ISSN: 2581-5989

International Journal of Dental Science and Innovative Research (IJDSIR)

IJDSIR : Dental Publication Service

Available Online at: www.ijdsir.com

Volume – 1, Issue – 1, May – June - 2018, Page No. : 14 - 22

Detection of antibiotics resistant faecal Coliform and Streptococci isolates from ground water

Shaista Suhail, Department of Oral Pathology and Microbiology, King George's Medical University, Lucknow, Uttar Pradesh

Shruti Singh, Department of Oral Pathology and Microbiology, King George's Medical University, Lucknow, Uttar Pradesh

Correspondence Author: Shaista Suhail, Department of Oral Pathology and Microbiology, King George's Medical University, Lucknow, Uttar Pradesh, India

Type of Publication: Original Research Paper

Conflicts of Interest: Nil

Abstract

The non therapeutic use of antibiotics in swine feed can select for antibiotic resistance in swine enteric bacteria. Leaking swine waste storage pits and the land-application of swine manure can result in the dispersion of resistant bacteria to water sources. However, there are few data comparing levels of resistant bacteria in swine manure impacted water sources versus unaffected sources. The goal of this study was to analyze ground water situated up and down gradient from a swine facility for antibiotic-resistant enterococci and other fecal indicators.

Methods: Ground water samples (n = 12) were collected from different locations of lucknow. From Jan to May 2013. Fecal indicators were isolated by membrane filtration, and enterococci (n = 200) were tested for susceptibility to erythromycin, amoxicillin, kanamycin, chloramphenicol, novobiocin, streptomycin, ciprofloxacin, teicoplanin, gentamycin, norfloxacin.

Results: Median concentrations of enterococci, fecal coliforms, and *Escherichia coli* were 4 to 33-fold higher in down-gradient versus up-gradient surface water and groundwater. We observed higher minimal inhibitory concentrations for four antibiotics in enterococci isolated from down-gradient versus up-gradient surface water and groundwater. Elevated percentages of erythromycin- (p = 0.02) and Norfloxacin -resistant (p = 0.06) enterococci were detected in down-gradient surface waters, and higher percentages of Norfloxacin - (p = 0.07) and Ciprofloxacin-resistant (p < 0.001) enterococci were detected in down-gradient groundwater.

Conclusions: We detected elevated levels of fecal indicators and antibiotic-resistant enterococci in water sources situated down gradient from a swine facility compared with up-gradient sources. These findings provide additional evidence that water contaminated with swine manure could contribute to the spread of antibiotic resistance.

KEY WORDS: antibiotic resistance, *E. coli*, enterococci, fecal coliforms, fecal indicators, groundwater,

Introduction

Water is essential to sustain life. Safe drinking water is one of the prime necessities of human health. Ever increasing population has caused microbial contamination of drinking water sources (industrialization, urbanization, agriculture and other human activities) have caused deterioration in quality of various natural water bodies, particularly in developing countries like India. A wide variety of viruses, fungi, bacteria, protozoa the major group of pathogens of common occurrence in contaminated water impart a great deal of water born

diseases like gastroenteritis, cholera, dysentery, typhoid etc., are the common health problem. The sewage disposal and land run off with faecal waste adversely affecting the ecology and characteristics of water microbiota such as coliform, *Enterococci*, etc. which generally indicates the extent of water pollution. The presence of these pathogens in water can be assessed by determining the density of a group of certain pollution indicator bacteria belonging to family Enterobacteriaceae. The coliforms are the prime indicators and consists of *Klebsiella* sp., *E. coli*, *Citrobacter* sp., *Enterobacter* sp.

The coliforms are ubiquitous in nature and is currently defined as gram negative rods which ferment lactose with gas production within 48^0 C (APHA, 2012). According to Indian Standard for drinking water recommends an average MNP of coliform (BIS, 2012). Coliform bacteria are the natural part of microbial flora of intestinal tract of warm blooded mammals including man. Coliform bacteria are also found in non-faecal sources, soil and vegetation. The coliform group is relatively easy to culture in the lab and is therefore, has been selected as prime indicator of pollution in water. Coliform bacteria are normally non-pathogenic and are only mildly infectious.

E.coli is normal inhabitant of the intestine and the most strain are non-pathogenic. Isolation of *E.coli* from water supply indicates faecal contamination. Members of lactose fermenting species of this group which may be referred as COLIFORM. The picture is further complicated by the fact that other members of coliform group are also found in the intestine.

Faecal *Streptococci* are other indicator bacteria. Their presence indicates the water is contaminated. In the genus *Streptococcus* only *S.bovis* and *S.equines* are included in the faecal streptococcus group. Streptococci rarely multiply in polluted water and they are more persistant than *E.coli* and other coliform bacteria. *Streptococci* ferment several sugar producing acids and no gas. *Streptococci* are catalase negative.

Along with human, other sources of streptococci are excreta from various domestic and wild animals, birds, reptiles, fishes and insects etc. These organism have also been considered to be an index of feacal pollution i.e. excreta from human being or from homeothermic animals. This is due to the presence of more coliforms than human faeces (Pathak et al. 1992).

In many countries increased prevalence of antibiotic resistance has been reported among microorganism derived from man and from a variety of sources in environments ranging from rivers to sewage. This is generally attributed to the indiscriminate use of antibiotics which encourages the spread of transmissible plasmids (R-factors) carrying antibiotic resistance among susceptible population of bacteria.

Even though this phenomenon is probably rather insufficient in nature a significant impact on the incidence of resistance has been reported causing considerable concern when epidemic of multi-resistant organism occur as was recently seen in India. Microorganisms resistant to antibiotic and tolerant to metal make their appearance as a result of exposure to metal contaminated environments of native water resources receiving effluent from metal associated industries which is coincidental for resistance factors antibiotics.

Increased antibiotic therapy in recent decades has caused the emergence of antibiotic resistance among enteric bacteria. Antibiotic resistant bacteria make their appearance as a result of exposure to aquatic environment contaminated with metals, industrial waste and municipal sewage. Drug resistance in bacteria may be brought by the production of drug destroying enzyme.

Mechanism by which organism become antibiotic tolerant vary with different antibiotic but usually involve the use

of an antibiotic. The mechanism by which antibiotic resistant organism becomes predominant in a community has often been considered to by mutation to antibiotic resistance.

An essential goal for provision of safe drinking water is that it should essentially free of (at low risk of contamination) disease causing organisms. Since the beginning of 20th century, the detection of faecal indicator bacteria in ground water has been used as the basis criteria, guideline and standard for acceptable limit of faecal contamination and as the basis for judging or predicting the possible presence or absence of pathogenic microorganisms. World Health Organization (WHO) guidelines for drinking water quality and many other authorities continue to support the use of bacterial indicator or levels and their measurement as basis for judging and verifying drinking water quality. However, such faecal indicator analysis of drinking water as a measure of end product quality and determinant of microbial disease risk is only one of many measures and activity in an overall system for providing safe drinking water (Sobsey et al.2002).

E.coli is the only coliform that is an undoubted inhabitant of the gastrointestinal tract. *Klebsiella* sp. *Citrobacter* sp. and *Enterobacter* sp. have also been isolated from human faecal samples but in small numbers (Prescott et al. 1947, Davis and Masten, 1947).

Antibiotic resistance in bacteria may be mediated in a variety of ways, but whatever the mechanism or its genetic control, it is usually found that in the absence of antibiotic usage, antibiotic- resistant organisms from only in a minority population. They are found, however even in antibiotic virgin population. Gardener et al. 1969 isolated antibiotic resistant organisms from 2 of 40 specimens obtained from soil and human faeces in Solomen Island. Dean and Hinshelwood (1964) have provided evidence that bacterial resistance to antibiotics can raise by process in Japan in 1955, (Watanabe, 1963).

The β-lactum groups of antibiotics such as Novobiocin and Kanamycin have more pronounced effect on antibiotic resistant bacterial profile in the primary water sources than those antibiotics used as feed additives (Mulamattahil et al., 2002).

Faecal contamination of natural drinking water source is the most serious threat to human health by causing various water borne infectious diseases. Coliform and faecal coliforms are established indicator organism is reliable and a very sensitive method for detection of faecal contamination in water due to sewage disposal or through other means. These microorganisms are widely distributed in nature; and their presence and diversity may be used as also a test for suitability of water (Okpokwasili and Akuugobi, 1996). Therefore, these indicator organisms have been recommended by regulatory agencies for water quality monitoring. According to WHO guidelines for drinking water, there should be <10 coliform and no faecal coliforms or faecal streptococci in 100 ml of any potable water sample (WHO, 2004).

Material and Methods

Water samples were most preferably collected in sterilized stopper glass bottles of heat resistant, borosilicate glass from hand pump and well water from different areas of Lucknow Viz. Hardoi Road and Mohanlalganj. Water was collected in sampling bottle.

Each bottle labeled with necessary information such as site, sources, date, time or any other specific remark. Water samples should be filled once without rinsing leaving 1/3rd or 1/4th air space in bottles. Sample bottles should be immediately transferred into ice box (below 10^0 C) and transported to laboratory. Samples were analyzed within 6 hrs of collection.

Total count of viable aerobic and facultative anaerobic bacteria in a certain volume of sample is enumerated by spread plate count method. In this method appropriate agar medium plates were prepared by pouring 25+-5 ml and drying it for 30 minutes at 45^0 C. 0.1 ml of liquid sample was poured on agar surface of plate and inoculums was spread over immediately after pouring in rotating manner gently to dryness with an "L" shape sterilized with spirit and flame and cooled round glass rod spreader. The inoculated agar plates were incubated at 37^0C for 24 hrs. The colonies grown on the agar plates were counted manually. Bacterial counts between 30-300 colonies per plate were statistically acceptable and reported as "colony forming units" (c.f.u) per ml. plates with dense colonies were counted after dividing the plate area in convenient number of squares.

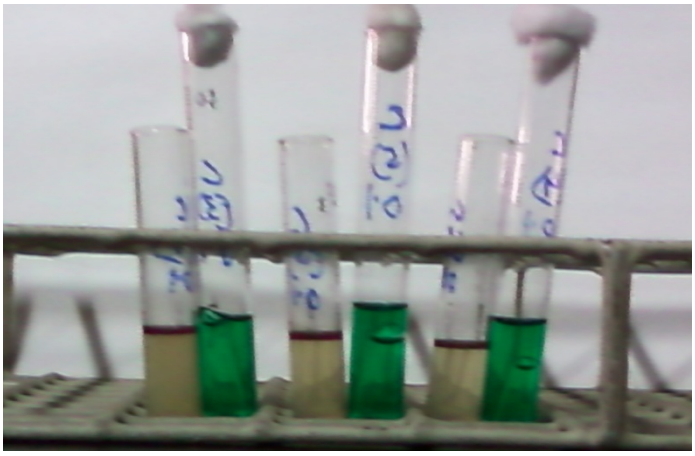
Coliform and faecal coliform enumeration was performed by **presumptive** coliform test: initially various tubes of MacConkey broth are inoculated with a standard volume of test water. Each tube of broth contain a vial in an inverted position called Durham's tube which detect the production of gas, the acid was recognize by the color change from red to orange yellow. Three sets of test tubes were arranged i.e. one set of 10ml. double strength and two sets of 5 ml. of single strength broth tubes were inoculated with 1ml. and 0.1 ml of water sample respectively. The inoculated tubes were agitated gently to mix the inoculums and the medium and then were incubated at 37^0C for 24 hours. Each tube was shaken gently and examined for gas, filled in Durham's tube with/without acid production and considered for positive results. The tubes with negative result were re-incubated and re-examined after 48 hours for the final observation and MPN of the

Confirmative Test

The tube with positive result in presumptive test sub cultured with a sterile loop in to Brilliant Green Lactose Bile Broth (BGLB broth) tubes containing with inverted Durham's tube. The inoculated BGLB tubes were incubated at 37^0C. Gas formation in any amount in the inverted vials of the BGLB broth fermentation tube within 24-48 hours indicates a positive result. For enumeration of faecal coliform, each confirmed coliform tubes was subcultured with flame sterilized loop into tubes of BGLB broth and peptone water. The inoculated BGLB broth and peptone water tubes were incubated for 24 hours at 44^0 C. The growth with gas formation in BGLB broth was observed and then 0.2ml of Kavoc's reagent was added into the peptone water tubes. Production if indole, which observed the red/pink color on upper layer of peptone water. Observation of yellow color constitutes negative results. From the number of tubes which show gas formation in BGLB broth tubes and indole positive peptone water tubes, the MPN of faecal coliform i.e. *E.Coli* per 100 ml was determined from the same probability table.

Faecal coliform Enumeration

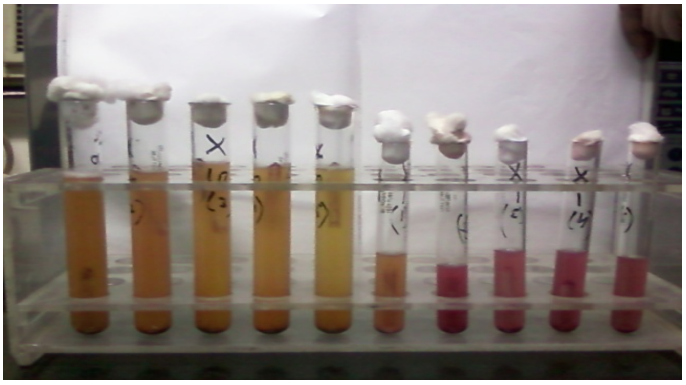
Each confirmed coliform tube was subcultured with flame sterilized loop into tubes of BGLB broth and peptone water tubes were incubated for 24 hours at 44^0C. The growth with gas formation in BGLB broth tubes was observed and then 0.2ml of Kavoc's reagent was added into the peptone water tubes. Production of indole indicated by red/pink color on upper layer of peptone water. Observation of yellow color constitutes negative result. From the number of tubes showing gas production in BGLB broth tubes and indole positive peptone water tubes, the MPN of fecal coliform per 100ml as determined from the same probability table.



Faecal Streptococci Enumeration:

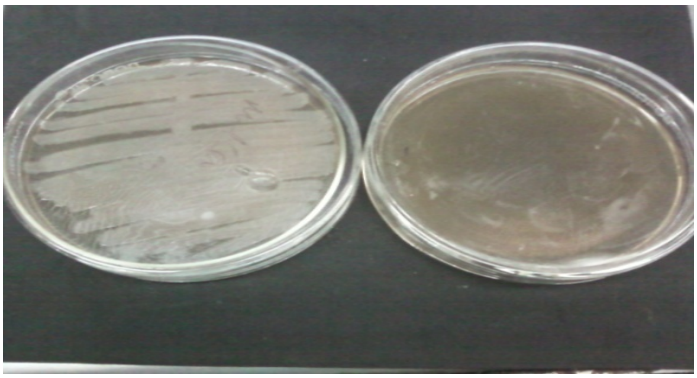
Inoculated a series of three test tubes of Azide Dextrose broth of double strength (10ml) and single strength (5ml) with 10ml. 1ml. and 0.1ml of water sample. Inoculated tubes were incubated at 37^0C. Each tube was examined for turbidity after 24-28 hours. If no definite turbidity was present, the tubes were re-incubated again after 48 hours. Each positive azide Dextrose broth tubes was sub cultured by streaking on to the Bile Esculine Azide medium and after 24 hours plates were observed for appearance of brown black color around the colonies and recorded as positive result.

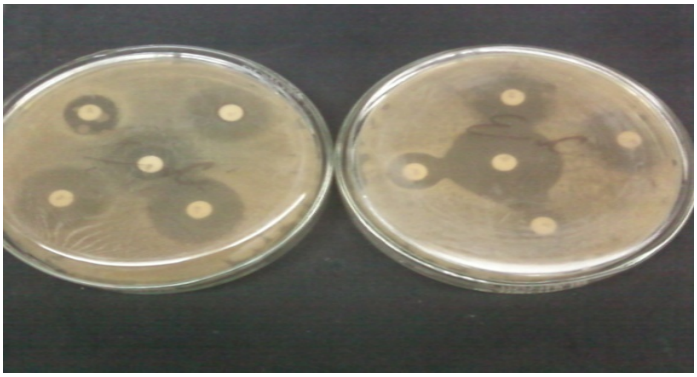
From each positive sample bacteria was selected. The streaking was done on MacConkey agar plates to obtain a single isolated colony of the desirable bacteria. These single colonies were then maintained on MacConkey agar plates and subjected to various necessary biochemical tests to identify. This study has been performed with the bacterial isolates from drinking water. The pure culture obtained on the streaked plates was then maintained by transferring the culture on the nutrient agar slant. The slant was incubated at 37^0C over night and was stored at 4^0C. The sub culturing of the culture was done every month.



Antibiotic Susceptibility Test

Antibiotic susceptibility test was performed to determine the sensitivity and resistivity of a pathogenic organism to various antimicrobial agents. The coliform isolation was subjected to antibiotics resistance test by disc diffusion method discs of ten antibiotics (microgram) viz. Amoxycillin (10), Gentamycin (10), Chloramphenicol (10), Ciprofloxacin (10), Kanamycin (30), Norfloxacin (10), Streptomycin (10), Tetracycline (30), Erythromycin (10) and Novobiocin (30) were used in this study.





Fresh culture of all test strains was prepared in nutrient broth after overnight incubation at 37^0C. Fresh broth cultures were sowed separately by sterile cotton swab on surface of Muller-Hinton Agar plates. Discs impregnated with appropriate concentration of each antibiotic were dispensed on the surface of sowed agar plates (5 discs per plates), which were incubated overnight at 37^0C temperature. Antibiotic resistance was estimated by measuring their respective zone of inhibition around each antibiotic disc. Antibiotic resistance index (ARI) of each test strain was also calculated by formula ARI=y/nx, where y= total number of resistant strain; n= total number of test strain and X= total number of antibiotics tested (Hinton and Linton, 1983). The level of bacterial contamination has been assessed by determining the plate count for heterotrophic as well as MPN coliform and faecal streptococci, coliform were observed to be <3->1100MPN/100ml where as faecal streptococci were observed to be <3->460 MPN/ 100ml. The isolation, purification and biochemical characterization of 20 bacterial strains from the water samples of different areas showed a wide range of bacterial species. Only few strains are belonging to the category of pathogenic bacteria, otherwise most of them were probably non- pathogenic or opportunistic pathogens. Worked by Cherry, Gutherie and Harvey (1976) showed that most of the coliforms are non pathogenic or opportunistic pathogens.

Antibiotic resistance was observed by disc diffusion method. Discs of ten antibiotics (micrograms) viz. Amoxycillin (10), Chloramphenicol (30), Ciprofloxacin (10), Kanamycin (30), Gentamycin (10), Novobiocin (30), Norfloxacin (10), Streptomycin (10), Erythromycin (10), Teicoplanin (30) were used.

About two or three bacterial strains in my study have shown sensitivity for one or two antibiotics as well as other bacterial strains were showed multiple antibiotics resistance.

Results and Conclusion

The ever growing urbanization and industrialization are continuously polluting the water bodies containing pathogenic microorganisms and toxic chemicals. The level of bacterial contamination in drinking water has been assessed by plate count method and by determining the MPN of the established pollution indicator organism's i.e. coliform, faecal coliform and faecal streptococci. Plate count of bacteria varied from 4.0 × 101 c.f.u to too numerous to count (TNTC) (Table.1). Coliforms were observed to be <3->1600/100 ml faecal coliform in <3-35/100ml where as faecal streptococci were observed to be <2->16/100 ml (Table.1). Coliform contamination was maximum (>1600/100ml) in water sample, 50% of the samples. Faecal coliform and faecal streptococci were present in 20% of the samples. Overall coliform, faecal colifrom and faecal streptococci contamination were observed in 50%, 40% and 10% of water samples, respectively.

The total coliform count was observed with multiple tube fermentation method as most probable number (MPN)/100ml. In this study the total coliform count ranged 280 to >1600/100ml. The higher the count of the entire pollution indicator organisms shows that water samples were highly contaminated with faecal waste of human as well as animal origin.

Observation of susceptibility test for 5 frequently used antibiotic revealed 60-80% of *E.coli* isolates showed resistant for all the test antibiotics while only 20% of *streptococci* isolates showed resistance.

The biochemical characterization of coliform isolates showed a wide range of bacterial species. Identification of bacterial strains was based on the basis of their biochemical characteristics. A battery of test (IMVic) specific for identification of coliform bacteria was used (Table.1). The maximum coliform contamination was due to *Citrobacter freundii* (30%). It was followed by *Klebsiella pneumonia, E.coli and Citrobacter koseri,* each was observed in 10% of the coliform contaminated drinking water samples. Thus we could see a considerable amount of the drinking water samples are contaminated with one or the other faecal pollution indicators. This is due to the increasing contamination of water sources with municipal sewage containing faecal bacteria.

Table: 1 Enumeration of plate count, coliform and *Faecal streptococci* present in water sample:

Strain No.	Platecount (c.f.u/100ml)	Coliform (MPN/100ml)	Faecal ccoliform (MPN/100ml)	Faecal streptococci (MPN/100ml)
1	3.2×10^3	<1.8	<1.8	6.8
2	1.36×10^6	4.0	4.0	2.0
3	2.08×10^6	>1600	>1600	>1600
4	4.97×10^3	>1600	>1600	>1600
5	2.2×10^4	>1600	350	>1600
6	3.2×10^2	>1600	130	210
7	1.62×10^4	5.5	<1.8	31
8	1.5×10^2	<1.8	<1.8	110
9	1.7×10^5	>1600	110	>1600
10	2.0×10^2	<1.8	<1.8	540
11	4.4×10^4	>1600	>1600	>1600
12	3.46×10^3	>1600	>1600	79

c.f.u= Colony forming unit, MPN= Most Probable Number

Conclusion

Since these days a wide range of antibiotics are used indiscriminately in treatment of different infectious diseases. The gut flora homeotherms including human beings after long exposure to such therapeutic chemicals, a vast bacterial population may affect the plasmid in bacterial cells. Among the identified coliform species, *E.coli* was found to be maximum.

Increased antibiotic therapy in recent decades has caused the emergence of antibiotic resistance among enteric bacteria (Bhattacharjee et al., 1998). More often the resistance method is plasmid born and transferable in nature resulting in spread among the sensitive aquatic bacteria including coliforms. Metal tolerant and antibiotic resistant bacteria make their appearance as a result of exposure to aquatic environments contaminated with metals and faecal waste from homeotherms by co-incidental co- selection of resistance factors (Timoney et al. 1978; Sterritt and Lester 1980; Calomiris et. al. 1989).

Bacterial resistance may be due to the presence of R-plasmid containing for metal tolerance (Timoney et al., 1978, Calomiris et al., 1984). Transmissibility of such plasmid mediated resistance to any sensitive bacteria is significant. It appears that the emergence of resistant bacteria is by the physicochemical characteristics of water and several environmental factors like constituents of ecosystems, ion interaction, pH, the form and the availability of metals to microbes (Rhodes et al., 2000). Thus bacterial tolerance for metal toxicity is clearly a significant environmental phenomenon (Steritt and Lester, 1980; Jones et al., 1986). Metal tolerances are transferable

in nature through plasmid and results in its spread among the sensitive bacteria. This resistance phenomenon may occur from non specific mechanism with gene regulation of plasmid and chromosomes which may be heritable or transferable due to the presence of resistance factor (Bhattacharjee and Ray, 1989; Silver and Walderhaug, 1992). Transmissibility of such plasmid mediated resistance to any sensitive bacteria is significant. In this way the population of resistant bacteria increases and this phenomenon has posed a therapeutic problem in treatment of infectious disease in man and animal.

The microbes resistance to metal ions is thus a potential health hazard. These findings indicate that sewage and industrial (metallic) pollution were responsible for the deterioration of water quality, along with posing risk to biodiversity of the hydrobionts and the human health. Therefore, the observations of this concern should be considered in dealing with water borne health problems.

So as far as possible, water sources must be protected from contamination by human and animal wastes, which contain a variety of pathogens and parasites. Lack of adequate protection and effective treatment will expose the community to the risk of outbreaks of intestinal and other infectious diseases.

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