpur Sukkur and Rohri throughout study period 2005-2007 was out of 216(100%) 180(83%) was found without chlorine, 30(14%) containing .01 mg/l, and 6(3%) contained 0.2 mg/l residual chlorine as shown in Fig 35. The Fig.36 illustrates the overall use of chlorine disinfection treatment of the drinking water of three cities from which the samples were collected.

Fig. 34a
Percentage of Drinking Water Samples Observed with and without Free Residual Chlorine in Drinking Water of Rohri City(n =72)



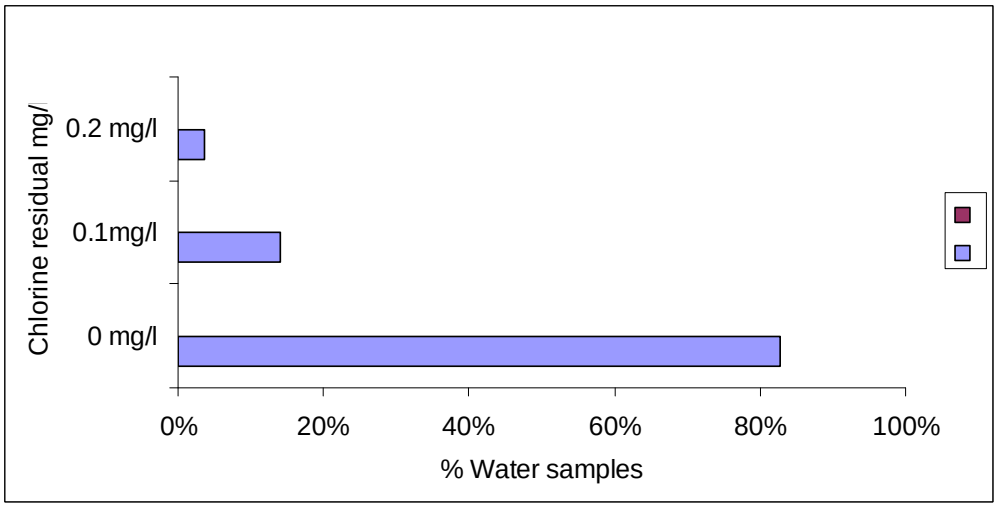
The year wise comparative percentage of drinking water samples containing varying amount of free residual chlorine during study period collected from drinking water of Rohri city.

Fig. 34b
Total Percentage of Drinking Water Samples Found with and without Free Residual Chlorine in Drinking Water of Rohri City during 2005-2007(n =72)



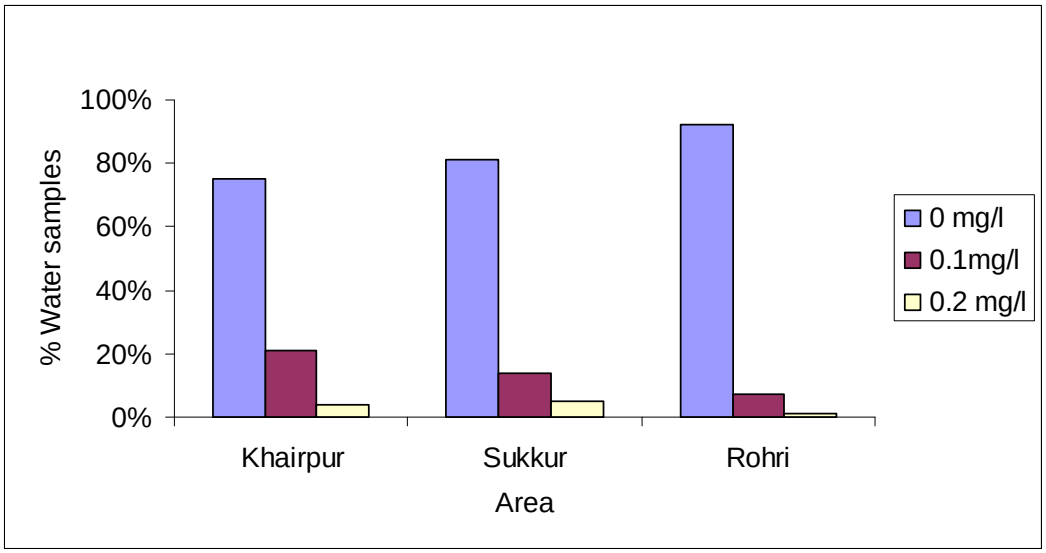
The mean percentage of drinking water samples containing varying amount of free residual chlorine during study period.

Fig.35
Total Percentage of Drinking Water Samples Found with and without Free Residual Chlorine in Drinking Water of Study Area (2005-2007) (n =72)



The mean percentage of drinking water samples containing varying amount of free residual chlorine throughout study period from three cities.

Fig. 36
The percentage of Drinking Water Samples with and without Free Residual Chlorine from Drinking Water of Study Area during Study Period (n =72)



The site wise comparative percentage of drinking water samples containing varying Amount of free residual chlorine throughout study period collected from municipal water of study area.

4. DISCUSSION

Enumeration of Fecal coliform (*Escherichia coli*) of Drinking Water of Khairpur, Sukkur and Rohri City 2005-2007.

It has been the goal of diagnostics to detect and enumerate target organisms in clinical and environmental samples rapidly and with sensitivity. Over the past ten years effective efforts have been made in this field (Cengi *et al.* 1993). Within the coliforms family *E coli* is of the prime interest and reliable indicator for fecal contamination rather than total coliform (Stevens *et al.* 2003), because its presence indicates recent fecal contamination with the possibility of enteric pathogens (Jay 1996). Assessment of water quality depends on detection of indicators in water samples (Baudart *et al.* 2002).

Qualitative and quantitative composition within bacterial pathogens in samples of drinking water of Khairpur, Sukkur and Rohri were probably due to contamination of sources of drinking water by human or animal excreta. Bacteriological contamination of drinking water has remained one of the major problems in the country in rural as well as urban areas (Abid & Jamil, 2005; Kahlown, Tahir, & Sheikh, 2004). Due to leakage of pipes, pollution from sewerage, biofilm formation in the distribution system, intermittent water supply, and human activities the chemical and biological contamination is likely to occure (Tahir & Bhatti 1994; Sajjad & Rahim, 1998; Chandio, 1999). The extremely hazardous sources of contamination are excessive monsoon rains, floods, herbicides, fungicides, untreated municipal waste, and coastal water pollution due to waste discharges and oil spills. To protect the consumers health it is of crucial importance to establish drinking water quality standards and criteria that are chemically balanced and medically safe (Health Services Academy 2008). General public, in Pakistan, use subjective quality criteria like brackish, foul smelling, bad tasting, turbid or colored water to determine that it is not suitable for drinking. The agencies responsible for monitoring of water quality perform periodic checks of the basic water parameters against certain recommended standards. This study was limited to the urban areas of Khairpur, Sukkur and Rohri the rural area were not included in this study**.**

The enumeration of fecal coliform (*E. coli*) bacteria from drinking water of Khairpur, Sukkur and Rohri municipal water in higher cfu per 100 ml, suggests the fecal contamination of drinking water of these cities. Since the presence of bacteria of fecal origin reflects the deteriorating

bacteriological quality of drinking water. In the present study the number of fecal coliform (*E. coli*) was recorded higher in Khairpur, Sukkur and Rohri. There were also very large numbers of fecal coliform (*E. coli*) per 100 ml found in warmer months than in winter months as given in Table.5, 6 and 7. In summer months when temperature of water reaches up to 30 ^{o}C the cfu/100 ml were higher than cfu/ 100 ml in winter months. The incidences of diarrhea cases were also reported higher in the warmer months however, data of diarrhoeal cases were obtained from district hospitals and may not present the true picture, because many cases of diarrhea go unreported. In same way the occurrence of waterborne pathogens could also be seasonal. In present study the enumeration of fecal coliform (*E. coli*) was conducted during 2005-2007 by using membrane filtration method. It was observed that the number of fecal coliform (*E. coli*) per 100 ml was thousand times higher in the drinking water of Khairpur Sukkur and Rohri cities than the World Health Organization (WHO) guideline values for drinking water. The results are consistent with study of Leony *et al.* 2005, who reported high prevalence of coliform (total and fecal) in warmer months. The main source of drinking water of Khairpur city is surface water from the Mirwah canal which is a tributary of river Indus and it is unprotected from the all kinds of contamination. This is pooled in a main reservoir before distribution. All 90 samples (100%) were found contaminated with total coliform and fecal coliform (*E. coli*) (Table 6) which indicates possible fecal contamination from human and animal sources. The quality of drinking water of Khairpur city is not an exception as results from various investigations provide evidence that most of the drinking water supplies are fecally contaminated (Javed 2003). Haleem (1996) reported that drinking water of G-10 sector Islamabad was more polluted. Ihsanullah in (1999) isolated *E. coli* in drinking water of Risalpur, Pubbi and Tarnab while he was working on drinking water. The present study also supports the above reports in regards that the quality of drinking water in many parts of the Pakistan is not up to WHO standard. The detection of total coliform and *Escherichia coli* in a large number implies that the contaminated drinking water may be responsible for increasing number of water born diseases in the country. The moderate correlation was observed between the diarrhea cases reported in district hospitals and total and fecal coliform present in the drinking water of study area. The burden of disease directly associated with incomplete water and sanitation provision in refugee camps in sub-Saharan African countries has been evaluated by Cronin *et al*. (2009). Around 132,000 cases reported of diarrhea attributed to incomplete water and sanitation provision. Another indirect correlation has

been established between diarrhea cases and drinking water quality was that of a report by H:\Water-related diseases « WASH Research News.htm in which it was clearly demonstrated that sanitation intervention improved the child health by reducing diarrhea cases. According to WHO drinking water quality standards there should be no coliform as well as fecal coliform present in 100 ml or 0 colony forming units (cfu) per 100 ml (Health Services Academy 2005). However, the result of our study is far from WHO standards and therefore, water under study was not suitable for drinking purpose. The level of drinking water pollution due to the presence of total and fecal coliform was also assessed at the point of consumers. Different sampling points i.e. households, hotel, schools and hospitals were included in this study. It is evident from the study that water quality further deteriorated at the consumer level**.** This demonstrated the importance of proper management of municipal water from storage and supply to households (point of use). The microbiological quality of the municipal water supplied to the people in Sukkur city was not according to World Health Organization (WHO) standards. The number of total coliform and fecal coliform were very high at all levels i.e. main reservoir, distribution line, households, hotels and hospitals (Table 10). The residual chlorine was not found in any sample which shows the lack of chlorine disinfection of municipal drinking water. The Sukkur city the thickly populated with 0.7million people has the drinking water source from River Indus and is totally unprotected from traditional sources of contamination i.e. sewage water disposal, garbage, animal carcass dumping , and entrance of human and animal excrements in the (River Indus)source water. All 120 samples (100%) and 98(81.66%) were found contaminated with total coliform and fecal coliform (*Escherichia coli*) respectively, which shows possible fecal contamination from human and animal sources. The supply of contaminated water to the people in Sukkur is not a single example but the results from various studies prove that most of the drinking water supplies in Pakistan are fecally contaminated. The quality of drinking water in most other cities such as Karachi has been reported contaminated and not fit for human consumption (Luby *et al*. 2001). In present study it is evident from the results that similar to other developing countries, a wide range of sources for drinking and other domestic purposes are being used in Pakistan. These sources are not adequately protected and containing higher number of fecal or Tthermotolerant coliform, this study has also demonstrated the same conditions during the investigation of municipal drinking water of area under study. The results are in the agreement with the data given by Hunter *et al.* (2003) in which it was reported that the drinking

water quality in most of the developing countries was very poor with 100000 fecal coliform or Thermotolerant coliform per 100 ml of drinking water. WHO guidelines for drinking water recommend that the total coliform or Thermotolerant fecal coliform must not be detected in any 100 ml of drinking water (Hunter *et al.* 2003). Tambe *et al*. (2008) reported that in many areas of India half the time the available drinking-water was contaminated with organisms whose ecological niche is the human intestine. This finding is common throughout South Asia including Pakistan where both urban and rural water supplies are frequently contaminated with human faecal organisms. Although 85% of drinking-water in South Asia meets the target of the Millennium Development Goal of coming from an improved source (United Nations 2006), this water is, in fact, frequently contaminated with human faecal organisms (Tambe *et al*.2008., Anwar et al. 2004., Sirajul Islam 2007). Indeed, the frequency of water contamination with human faeces is so common throughout South Asia that it is accepted as the norm. Those who can afford it buy bottled water (of dubious quality), and the majority are left to drink the available contaminated water. The commonality of this contamination risks preventing us from appreciating the seriousness of the problem. In a meta-analysis of studies of the community-based approaches to improve water supply, the type of intervention that Tambe *et al*. (2008) described in their article, these interventions have not been associated with a significant reduction in diarrhoeal disease (Clasen *et al*. 2007). We need to confront the dogmatism that current interventions represent improvement. We need more of what Tambe *et al*. (2008) have done, i.e. we need to evaluate the outcomes of interventions on water quality. We need to identify which interventions on water quality improve health and how these can be implemented at a large scale. Importantly, the problem of water quality does not end with microbiological contamination. Groundwater, especially shallow groundwater, in many sites in South Asia is contaminated with dangerously high levels of arsenic (Smith *et al*. 2000). The contamination of municipal drinking water of area under study seems to have the same pattern like other cities in Pakistan. The pollution at source level was very common in Khairpur, Sukkur and Rohri because the storage ponds were open and uncovered, and not protected from human and animal activities. The storage areas were bushy and grassy; plants and grass were present in storage ponds, rodent burrows, and bird's habitats were very common. These unhygienic conditions may have the contribution in regular transmission and growth of *E. coli* in such waters. This study was carried out using membrane filtration technique and API 20E for the isolation and identification of

waterborne bacterial pathogens. These are standard microbiological methods with high sensitivity and specificity reported in the literature. However, 16s rRNA technique could be used to further support the results of this study.

Multi-drug Resistance of Thermo-tolerant *E. coli*.

Multi-drug resistance assessed for the thermo-tolerant *E. coli*, isolated in present study from drinking water samples was subjected to antibiotic sensitivity testing. The isolation of multi-drug resistant thermotolerant *E. coli* is alarming**.** The emergence of antimicrobial resistance in bacteria has been a problem throughout the world (Cohen 2000). Tambekar *et al*. (2006) reported the resistance of *E. coli* isolates to ofloxacin followed by novabiocin, cefdixin and Ciprofloxacin. It has been reported that Beta- lactamase producing *E. coli* which had become resistant to ceftriaxome can become sensitive to the same antibiotic when the inhibitor sulbactam is added (Abdul *et al*. 2005). Alhussain *et al*. (2005) reported the excellent susceptibility to meropenium and variable suseptibility to aminoglycosides fluoroquinolines but greatly reduced suseptibility to beta lacatam inhibitors combination, trimethoprime and sulfa methoxazole in extended spectrum beta-lacatamase producing *E. coli* (ESBL) producing *E. coli*, during the work on in-vitro susceptibilities of *E. coli* ampicillin-sulbactam and amoxicillin-clavulanic acid. Birgule and Nedim (2007) found that more organism were susceptible to amoxicillin-clavulanic acid than ampicillin-sulbactam. Wimmerstedt and Kahlmets (2008) investigated the trimethoprime resistance in ampicillin resistant than ampicillin susceptible isolates of *E. coli*. The results of the multi-drug resistance study (Table 8) indicate that *E. coli* resistant to sixteen different antibiotics is widespread in municipal water of Khairpur included in this study. The emergence of multi-drug resistance among these bacteria and their presence in drinking water is the matter of grave concern. In Khairpur the untreated domestic sewage is being disposed off in canals (source water). The presence of drug residues in such waters can not be ruled out because that sewage also contains hospital wastes, further surface runoff during rainy season might introduce these bacteria into the fresh water environment. Hence it is possible that municipal water taken from surface water of river could be contaminated with *E. coli.* The presence of penicillin amoxil streptomycin and other antibiotic resistance in certain strains of *E. coli* might suggest a hospital or veterinary origin of such strains. *E. coli* in aquatic environment is exposed to sub-lethal doses of antibiotics present in that environment brought in by disposed waste. The use of antibiotic is

widespread in animal industry. It is estimated that the use of antibiotics in animals is 100-1000 times more than that in human population (Feimen 1998, Levy 1998, Witte 1998). No study so for has been done to evaluate the impact of frequent antibiotic use in animals as well as in humans in Pakistan. The uncontrolled usage of antibiotics in Pakistan contributes significantly antibiotic residues in fresh water environments. The antibiotic resistance patterns of strains of *E. coli* isolated from drinking water observed in present study suggests a greater risk in form of transfer of resistance to other pathogenic bacteria. The possible exchange of plasmids between *E. coli* and other bacteria has been previously reported (Grabow 1976). The antibiotic resistance could be transferred from non-pathogenic to pathogenic bacteria by transfer of resistant genes in closely related bacteria such as members of Enterobacteriaceae family, It is remarkable that Alhussain and Naeem (2005), reported in their studies where *E. coli* was observed susceptible to meropenem, but the present study reported the resistance in *E. coli* to this antibiotic. The sources and routes of contamination in water are difficult to establish, as fresh water receives bacterial population from diverse sources. An effective surveillance infrastructure is needed to be made to determine the presence and distribution of antibiotic resistant strains of *E. coli* in municipal water in Pakistan.

Identification of Waterborne Bacterial Pathogens from Drinking Water

Qualitative and quantitative composition of Enterobacteriaceae and other pathogenic bacteria in fresh water samples from main storage reservoirs of Khairpur, Sukkur and Rohri cities of Sindh Pakistan was probably due to insufficient preventive measures in drinking water sector. The difference in the quantitative frequency of waterborne pathogens depending upon the conditions prevailing around the sources of water, protection of source of water, treatment and the well being of supply system. The agricultural organic pollutant and potentially pathogenic bacteria may migrate with surface runoff in the river or stream (Brenner *et al.* 1999., Brenner *et al.* 1996., Miles 1993), thereby deteriorating the bacteriological state of waters and increasing the risk of transmission of various diseases (Boyer and Pasquarell 1999).

The presence of pathogenic bacteria in drinking water is proportional to the sources of their origin and influenced by input of contaminants. In bacteriological analysis of municipal water collected from main storage reservoirs of area under study, including the physico-chemical factor determination, higher numbers of thermo-tolerant *E. coli* which are the indicators of fecal

contamination were obtained, ranging from 3.0-3.8 log cfu/100 ml of drinking water, this situation may corresponding the health risk from waterborne bacterial pathogens in drinking water of area under study.

E. coli

The *E. coli* was isolated at highest isolation rate of 70%, in drinking water samples tested in this study. Through studies it has been reported that *E. coli* is the only coliform which is mostly associated with a fecal source (Bej *et al.* 1990; Dogan- Halkman *et. al.* 2003; Petit *et al.* 2001). It was also reported that the other thermo-tolerant coliform and total coliform were also capable of growth in non-polluted waters. This supplements a recommendation for *E. coli to* be used as the prime indicator bacteria for recent fecal contamination (Baudizsova 1997). *E. coli* is typically sensitive to environmental stress. Its survival time in the environment is dependant on many factors, including temperature, exposure to sunlight, presence and types of microflora, and the type of water involved (e.g., groundwater, surface water, or treated distribution water). Moderate microflora *E. coli* survives for about 4-12 weeks at temperature range of 15-18 ^{o}C (Kudryavtseva, 1972; Edberg *et al.* 2000). The isolation rate of *E. coli* 1was significantly greater in summer months than in winter months in Khairpur, Sukkur and rohri city ($p<0.014$, $p<0.018$, $p<0.011$) respectively. The results show continuous presence of *E. coli* in drinking water indicating the continuous source of fecal contamination in these waters. The conditions around the sampling sites were very unhygienic; the area where municipal water was stored for supply was open for the human and animal's access. The old service rooms which were used for preventing the animal and human from entering into the settling tanks were being used as open and dry toilets at main storage reservoirs of Khairpur municipal water. This may be the immediate and continuous source of *E. coli* and other waterborne bacterial pathogens in municipal water of Khairpur.

P. mirabilis

Our findings reveal the presence of *P. mirabilis* in 67% drinking water samples colleted and tested from main reservoirs of three municipalities of Khairpur, Sukkur and Rohri cities. The fecal contamination of drinking water, raw sea food and other foods by indicator organisms and opportunistic pathogenic bacteria represents a public health concern (Fernandez-Delagado *et al.* 2007). *Proteus mirabilis* as an opportunistic pathogenic bacterium has been found in bacteremia (Berger1985. Watanakunakorn1994), neonatal meningoencephalitis (Grahnquist, *et al.* 1992),

empymeia (Isenstein and Honig 1990), and osteomyelitis (McDonahg *et al.* 1994). In the care of the patients undergoing long term bladder catheterization *P. mirabilis* infections have been reported (Sabbuba *et al.* 2003). The isolation rate of *P. mirabilis* was significantly greater in summer months than in winter months in drinking water of Khairpur, Sukkur and Rohri city ($p<0.009$, $p<0.009$,$p< 0.012$) respectively**.** The study showed that the drinking water of area under study was containing *Proteus mirabilis* during three consecutive years with different isolation rate. The continuous presence of this organism may indicate the unhygienic drinking water quality in the area. The source of contamination by this organisms may the both anthropogenic and from animals, because the source water was unprotected in terms of protection from animal entrance for grazing around the storage reservoirs and unnecessary human activities. Furthermore the purification of water was inadequate, the chlorine treatment was not proper. The storage settling tanks contained silt, grass, and floating material i.e. wood sticks and plastic waste.

P. rettgeri

P. rettgeri was isolated in present study in 67% of drinking water samples. The presence of this organism in drinking water may be attributed to unhygienic condition of at source water, at storage points and also in distribution pipes of distribution system. As this organism is the member of family *Enterobacteriaceae* may present in the fecal material of human and animal may provide the evidence of fecal contamination of municipal water. *P. rettgeri* can be isolated from poultry, reptiles, amphibian's excrements and surface water (Stock and Wiedemann 1998). The isolation of *P. rettgeri* also was reported from waste water and sludge (Urbanova *et. al.* 2000). The strains of *P. rettgeri* can cause diarrhea (Yoh *et. al.* 2005). There are incidents of *P. rettgeri* causing nosocomial infection. An outbreak caused by a highly resistant, lactose fermenting strains of *P. rettgeri* was reported in 1971 (Traub *et al.*). Edwards *et al.* in (1974) also reported 10 cases with one death from urinary tract infection. The isolation rate of *P. rettgeri* was significantly greater in summer months than in winter months in drinking water of Khairpur, Sukkur and Rohri city ($p<0.017$, $p<0.014$, $p<0.016$).

P. stuarti

P. stuarti has been isolated from 63% water samples investigated in this study. As these bacteria are the members of family *Enterobacteriaceae* can be present in excreta of animals. The presence of this organism in drinking water is the indication of fecal contamination of water.

However human isolates of *Providencia* have also been recovered from urine, throat, perineum, axilla, stool, blood and wound specimens. *P. heimbachae* and *P. rustigianii* have also been isolated from penguins (Hichman *et al.* 1983 and Pompei *et al.* 1990). *P. stuarti* has been isolated from patients of UTI. A total of 21 to 61% of urinary tract specimens in this population contain either *P. mirabilis* or *P. stuarti* and organism may even result in fatal bacteremia (Warren 1986). The organism may have the etiologic role in diarrheal diseases; Yoh *et al*. isolated in 2005 the *P. stuarti* from patients with traveler's diarrhea. The isolation rate of *P. stuarti* was significantly greater in summer months than in winter months in drinking water of Khairpur, Sukkur and Rohri city ($p<0.017$, $p<0.015$, $p<0.015$) respectively**.**

K. oxytoca

K. oxytoca was isolated in present research in drinking water of the area under study. The isolation rate was 49%, in drinking water samples from municipal water. *Klebsiella oxytoca* is a Gram negative member of family Enterobacteriaceae commonly found as the gastrointestinal inhabitants of rodents as well as humans. *Klebsiella spp.* is opportunistic human pathogens. They can be recovered from various clinical samples (Podschun an Ullmann 1998). In neonatal and pediatric-Intensive Care Units infections are frequently associated with *K. oxytoca* (Hable *et al.* 1972, Kayali *et al.* 1972, Hill *et al.* 1974). New born babies colonized with *Klebsiella species* become sources of infection in hospitals. Other sources could be containers, apparatus, and hand washing scrubbers. In 2001 Podschun *et al.* isolated *Klebsiella sp.* from 53% surface water samples. In present study the *Klebsiella oxytoca* was isolated from drinking water of Khairpur, Sukkur and Rohri cites of Sindh Pakistan. The water which is also used in the Hospital for cleaning, washing of surgical instruments and hand washing the patients who come into contact with such contaminated water can get infected by *Klebsiella spp*. Many investigators have reported that the *Klebsiella* bacteremia mostly having nosocomial origin (McCabe and Jakson 1962, Montgomerie 1980). As this organism can cause the nosocomial infection or opportunistic infection it is likely that it may be present in the water which is used for various purposes because any person who comes in the contact with contaminated water with *K. oxytoca* might be infected with this organism. The municipal authorities should take care of the purification of municipal water and prevent such incidences by *K. oxytoca.* The presence of *K. oxytoca* in drinking water indicates the contamination of water with fecal material of humans and rodents. The members of the genus *Klebsiella* are therefore a frequent cause of nosocomial infections

(Horan *et al.* 1988). The sources of *Klebsiella* could be due to their habitat that includes gastrointestinal tract of mammals as well as environmental sources such as soil, surface waters and plants (Bagley 1985). Such habitat could be potential reservoirs for the growth and spread of these bacteria (Knittel *et al.* 1977). *Klebsiella pneumonae and Klebsiella oxytoca* are clinically the most important species (Farmer *et al.* 1985, Podschun and Ullmann 1998). They may be associated with Klebsiella bacteremia with a high rate of morbidity and mortality, and are responsible for nosocomial infections particularly in intensive care units (Kreger *et al.* 1980 and Montgomerie 1979). Most of the Klebsiella strains implicated in invasive infection have been *K. pneumoniae* (Torre *et al.* 1985). However the frequency of isolation of the indole positive Klebsiella species *K. oxytoca* has recently increased (Mori 1989). The difference in isolation rate of *K. oxytoca* in different quarters was not significant in Khairpur city, but the isolation rate of *K. oxytoca* was significantly greater in summer months than in winter months in drinking water of Sukkur and Rohri city ($p<0.029$, $p< 0.036$) respectively

C. youngae

C. youngae was isolated from the drinking water at isolation rate of 65%. It has been reported that these bacteria are the inhabitants of intestinal tract and are found in sewage, surface waters and food contaminated with fecal material and cause gastroenteritis and opportunistic infections (Lanyi 1984). *Citrobacter* can also cause UTI and infection of respiratory tract especially in immunocompromised patients (Badger *et al.* 1999). Currently, the genus is divided into 11 species with 43 O-serotypes (Lanyi 1984, Bernner *et al.* 1999 and Keleti 1971) However, some O-antigens from different species closely related and antigenic heterogeneity of strains belonging to the same serogroup is observed (Miki *et al.* 1996**).** The isolation rate of *C. youngae* was significantly greater in summer months than in winter months in drinking water of Khairpur, Sukkur and Rohri city ($p<0.008$, $p<0.005$, $p<0.013$) respectively. The presence of *C. youngae* in municipal water of area under study may be due to the addition of contaminated sewage water into the water sources and uncontrolled access of human and animals in settling tanks of municipalities. Another factor is the lack of chlorine disinfectant in the drinking water.

Non-fermenter species

Non-fermenter spp. was isolated at isolation rate of 59% in drinking water samples tested in present study. These aerobic, non-fermenting, gram-negative bacilli (*Non-fermenters*) are a heterogeneous group of organisms that are either incapable of utilizing carbohydrates as a source

of energy or degrade them via oxidative rather than fermentative pathway.(Koneman *et al.* 1988) This group includes organisms from diverse genera like *Pseudomonas, Acinetobacter, Alcaligenes, Flavobacter, Oligella, Flavimonas, Agrobacter Weeksiella,* etc. A few of these have not been named and are designated by CDC numbers like *CDC11g.* These organisms are common inhabitants of soil and water. They also exist as harmless parasites on the mucus membranes of humans and animals. Though primarily regarded as contaminants or incidental organisms, they are becoming increasingly important as opportunistic pathogens in immunocompromised patients. They can also cause infection by gaining access to normally sterile body sites through trauma (Bailey and Scott 1990). Though these organisms vary in their potential to cause infection and antibiotic susceptibility, there are common disease manifestations and risk factors. These organisms have been implicated in causing septicemia, meningitis, osteomyelitis, wound infections, pneumonias and urinary tract infections. Risk factors include immuno-suppression, trauma, foreign body, broad-spectrum antibiotic use and infused body fluids like saline irrigations (Hall, 1995). Several of the clinically important non-fermenters are multi-resistant organisms (Cisneroset *et al.* 1996, Hancock 1998, and Seifert 1995), and treatments for infections caused by non-fermenter are somewhat different from those for infections caused by fermenters. It is generally recognized that narrow- and expanded-spectrum cephalosporin are minimally active against *Nonfermenters* (Appelbaum *et al.* 1983, Chang, *et al.* 1995). The isolation rate of *Non-fermenter species* was significantly greater in summer months than in winter months in drinking water of Khairpur, Sukkur and Rohri city ($p<0.007$,$p<0.028$, $p<0.016$).

C. meningosepticum

This study shows that *C. meningosepticum* was isolated in 55% drinking water samples tested in the present study and these bacteria were present with high frequency in the municipal water of three cities of Sindh Pakistan. The isolation rate of *C. meningosepticum* was significantly greater in summer months than in winter months in drinking water of Khairpur, Sukkur and Rohri city ($p<0.015$, $p<0.014$, $p<0.012$) respectively.

C. meningosepticum is a ubiquitous, waterborne saprophytic bacterium. It is an opportunistic pathogenic bacterium; the intermittent epidemics in NICUs have been reported (Hoque *et al.* 2001). In chlorine treated water *C. meningosepticum* can survive, colonizing sinks and taps which serve as reservoirs (Kirby *et. al.* 2004). Most sources are derived from water containing

materials and water itself (Gungor *et al.* 2003). In 2007 two cases of *C. meningosepticum* in Seoul, Korea were identified in neonatal intensive care units where the bacterium was isolated from water inside suction bottles and suction bottle caps. The origin infection was established as the biofilm on the suction bottle caps which were made of steel (Yoon 2007). This organism was isolated in the present study from drinking water of three cities of Pakistan. The entrance of this organism in the drinking water may be from surrounding area of the main storage reservoirs which were open for animal, humans, and also for wild animals and reptiles and rodents which can contaminate the stored water which was collected in uncovered tanks at the main storage reservoirs of municipal water of area under study. The importance of this organism in municipal water is that the water contaminated with this organism might be the source infection in hospital because this organism is widely distributed in nature so its role in pathogenicity of community acquired infection is possible in immunocompromised patients (Bloch *et al.* 1997, Lim *et al.* 1999, Lin *et al.* 2004).

C. meningosepticum is the most clinically important human pathogen among the *Chryseobacterium* and *Flavobacterium* genera it is responsible for neonatal meningitis, with a mortality of up to 50% (Yoon 2007, George *et al.* 1961). This organism typically is found in plants, soil and hospital environments. It is very likely that its presence in drinking water could be due to the dumping of sewage water in source of municipal water.

V. mimicus

Vibrio mimicus was isolated from drinking water of three cities of Pakistan with different levels of prevalence with isolation rate of 51%. The isolation rate of *V. mimicus* was not significant in Khairpur city, but the isolation rate was significantly greater in summer months than in winter months in drinking water of Sukkur and Rohri city ($p<0.0.014$, $p<0.016$) respectively.

.The importance of this bacterium as a pathogen was elaborated when in 1991 a large cholera outbreak started in Latin America and etiologic agent was *V. cholerae* O1 but during this epidemics cases of severe diarrhea associated with the *V. mimicus* were reported in Costa Rica (Campos *et al.* 1996). *V. mimicus* is found in sea water and fresh water where they may pose the threat to aquatic animals and the human who come in the contact with the water contaminated with these bacteria. The prevalence of these bacteria in fresh water of municipalities of area under study may be due to the pollution of source water and inadequate disinfection of water. Due to the unclean storage tanks, water distribution pipes, the chances of biofilm formation can

not be ruled out. In this situation the growth of *V. mimicus* may be high and the transmission of organism through such types of water is likely. Outbreaks of moderate to severe cholera and cholera-like diarrhea caused by non-O1 *V. cholera* in Bangladesh and other parts of the world have been described (Hughes *et al.* 1978; Zafari *et al.* 1973). Fanning *et al.* 1981 suggested that both *V. cholera*-O1 and non-O1 as well as *V. mimicus* are potentially important in terms of public health in areas where sanitation and personal hygiene are very poor. Seafood-associated gastroenteritis caused by *V. mimicus* has been reported in Japan (Muramatsu *et al.* 1981). Association between *V. mimicus* and fresh water and aquatic plants has been described (Chowdhury *et al.* 1986) *V. mimicus* is a species closely related to the *V. cholerae.* Phenotypically most of the features of this organism are identical or similar to those found in *V. cholerae* and sucrose fermentation is main trait differentiating them biochemically (Shi *et al.* 1998; Desmarschelier and Reichelt 1984).Both species cell wall share antigens and virulence related genes and are associated with sporadic and epidemic cholera and diarrhea (Boyed *et al.* 2000, Davis *et al.* 1981, and Shi *et al.* 1998).

V. cholerae

V. cholerae was isolated in 28% drinking water samples tested in present study. The isolation of *V. cholerae* from drinking water in Khairpur, Sukkur and Rohri is the evidence of poor sanitation and monitoring of drinking water quality. The prevalence of *V. cholerae* showed seasonal variation. In summer months isolation rate was higher than in winter months The isolation rate of *V. cholerae* was significantly greater in summer months than in winter months in drinking water of Khairpur,Sukkur and Rohri city ($p<0.025$, $p<0.016$, $p<0.015$).

The presence of *Vibrio cholerae* in water is influenced by many factors which help the organisms to adhere to certain surfaces. The factors responsible for adhesion are lectin fibronectin, collagen binding and the presence of bacterial fimbriae (Matthysse 1992) or microbial surface antigens which bind to a specific receptor of the host cells (Marshall 1976). Several bio-physico-chemical parameters are also involved in bacterial adhesion to the plant and animal surfaces such as charge properties and hydrophobic interactions. Along with bacterial adhesion factors *V. cholerae* secrets the degrading enzyme that degrades mucin and mucinlike substances in plant cells and contributes to the association between *Vibrios* and plant surfaces especially in aquatic plant root (Schneider and Parker 1982). The plant surface may act as a habitat or a reservoir for *V. cholerae* through a non-specification association or by commercial

interaction (Islam *et. al.* 1990). The presence of even non-pathogenic *V. cholerae* in water used for drinking purposes is also matter of concern because the environmental factors regulate genes (Shears 2001). These genes switch on due to maybe the horizontal gene transfers and allow a benign bacterium into a dangerous pathogen in response to environmental factors because cholerae toxin genes are integrated in the genome of lysogenic bacteriophage and transcription of this bacteriophage is reported to be influenced by environmental factors like sunlight, temperature and salt in water (Shears 2001., Reidle and Klose 2002).

A. hydrophila

A. hydrophilia was isolated from 54% of drinking water samples tested in this study. The isolation rate of *A. hydrophila* was significantly greater in summer months than in winter months in drinking water of Khairpur, Sukkur and Rori city ($p<0.049$, $p< 0.001$, $p<0.002$)**.**The literature contains numerous reports of the presence of *A. hydrophila* from fresh water. Sato *et al.* (2007) reported 36% water samples positive with *Aeromonas spp*. In less protected drinking water source. In Italy *Aeromonas spp*. was isolated from 25% well water samples (Massa *et al.* 2001). The data are similar to the results obtained in this study (30.3%) where isolation was reported by Ghenghesh *et al.* 2001 in Tripoli, Libya. In the aquatic environments Aeromonas is normally found drinking water (Holmes *et al.* 1996).This bacterium has been isolated from different sea food products, unprocessed meat, vegetables and raw milk (Palumbo, 1996). Aeromonads are now listed as emerging water and food-borne pathogens and it has been shown that some *Aeromonas* can produce different virulence factors, both at optimum growth temperature and refrigeration temperatures (Merino *et al.* 1995). However, it is not yet clear whether Pathogenesis of motile *Aeromonas* is due to these virulence factors. It has been reported that Aeromonas were isolated from the cases of human gastroenteritis cases of human gastroenteritis, frequently in children less than two years of age, elderly persons and immunocompromised patients (ICMSF 1988; Joseph, 1996). *Aeromonas* species have been also associated with traveler's diarrhea (Hanninen, 1993; Yamada *et al.* 1997). Due to ubiquitous nature of *Aeromonas spp.* in environment one might expect the occurrence of these organisms in surface drinking water sources, but it witnesses the ignorance of drinking water suppliers for the consumers in study area.

P. aeruginosa

The isolation rate of *P. aeruginosa* was 75%, drinking water samples tested in this study. The isolation rate of *P. aeruginosa* was significantly higher in all season in drinking water of Khairpur, Sukkur and Rohri city ($p<0.002$, $p<0.001$, $p<0.003$) respectively. The high isolation rate of these bacteria in the water samples indicate that the water examined was not really poor in organic matter. In the most water samples tested in this study the prevalence of *P. aeruginosa* was very high in all seasons, possibly due to mesophilic nature of the organism (Kooij *et al.* 1982). The possible source of *P. aeruginosa* in drinking water of area under study might be environmental because *P. aeruginosa* is widely distributed in nature (water soil, plants, animals and man). Colonization in human occurs mostly at moist places such as perineum, axilla and the ear. The moisture loving characters contributes to the presence of *Pseudomonas* in respiratory equipments, antiseptic solutions, soaps, sinks, mops, vegetable, flowers, hydrotherapy equipments and swimming pools, *Pseudomonas* could be found inside and outside hospitals (Pollak 1995). *Pseudomonas aeruginosa* is usually considered a nosocomial pathogen. The overall incidence of *P. aeruginosa* infection in US hospital between 1985-and 1991was reported as 4.0 per 100 discharges, listing it the fourth most frequently isolated nosocomial pathogen (Pollak 1995). In hospitals, nosocomial pneumonia and ICU related respiratory tract infection are attributed to the *Pseudomonas* (Jarvis and Martine 1992). *Pseudomonas* in municipal water of area under study indicates that the water which is used for drinking in Khairpur, Sukkur and Rohri is of poor quality and not safe from domestic wastes, hospital waste, and also wastewater effluents. The minor difference in isolation rate from various samples from different reservoirs may be due to varying sources of contamination. The very high isolation rate of *P. aeruginosa* to a bacterial flora of water suggests that in these situations the organism will also be the major part of bacterial flora of municipal water.

Salmonella spp.

Salmonella species was not isolated in this study throughout study period from drinking water of Khairpur, Sukkur and Rohri city. In developed countries, isolation of *Salmonellae* from drinking water and piped water supplies (treated or untreated) is rare and their presence indicates a serious fault in the design or management of the system (Chiu *et al.* 2006, LIoyd 1983). Salmonella was not isolated from drinking water of area under study because the possible poresence of bacterial species other than enteriobactewriaceae which may have antiproliferative effect on the

salmoenlla. The origin of such species may be due to the animal's activities in the water reservoirs. Enterococcus feaium and lactobacillius fermentum have been reported to inhibit eight foodborn pathogens including salmonella typhi (Thirabunyanon *et al.* 2009).

Physico-chemical Factors

Temperatures

The temperature of drinking water from main reservoirs of Khairpur, Sukkur and Rohri varied from January- December of the study period (2005-2007). The range was 17°C-23°C, during January-March, 26°C-29°C during April-June, 29°C-31°C during July-September and 21°C-29°C during October-December reported in this study (Table 21) is comparable to the temperature ranges reported by other authors in similar study as Alabaster and Lloyd (1980) reported a temperature ranges of 26°Cand 30°C. The temperature range reported in this study of water samples and the water body is presumably due to the intensity of sunlight as temperature elevates from 17°C in cold season to 50 °C in summer season in upper Sindh Pakistan. The growth environment, particularly the incubation temperature, have an influence on the survival ability of the bacteria in nutrient poor-water (Sautour *et al.* 2003). For temperate climates, *Aermonads* were isolated in high numbers in late summer when the temperature were around 20-25°C and rarely detected during the cold months (Kertsters *et al.* 1995; Gavriel *et al.* 1998). Similar phenomenon was observed for *Vibrio vulnificus* (Wolf and Oliver 1992). On average the occurrence of coliform bacteria was significantly higher when temperatures were above 15°C. As reported by Straskrabova in (2007) in similar studies that in warmer months the *Enterobacteriaceae* and *Aeromonas*, were found in higher numbers, while in cold months the *Pseudomonads and Acinetobacter* were found in increased densities. Temperature is widely recognized as an important controlling factor in influencing bacterial growth. Climates where water temperatures are high, bacterial growth may be fast; however, the minimum temperature at which microbial activity is observed varies from system to system (WHO 2003). Kaplan and Bott (1989) reported that during algal blooms in White Clay Creek increases in bacterial activity occurred in the day. The bacteria respond more quickly and at greater extent to dissolved organic matter (DOM) additions at higher temperatures (Kirchman and Rich 1997). Solar radiation both degrades and alters the quality of natural organic matter as well organic pollutants in surface water. Only recently has indirect influence of photochemical process on aquatic organisms (e.g. bacteria) received attention (Stefan and Anneli 2002). High frequency of waterborne bacterial

pathogens was obtained in summer seasons but not in winter, which indicates the possible effect of temperature on prevalence of waterborne bacteria inside biofilm environment.

pH

The pH values of water samples were within the acceptable range. This confirms to the pH range reported by most authors (Okonko *et al.* 2008). According to Medera *et al.* (1982) the pH of most drinking water ranges between 6.5-8.5 while deviation from the neutral 7.0 in most natural waters is as result of carbon dioxide, bicarbonate-carbonate equilibrium system. An increased carbon dioxide concentration will therefore lower pH, where as a decrease will cause it to rise. Temperature will also affect the equilibrium and the pH. pH values of water are inversely proportional to temperature, water pH drops about 0.45 as the temperature elevated by 25 ^{o}C. Water where a buffering capacity imparted by bicarbonate, carbonate and hydroxyl ions, this temperature effect is modified (APHA 1989). The pH determines the corrosivity of the water; otherwise pH has no direct effect on consumers. To ensure satisfactory water clarification and chlorine disinfection the pH should preferably be below 8.0 (WHO 2007). The prevention of corrosion of water mains and pipes in household system requires the proper monitoring of the pH of water prior to entering the distribution pipes. The contamination of drinking water and in change of its taste, odor and appearance may result if pH of water is not properly maintained (WHO 2007). However Lars *et al.* (2002) reported that the tap water (pH 6.2-6.9) posed fourfold greater risk of type 1 diabetes compared with pH $\geq$7.7. In present study the pH of water has been analyzed in order to observe the condition of drinking water of Khairpur Sukkur and Rohri. It was observed that the pH of drinking water was within the recommended range (6.5-8.5) advocated by World Health Organization (WHO) guideline standards for drinking water (WSC 2004).

Chlorine Analysis

Chlorine based disinfection has a long history. Bleach was used to disinfect water in 1897 to combat typhoid out break in Maidstone, Kent (UK) (Christman 1998). Water chlorination has played a role in extending life expectancy from 45 years in the early 1900s to 76 years to day (CCC 2005). One of the great achievements in health section is the distribution of safe drinking water. Treating drinking water with chlorine (starting with Chicago and Jersey City in 1908) prevented cholera, typhoid fever, dysentery and hepatitis that killed thousands of U.S residents annually. In developing countries, drinking water chlorination and filtration have helped virtually

eliminate these diseases (CCC 2005). Ensuring the safety from microbiological pollution of drinking water is of prime importance. Normally drinking water treatment includes the use of chlorine or other oxidant for final disinfection. But if the drinking water source is surface water, the pre-treatment such as coagulation, sedimentation and filtration is used to clear the water before disinfection (FWR 1999). Oxidizing disinfectants (chlorine, chloramines and ozone) are the final barrier. Environmental protection agency recommended a multi-barrier approach to provide pathogen-free water to consumers. They are the most commonly used disinfectant for drinking waters (Margolin 1997). Use of chlorine in drinking water treatment is effective because hypochlorite is lethal for most microbes (Rutala and Weber 1997) reported that *H. pylori* gains entry into distribution system via either a break in treatment or infiltration into the system it self, it may be able to survive within distribution system where the level of oxidizing disinfectant is reduced (Katherine *et. al.* 2002). The absence of residual chlorine in any of drinking water samples which indicates faulty chlorination process or chlorination was not done at all. It is evident from the results (high coliform and faecal coliform count at all 3 levels) that the quality of drinking water is further deteriorated in the distribution system may be due to the leakage of pipes, where sewage water enters into the municipal water. At the consumer level the drinking water is getting more contaminated due to the unhygienic handling and uncovered storage tanks. The results of this study leads to conclusion that the chlorination process at main reservoir is not working efficiently thereby giving high coliform count even at consumer level. It is therefore, recommended that following steps may be taken by the relevant authorities to curtail the bacterial contamination of water supplied to Khairpur city for drinking purpose: Most water supply systems in Pakistan and other developing countries are not working according to design and many are completely dysfunctional (Jensen *et al.* 2003). The situation in the area under study was also observed same. Chlorine was not used adequately; that is why it was present in meager amount or completely absent in water samples tested in this study (Fig. 36). In present study it was observed that the residual chlorine was rarely found or when found was in very low concentration below the WHO and Pakistan guideline values for drinking water. The provision of untreated or inadequately treated water to the population results in the waterborne diseases in area. The health effects of that unsafe water are clear from data presented in this study. These are high number of diarrhea cases in the area during the study period (Fig 6, 11, 16). Siddiqui *et al.* (2010) reported that the microbiological quality of water was not bsatisfactory 36% samples

were found to be contaminated with waterborne pathogens during a microbiological study of drinking water supplied to the schools in Karachi. An other study on ground water quality conducted by Habib *et al*. (2009), the fecal coliform as isolated in most of the sample of ground water in kasur pakistan. In general a higher isolation rate was obtained because the waterborne pathogens are mesophilic, can best grow at the temperature ranges of the drinking water of area under study. However, other factors such as organic matter, water level and drought may influence the bacteriological quality of water. It will be interesting to evaluate and determine these parameters for proper understanding of bacterial pathogens in drinking water.

Conclusions

1. The water bodies used for drinking purpose in Khairpur, Sukkur and Rohri are heavily contaminated with fecal and total coliform bacteria.
2 The number of total and fecal coliform in summer months increases as compared to winter months.
3 Number of total and fecal coliform bacteria per 100 ml of water and water temperature is in positive correlation and directly proportional
4 Diarrheal cases and number of total and fecal coliform in drinking water are significant and in positive correlation.
5 Absence of residual chlorine is one of the reason of high values of fecal and total coliform in drinking water
6 This is the 'First report' of isolation of pathogenic and emerging opportunistic bacteria in drinking water area under study
7 Twelve different bacterial species isolated from drinking water of Khairpur, Sukkur and Rohri in present study,

   The species isolated are: *E. coli,* 64%, *P. mirabilis,* 67% *P. rettgeri* 60%, *P. stuarti* 67%, C *youngae* 64%, *K. oxytoca* 49%, *V. cholerae* 29.16% *and V. mimicus* 47.22%, *A. hydrophilia* 53%, *P. aeruginosa* 69.44%, *C. meningosepticum* 57% and *Non- Fermenter* species 61%

Recommendations

Keeping in view the quality of drinking water of area under study following recommendations have been made

The drinking water of area under study should be boiled before drinking it

- This reported case of waterborne bacterial pathogens in drinking water is alarming.
- Government should fulfill its basic complacence of providing safe drinking water to community.
- The regular chlorine disinfection treatment of drinking water may be ensured.
- The quality of drinking water may be checked in light of drinking water guideline established by WHO and Pakistan
- The source of drinking water may be protected from un-necessary human and animal access.
- The general cleanliness and hygiene of water main storage reservoirs may be maintained.
- Sewage water should be treated and disinfected before disposing it.

Future work

Presently little is known about survival /persistence of bacterial, viral and protozoan pathogens in municipal water under different environmental conditions, which is most important in pathogen impact. The better understanding of the source of microbial contaminants (human versus animals), their transport, prevalence and fate in water environment and the resulting public health risk is urgently needed. The distribution of host specific genetic markers including humans has not been extensively investigated yet leaving a large space for additional future researches on identification of sources pathogens in municipal water. In addition to establish the link between waterborne bacterial pathogens and waterborne diseases is an important task for microbiologist to provide more futuristic vision of drinking water quality monitoring.

APPENDIX 1 CULTURE MEDIA

Eosine Methylene Blue (EMB) agar (Oxoid)	Grm/Ltr
Peptone	10
Lactose	10
Dipotassium hydrogen phosphate	2
Eosin Y	0.4
Methylene blue	0.06
Agar	15
Distilled water	1 liter

All contents were mixed in required volume of distilled water and homogenized and the medium was autoclaved at 121oC for 15 minutes at 15 PSI.

Deoxycholate Citrate Agar (DCA BioM USA)	Grm/Ltr
Lactose	10
Proteose peptone	10
Heart infusion	330
Sodium citrate	20
Agar agar	15
Neutral red	0.033
Ferric ammonium citrate	2
Sodium desoxycholate	5
Distilled water	1 liter
pH	7.5

All contents were mixed in required volume of distilled water and homogenized and the medium was autoclaved at 121oC for 15 minutes at 15 PSI.

Xylose Lysine Deoxicholate Agar (XLD BioM USA) Grm/Ltr

Yeast extract powder	3
L-Lysine HCL	5
Xylose	3.75
Lactose	7.5
Sucrose	7.5
Sodium deoxicholate	1.0
Sodium chloride	5
Sodium thiosuphate	6.8
Ferric ammonium citrate	0.8
Phenole red	0.08
Agar	12
Distilled water	1 liter

All contents were mixed in required volume of distilled water and homogenized pH was adjusted and the medium was heated to boil.

Ampicillin Dextrin Agar (BioM USA)	**Grm/Ltr**
Dextrin	10.0gm
Tryptose	5.0gm
Sodium Chloride	3.0gm
Yeast Extract	2.0gm
Potassium Chloride	2.0gm
Magnesium Sulphate Heptahydrate	0.2gm
Ferric Chloride	0.1gm
Sodium Deoxycholate	0.1gm
Bromothymol Blue	0.08gm
Ampicillin	10.0mg
Agar	15.0gm
pH	8.0

All contents were mixed in required volume of distilled water and homogenized pH was adjusted and the medium was heated to boil.

Alkaline peptone water (OXOID)	Grm/Ltr
Peptone	10
Sodium chloride	10
Distilled water	1 litre
pH	8.6

All ingredients were dissolved in water the pH was adjusted 8.6 by 1 N NaOH and sterilized by autoclaving at 121^{o}C for 15 minutes.

Buffered peptone water	Grm/Ltr
Petone	10
Sodium chloride	5
Disodium hydrogen phosphate	3.5
Potassium dihydrogen phosphate	1.5
Distilled water	1 liter

All ingredients were dissolved in distilled water. The resulting solution was dispensed in 90 ml in screw caped tubes and sterilized by autoclaving at 121^{o}C for 15 minutes.

Thiosulfate Citrate Bile salt Sucrose (TCBS) Agar (BioM, USA)

	Grm/Ltr
Yeast extracts	5
Proteose peptone	10
Sodium thiosulfate	10
Sodium citrate	10
Ox bile	8
Sucrose	20
Sodium chloride	10
Feric citrate	1.0
Bromothymol blue (1% m/v aqueous solution)	0.04
Thymol blue (1% m/v aques solution)	0.04
Agar	15
Distilled water	1 liter
pH	8.6

All contents were mixed in required volume of distilled water and homogenized pH was adjusted and the medium was heated to boil.

Nutrient agar (OXOID)	Grm/Ltr
Lablemco powder	1
Yeast extract	2
Peptone	5
Sodium chloride	5
Agar	15
Distilled water	1 liter

All contents were mixed in required volume of distilled water and homogenized pH was adjusted and the medium was autoclaved at 121oC for 15 minutes at 15 PSI.

Pseudomonas agar F (BioM USA)	Grm/Ltr
Proteose peptone	10
Tryptone	10
Dipotassium	1.5
Magnesium sulfate	1.5
Agar	15
Distilled water	1 liter
pH	7

Content were mixed in required volume of distilled water and homogenized pH was adjusted and the medium was autoclaved at 121oC for 15 minutes at 15 PSI.

Rapaport Vasidialis enrichment broth (OXOID)	Grm/Ltr
Soya peptone	4.5
Sodium chloride	7.2
Potassium dihydrogen phosphate	1.26
Dipotassium hydrogen phosphate	0.18
Magnesium chloride (anhydrous)	13.58
Malachite green	0.036
Distilled water	1 liter

All contents were mixed in required volume of distilled water and homogenized pH was adjusted and the medium was autoclaved at 121oC for 15 minutes at 15 PSI.

APPENDIX 11 REAGENTS

Gram stain reagents

Crystal violet	Grm/Ltr
Crystal violet	20
Ammonium oxalate	9
Methanol absolute	95 ml
Distilled water	to1 liter

Crystal violet was dissolved in 95 ml methanol absolute in 1 liter bottle; the ammonium oxalate was dissolved in 200 ml of distilled water and made up to 1 liter mark with distilled water

Gram's iodine	Grm/Ltr
Potassium iodide	20
Iodine	10
Distilled water	to 1 liter

Potassium iodide was dissolved in 250 ml of distilled water and then iodine was added to the potassium iodide solution and mixed to dissolution and was made to 1 liter by adding distilled water.

Decolorizer (95% ethyl alcohol)

Acetone	500 ml
Ethanol absolute	475 ml
Distilled water	25 ml

Distilled water was mixed with absolute ethanol, and then required volume of acetone was added to the ethanol solution and mixed well.

Neutral red	Grm/Ltr
Neutral red	1
Distilled water	1 liter

Neutral red was dissolved in 250 ml of distilled water and then the solution was made to 1 liter by adding distilled water.

Sodium chloride, 0.85% (Physiological saline)	Grm/Ltr
Sodium chloride	8.5
Distilled water	1 lites

Ingredient was dissolved in distilled water to 1 liter mixed well until salt was fully dissolved.

Oxidase reagent (Sigma chemical company) Grm/Ltr

To make 10 ml	
Tetramethyl-p-phenylenediamine dihydrochloride	0.1
Distilled water	10 ml

To prepare 10 ml 0.1 g of tetramethyl-p-phenylenediamine dihydrochloride was dissolved in distilled water the reagent was made freshly when required.

APPENDIX III Statistical Analysis by (SPSS version 13) software

Khairpur 2005 *E. Coli* counts

One-Sample Test

	Test Value = 0					
					95% Confidence Interval of the Difference	
	t	df	Sig. (2-tailed)	Mean Difference	Lower	Upper
E.coli	5.218	3	.014	1601.66675	624.7801	2578.5534

One-Sample Statistics

	N	Mean	Std. Deviation	Std. Error Mean
E.coli	4	1601.6668	613.92187	306.96093

T-Test Khairpur *E. coli* count 2006

One-Sample Statistics

	N	Mean	Std. Deviation	Std. Error Mean
E. coli	4	1625.0000	667.98507	333.99253

One-Sample Test

	Test Value = 0					
					95% Confidence Interval of the Difference	
	t	df	Sig. (2-tailed)	Mean Difference	Lower	Upper
E. coli	4.865	3	.017	1625.00000	562.0867	2687.9133

T-Test khp *E. coli* count 2007

One-Sample Statistics

	N	Mean	Std. Deviation	Std. Error Mean
E.coli	4	2066.6667	663.88308	331.94154

One-Sample Test

	Test Value = 0					
					95% Confidence Interval of the Difference	
	t	df	Sig. (2-tailed)	Mean Difference	Lower	Upper
E.coli	6.226	3	.008	2066.66667	1010.2805	3123.0528

T-Test Sukkur 2005 *E. coli* count T test

One-Sample Statistics

	N	Mean	Std. Deviation	Std. Error Mean
E.coli	4	3341.6668	849.12826	424.56413

One-Sample Test

	Test Value = 0					
					95% Confidence Interval of the Difference	
	t	df	Sig. (2-tailed)	Mean Difference	Lower	Upper
E.coli	7.871	3	.004	3341.66675	1990.5142	4692.8193

T-Test Sukkur *E. coli* count 2006

One-Sample Statistics

	N	Mean	Std. Deviation	Std. Error Mean
E. coli	4	2241.6665	1250.59263	625.29632

One-Sample Test

	Test Value = 0					
					95% Confidence Interval of the Difference	
	t	df	Sig. (2-tailed)	Mean Difference	Lower	Upper
E. coli	3.585	3	.037	2241.66650	251.6946	4231.6384

T-Test Sukkur *E. coli* count 2007

One-Sample Statistics

	N	Mean	Std. Deviation	Std. Error Mean
E.coli	4	2833.3335	1652.60742	826.30371

One-Sample Test

	Test Value = 0					
					95% Confidence Interval of the Difference	
	t	df	Sig. (2-tailed)	Mean Difference	Lower	Upper
E.coli	3.429	3	.042	2833.33350	203.6663	5463.0007

T-Test Rohri 2005 *E. coli* count

One-Sample Statistics

	N	Mean	Std. Deviation	Std. Error Mean
E.coli	4	1933.3333	879.81506	439.90753

One-Sample Test

	Test Value = 0					
					95% Confidence Interval of the Difference	
	t	df	Sig. (2-tailed)	Mean Difference	Lower	Upper
E.coli	4.395	3	.022	1933.33325	533.3511	3333.3154

T-Test Rori 2006 *E. coli* count

One-Sample Statistics

	N	Mean	Std. Deviation	Std. Error Mean
E. col	4	1550.0000	582.77771	291.38885

One-Sample Test

	Test Value = 0					
					95% Confidence Interval of the Difference	
	t	df	Sig. (2-tailed)	Mean Difference	Lower	Upper
E. col	5.319	3	.013	1550.00000	622.6706	2477.3294

T-Test Rohri 2007 *E. coli* count

One-Sample Statistics

	N	Mean	Std. Deviation	Std. Error Mean
E.coli	4	3016.6666	646.64374	323.32187

One-Sample Test

	Test Value = 0					
					95% Confidence Interval of the Difference	
	t	df	Sig. (2-tailed)	Mean Difference	Lower	Upper
E.coli	9.330	3	.003	3016.66658	1987.7121	4045.6211

T-Test *E. coli* 1 isolation rate in different quarters (2005-2007)

One-Sample Statistics

	N	Mean	Std. Deviation	Std. Error Mean
Khairpur	4	63.9000	24.57885	12.28943
Sukkur	4	69.4700	29.24895	14.62448
Rohri	4	76.3750	27.37205	13.68603

One-Sample Test

	Test Value = 0					
					95% Confidence Interval of the Difference	
	t	df	Sig. (2-tailed)	Mean Difference	Lower	Upper
Khairpur	5.200	3	.014	63.90000	24.7896	103.0104
Sukkur	4.750	3	.018	69.47000	22.9284	116.0116
Rohri	5.581	3	.011	76.37500	32.8200	119.9300

T-Test *E. coli* 2 isolation rate (2005-2007)

One-Sample Statistics

	N	Mean	Std. Deviation	Std. Error Mean
Khairpur	4	40.3775	28.52731	14.26365
Sukkur	4	62.6375	20.99982	10.49991
Rohri	4	48.8000	24.11141	12.05570

One-Sample Test

	Test Value = 0					
					95% Confidence Interval of the Difference	
	t	df	Sig. (2-tailed)	Mean Difference	Lower	Upper
Khairpur	2.831	3	.066	40.37750	-5.0158	85.7708
Sukkur	5.966	3	.009	62.63750	29.2221	96.0529
Rohri	4.048	3	.027	48.80000	10.4334	87.1666

T-Test *P. mirabilis* isolation rate in different quarters (2005-2007)

One-Sample Statistics

	N	Mean	Std. Deviation	Std. Error Mean
Khairpur	4	66.6350	21.74451	10.87225
Sukkur	4	65.2875	21.86894	10.93447
Rohri	4	68.0350	24.56365	12.28182

One-Sample Test

	Test Value = 0					
					95% Confidence Interval of the Difference	
	t	df	Sig. (2-tailed)	Mean Difference	Lower	Upper
Khairpur	6.129	3	.009	66.63500	32.0346	101.2354
Sukkur	5.971	3	.009	65.28750	30.4891	100.0859
Rohri	5.539	3	.012	68.03500	28.9488	107.1212

T-Test *P. rettgeri* isolation rate in different quarters (2005-2007)

One-Sample Statistics

	N	Mean	Std. Deviation	Std. Error Mean
Khairpur	4	59.7250	24.95748	12.47874
Sukkur	4	65.3100	25.03364	12.51682
Rohri	4	68.1000	27.70632	13.85316

One-Sample Test

	Test Value = 0					
					95% Confidence Interval of the Difference	
	t	df	Sig. (2-tailed)	Mean Difference	Lower	Upper
Khairpur	4.786	3	.017	59.72500	20.0121	99.4379
Sukkur	5.218	3	.014	65.31000	25.4759	105.1441
Rohri	4.916	3	.016	68.10000	24.0131	112.1869

T-Test *P. stuarti* isolation rate in different quarters (2005-2007)

One-Sample Statistics

	N	Mean	Std. Deviation	Std. Error Mean
Khairpur	4	66.6250	27.59123	13.79561
Sukkur	4	61.1000	23.94800	11.97400
Rohri	4	66.6750	26.38830	13.19415

One-Sample Test

	Test Value = 0					
					95% Confidence Interval of the Difference	
	t	df	Sig. (2-tailed)	Mean Difference	Lower	Upper
Khairpur	4.829	3	.017	66.62500	22.7212	110.5288
Sukkur	5.103	3	.015	61.10000	22.9934	99.2066
Rohri	5.053	3	.015	66.67500	24.6853	108.6647

T-Test *K. oxytoca* isolation rate in different quarters (2005-2007)

One-Sample Statistics

	N	Mean	Std. Deviation	Std. Error Mean
Khairpur	4	48.6800	30.55055	15.27528
Sukkur	4	54.2075	27.64541	13.82271
Rohri	4	46.0000	25.36402	12.68201

One-Sample Test

	Test Value = 0					
					95% Confidence Interval of the Difference	
	t	df	Sig. (2-tailed)	Mean Difference	Lower	Upper
Khairpur	3.187	3	.050	48.68000	.0673	97.2927
Sukkur	3.922	3	.029	54.20750	10.2175	98.1975
Rohri	3.627	3	.036	46.00000	5.6402	86.3598

T-Test *C. youngae* isolation rate in different quarters (2005-2007)

One-Sample Statistics

	N	Mean	Std. Deviation	Std. Error Mean
Khairpur	4	64.0000	20.55886	10.27943
Sukkur	4	62.5750	17.20976	8.60488
Rohri	4	69.4600	25.87474	12.93737

One-Sample Test

	Test Value = 0					
					95% Confidence Interval of the Difference	
	t	df	Sig. (2-tailed)	Mean Difference	Lower	Upper
Khairpur	6.226	3	.008	64.00000	31.2863	96.7137
Sukkur	7.272	3	.005	62.57500	35.1904	89.9596
Rohri	5.369	3	.013	69.46000	28.2875	110.6325

T-Test *Non- fermenter* isolation rate in different quarters (2005-2007)

One-Sample Statistics

	N	Mean	Std. Deviation	Std. Error Mean
Khairpur	4	61.1250	18.76333	9.38166
Sukkur	4	55.7375	27.83276	13.91638
Rohri	4	59.8250	24.14379	12.07189

One-Sample Test

	Test Value = 0					
					95% Confidence Interval of the Difference	
	t	df	Sig. (2-tailed)	Mean Difference	Lower	Upper
Khairpur	6.515	3	.007	61.12500	31.2684	90.9816
Sukkur	4.005	3	.028	55.73750	11.4494	100.0256
Rohri	4.956	3	.016	59.82500	21.4068	98.2432

T-Test I *C. meningosepticum* isolation rate in different quarters (2005-2007)

One-Sample Statistics

	N	Mean	Std. Deviation	Std. Error Mean
Khairpur	4	56.9750	22.42772	11.21386
Sukkur	4	51.4500	20.04769	10.02385
Rohri	4	52.8825	19.55755	9.77878

One-Sample Test

	Test Value = 0					
					95% Confidence Interval of the Difference	
	t	df	Sig. (2-tailed)	Mean Difference	Lower	Upper
Khairpur	5.081	3	.015	56.97500	21.2875	92.6625
Sukkur	5.133	3	.014	51.45000	19.5496	83.3504
Rohri	5.408	3	.012	52.88250	21.7621	84.0029

T-Test *V. mimicus* isolation rate in different quarters (2005-2007)

One-Sample Statistics

	N	Mean	Std. Deviation	Std. Error Mean
Khairpur	4	43.0000	27.58019	13.79009
Sukkur	4	52.8325	20.58961	10.29481
Rohri	4	54.2100	21.83039	10.91519

One-Sample Test

	Test Value = 0					
					95% Confidence Interval of the Difference	
	t	df	Sig. (2-tailed)	Mean Difference	Lower	Upper
Khairpur	3.118	3	.053	43.00000	-.8862	86.8862
Sukkur	5.132	3	.014	52.83250	20.0698	85.5952
Rohri	4.966	3	.016	54.21000	19.4730	88.9470

T-Test *V. cholera* isolated in different quarters (2005-2007)

One-Sample Statistics

	N	Mean	Std. Deviation	Std. Error Mean
Khairpur	4	29.3500	27.75734	13.87867
Sukkur	4	37.6100	25.51382	12.75691
Rohri	4	54.3325	21.57782	10.78891

One-Sample Test

	Test Value = 0					
	t	df	Sig. (2-tailed)	Mean Difference	95% Confidence Interval of the Difference	
					Lower	Upper
Khairpur	2.115	3	.025	29.35000	-14.8181	73.5181
Sukkur	2.948	3	.016	37.61000	-2.9882	78.2082
Rohri	5.036	3	.015	54.33250	19.9974	88.6676

T-Test *A. hydrophila* isolation rate in different quarters (2005-2007)

One-Sample Statistics

	N	Mean	Std. Deviation	Std. Error Mean
Khairpur	4	52.8550	32.84795	16.42398
Sukkur	4	50.2500	33.57951	16.78975
Rohri	4	51.3000	41.91690	20.95845

One-Sample Test

	Test Value = 0					
	t	df	Sig. (2-tailed)	Mean Difference	95% Confidence Interval of the Difference	
					Lwer	Upper
Khairpur	3.218	3	.049	52.85500	14.5866	105.1234
Sukkur	2.993	3	.001	50.25000	3.1825	103.6825
Rohri	2.448	3	.002	51.30000	15.3991	117.9991

T-Test *P. aeruginosa* isolation rate in different quarters (2005-2007)

One-Sample Statistics

	N	Mean	Std. Deviation	Std. Error Mean
Khairpur	4	69.4875	13.26464	6.63232
Sukkur	4	77.8500	12.03620	6.01810
Rohri	4	76.6000	18.13946	9.06973

One-Sample Test

	Test Value = 0					
					95% Confidence Interval of the Difference	
	t	df	Sig. (2-tailed)	Mean Difference	Lower	Upper
Khairpur	10.477	3	.002	69.48750	48.3805	90.5945
Sukkur	12.936	3	.001	77.85000	58.6977	97.0023
Rohri	8.446	3	.003	76.60000	47.7361	105.4639

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