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Faecal Contamination of Drinking Water From Deep Aquifers in Multan, Pakistan

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Abstract: A bacteriological analysis of hand pump water was undertaken for determining the quality and extent of contamination in water from different localities of Multan. A total of 100 hand pump water samples were studied for total streptococci (TS) and faecal streptococci (FS). Eighty percent of the samples were positive for total streptococci, 40% of which was in the range of 3-10, 46% in the range of 10-100, 10% were in the range of 100-1000, and 4% were between 1000-1500 streptococci/ml. FS were found in 67% of the samples of which 51% were in the range of 1-10, 4% in the range of 10-100, 7.5 in the range of 100-1000 and 1.5% in the range of 1000-1500MPN/ml. The minimum most probable number (MPN) was 3 and maximum was >2400 with a mean value of 81% for total streptococci and 57% for FS. The ratio of FS to TS was 10:14. Of 67% FS positive samples 63% formed red or pink colonies on KF-streptococcal agar, while 37% failed to grow. Of the 54 FS strains 39 (72.2%) were identified as enterococci, 32% of which were identified as *Ent. faecium* and 11% as *Ent. faecalis*. Enterococci were found to be resistant to most of the antibiotics used in this survey, though ampiclox and augmentin were effective against enterococci in 87% and 80%, respectively.

Key words: Faecal contamination, faecal streptococci, antibiotic resistance, drinking water.

INTRODUCTION

Unfortunately much of the developed worlds drinking water supplies are contaminated with enteric pathogens owing to inadequate sewage treatment and water purification facilities. The ground water usually contains very few bacteria because of the effective natural filtering action of the soil and several other controlling factors *i.e*. temperature, salt concentration and pH (Geldreich, 1990; Kimberly *et al.*, 2005). The lack of basic sanitation and lack of access to safe water supplies constitute the cause of water borne diseases in developing countries (Jensen *et al*., 2004; Haruna *et al.*, 2005; Nanan *et al.*, 2003). Large portions of the rural population in Pakistan are still without access to safe and clean drinking water and treated drinking water in urban centers frequently becomes contaminated in the distribution system during domestic storage. The spread of diarrhoeal diseases especially in infants is because of contamination of drinking water with organisms of faecal origin (APHA, 1975; Barabas, 1986).

Water borne bacterial pathogens most often detected in contaminated drinking water supplies include *Shigella*, *Salmonella*, *Campylobacter*, *Vibrio*, toxigenic *E. coli*, *Yersinia enterocolitica*, *Streptococci faecalis* which are also detected in faeces of infected individuals (Geldreich, 1991). Faecal streptococci (FS) are consistently present in the faeces of all warm-blooded animals and in the environment associated with animal discharges (APHA, 1975). FS provide a better indication of a negative test than coliform (Collin *et al*., 1988; Hornberger *et al.*, 1990).

FS or enterococci are considered as *Streptococcus spp.* that normally occur in faecal matter, which include *E. faecalis, E. faecium, S. bovis, E. avium, S. equines, S. mitis, S. salivarius* (Leclerc *et al*., 1996; Houston, 1900). Enterococci are Gram-positive cocci which grow at temperature range 10-45°C and survive exposure to 60°C for at least 30 minute, grow at pH 9.6 and also grow in 6.5% NaCl and can reduce 0.1% methylene blue (Sherman, 1938). They are nonmotile, occur in pairs or in short chains. The natural habitat of these organisms is the intestinal tract of man and animals. Enterococci have been studied extensively not only because of their being indicator of faecal

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contamination and their involvement of food spoilage, but also due to their influence on host physiology and nutrition, and because of their possible role as direct or indirect agent of disease in man (Esrey, 1996; Riaz, 2005). The objective of this study was to examine the drinking water reservoir in Multan for the incidence and extent of faecal contamination.

MATERIALS AND METHODS

Sample collection

Water samples were collected in 300ml sterile clean, labeled wide mouth container after working the hand pump until fresh water from deep aquifer started flowing. The water outlet was thoroughly cleaned before water sample was collected. Temperature and pH of flowing water was noted at the time of collection. Sample was processed within 2 hours or refrigerated at 5-7°C for not more than 12 hours.

Estimation of total and faecal streptococci

For enumeration of streptococci most probable number (MPN) technique was used (APHA, 1971; Collins *et al.*, 1989). Three tubes containing 10ml double strength Azide Dextrose broth (BBL) was inoculated with 10ml of water samples, while two other sets, each of 3 tubes containing 5ml single strength broth were inoculated with 1ml and 0.1ml water sample, respectively. Well mixed tubes were incubated at 35±0.5°C for 24-48hours. Turbidity was noted and interpreted from MPN table (Collins *et al*., 1989). Five ml of single strength Azide dextrose broth was inoculated with 1ml, shaken well and incubated at 44.5°C for 48hours. The tubes showing growth were considered positive for faecal streptococci. MPN was computed. For further confirmation KF-streptococcal agar was streaked with 0.01-0.05ml turbid culture, and incubated at 35±0.5°C for 48hours. Pink to dark red colonies were examined, purified and further identified by different biochemical tests.

Biochemical tests for streptococci

For biochemical tests fresh culture was obtained by growing pink or red colonies from the above plates into trypticase soy broth.

0.1% methylene blue Milk

Sterile 0.1% methylene blue milk (5ml), was inoculated with fresh broth FS culture and incubated at 35±0.5°C for 24-72hours. Methylene blue was reduced to leuco methylene blue (colourless) in the case of positive test (Brock and Brock, 1978).

Arginine dehydrogenase

Four ml of broth (5g peptone, 3g yeast extract, 1g D(+)glucose, 0.016g bromocresol blue and 0.5g arginine per liter) was inoculated with pure fresh FS culture, overlaid with sterile paraffin (viscous) and incubated at 37°C for four days. Tubes with violet colour were considered as positive and with yellow colour as negative. Control tubes containing culture medium base only also gave yellow colour (Merck, 1988).

Aesculine hydrolysis

Five ml of sterilized Aesculine broth (Peptone 2g, aesculine 0.1g, and ferric citrate 0.05g per 100ml) was inoculated with fresh culture. Olive green colour of the medium changed to brown in positive test after 12-hours incubation at 35±0.5°C.

Acids from carbohydrates

Different carbohydrates such as mannitol, sucrose, sorbitol and L-arabinose (1% each) dispensed separately in five ml of autoclaved Phenol red broth (BBL), were inoculated and incubated at 35±0.5°C for 24±2hours. Acid production from carbohydrates was indicated by phenol red.

Growth at 10℃ and 45℃

Five ml of Tryptose soy broth (BBL) was inoculated with fresh pure culture and incubated at 10°C in one case and 45°C in other for 24±2 hours.

Survival at 60℃ for 30 minutes

Twenty four hours fresh cultures were incubated at 60°C for 30 minutes, and then streaked on KF- streptococcal agar. Dark red colonies after 48 hours incubation at 35±0.5°C indicated faecal streptococci.

Growth at pH 9.6

Tryptose soy broth, pH 9.6, inoculated with fresh pure culture was incubated at 35±0.5°C for 24±2hours. Turbidity in tube was considered as positive test.

Table I.- Incidence of total streptococci and faecal streptococci in water from deep aquifers.

Organism	Number	Positive	MPN / ml				
			1 – 10	$10 – 10^2$	$10^2 – 5x10^2$	$5x10^2 – 1x10^3$	$10^3 – 1.5x10^3$
Total streptococci	100	80	32	37	5	3	3
Faecal streptococci	100	67	34	27	3	2	1

Growth at 6.5% NaCl broth

Trypticase soy broth with 6g/L NaCl added was incubated with fresh culture. Turbidity after 24±2hours at 35±0.5°C was considered as positive test (Garg and Mital, 1991).

Antibacterial susceptibility testing

Fecal streptococci from KF streptococcal agar plate were streaked on nutrient agar plate to have 24 hours fresh culture. A pure culture inoculation was prepared by emulsifying fresh growth in 1 ml sterile 0.1% peptone water (Gibco lab. Cat.# M38100). Turbid suspension was spread uniformly on the nutrient agar plate. Twelve antibiotics, Penicillin, Methicillin, Tetracycline, Orbenin, Augmentin, Trimethoprim, Erythromycin, Lincomycin, Velosef, Cefaclore, Ampliclox and Ceftriaxon were used for test. The plates were incubated at 37°C for 24 hours. Zone of inhibition were measured and results were interpreted according the table provided with antibiotics

RESULTS

Water samples were clear in appearance and without any smell or odor. Temperature was found to be in the range of 24-29°C and pH ranged between 7.29-8.69.

Total streptococci (TS) and faecal streptococci (FS)

Table I shows the incidence of total and faecal streptococci. The MPN ranged between 3->2400/ml. The total and faecal streptococci constituted 80% and 67% of the total number of the samples. Among positive samples 40% had streptococci in the range of 3-10, 46% in the range of 10-100, 10% were in the range of 100-1000 and 4% were between 1000-1500 streptococci/ml.

Out of total faecal streptococci 51% of the samples had a range of 1-10, 40% in the range of 10-100, and 7.5% were in the range of 100-1000 per ml. Only one sample gave MPN >2400 (Table 1). Mean values of MPN calculated for TS and FS were 81 and 57, respectively. The ratio of FS: TS was estimated to be 10:14. Hence according to our investigation 67% samples was not of potable quality.

Out of 67 FS positive samples 42(63%) formed red or pink colonies on KF streptococcal agar, the rest 25(37%) positive samples failed to grow after at least three trials. They were found Gram positive *Micrococci* on Gram staining. Hence according to standard MPN technique (APHA, 1971), 67% samples were positive for FS but if completed test on KF streptococcal agar was taken into consideration, then only 42% samples were actually FS positive.

Fifty four strains of FS were randomly obtained from 25 samples showing growth on KF streptococcal agar. These strains were subjected to physiological and biochemical tests (Table II). Thirty nine strains (72.2%) were identified as enterococci, of which 17(31.5%) strains were identified as *Ent. faecium*, 6(11%) as *Ent. faecalis*, 2 (3.7) as *Ent. durans* and 3(5.6%) as *Ent. avium.* Eleven (20.4%) strains could not be identified up to species level whereas 15 were remained unidentified.

Antibiotic susceptibility of faecal streptococci

Faecal streptococci isolated from hand pump water showed varying degree of resistance to twelve antibiotics used (Table III). Table III shows

decrease in susceptibility among enterococci in the order; ampiclox > augmentin > tetracycline > erythromycin > velosef > cefaclore > trimethoprim = ceftrriaxone > orbinin > penicillin > methicillin > lincomycin. No strain of *Ent. faecium* was susceptible to penicillin and orbenin whereas 6%

Table II.- Biochemical tests of faecal streptococo.

S.No.	Strain No.	Acid form				pH	NaCl	mbm	Growth (°C)			Hydrolysis		Strains identified
		so	su	ar	m	9.6	6.50%	0.10%	10	45	60	Arg	Aes	
1	1a	-	+	+	+	+	+	+	+	+	+	+	+	*E. faecuim*
2	1b	-	+	+	+	+	+	+	+	+	+	+	+	*E. faecuim*
3	1c	-	+	+	+	+	+	+	+	+	+	-	+	*E. faecuim*
4	2a	-	+	+	+	+	+	+	+	+	+	+	+	*E. faecuim*
5	2b	-	+	+	+	+	+	+	+	+	+	+	+	*E. faecuim*
6	2c	-	+	+	+	+	+	+	-	+	+	-	+	Ul *
7	3a	-	+	+	+	+	+	+	+	+	+	+	+	*E. faecuim*
8	3b	-	+	+	+	+	+	+	+	+	+	+	+	*E. faecuim*
9	3c	-	+	+	+	+	+	+	+	+	+	+	+	*E. faecuim*
10	5a	+	+	+	+	+	+	+	+	+	+	+	+	*Ent.* Spp.
11	5b	-	+	+	+	+	+	+	+	+	+	+	+	*E. faecuim*
12	5c	-	+	+	+	+	+	+	+	+	+	+	+	*E. faecuim*
13	12a	+	+	+	+	+	+	+	+	+	+	-	+	*Ent.* Spp.
14	12b	+	+	+	+	+	+	+	+	+	+	-	+	*Ent.* Spp.
15	12c	+	+	+	+	+	+	-	-	+	+	-	+	*E. avium*
16	15a	+	+	+	+	+	+	+	+	+	+	-	+	*Ent.* Spp.
17	15b	+	+	+	+	+	+	-	-	+	+	-	+	*E. avium*
18	15c	+	+	+	+	+	+	-	+	+	+	-	+	*Ent.* Spp.
19	21a	+	+	-	+	-	-	-	+	+	+	-	-	Ul *
20	21b	+	+	-	+	+	+	-	+	+	+	-	-	Ul *
21	21c	+	+	-	-	-	+	-	+	+	+	-	+	*E. durans*
22	18a	-	+	+	+	+	+	+	+	+	+	+	+	*E. faecuim*
23	18b	-	+	+	+	+	+	+	+	+	+	+	+	*E. faecuim*
24	18c	-	+	+	+	+	+	+	+	+	+	+	+	*E. faecuim*
25	23a	+	+	-	-	+	+	-	+	+	+	-	+	*E. durans*
26	23b	+	+	+	+	+	+	+	+	+	+	-	+	*Ent.* Spp.
27	23c	+	+	-	+	+	+	+	+	+	+	-	+	*Ent.* Spp.
28	16	+	+	-	+	+	+	+	+	+	+	+	+	*E. faecalis*
29	34a	+	+	-	+	+	+	+	+	+	+	+	+	*E. faecalis*
30	34b	+	+	-	+	+	+	+	+	+	+	+	+	*E. faecalis*
31	30	+	+	-	+	+	+	+	+	+	+	+	+	*E. faecalis*
32	32	-	+	+	+	+	+	+	-	+	+	-	+	*E. avium*
33	37	+	+	+	+	+	-	+	-	+	+	-	-	Ul *
34	39	-	-	-	-	+	+	+	+	+	+	+	-	Ul *
35	40	-	+	+	+	+	-	-	+	+	+	-	+	Ul *
36	29a	-	+	-	-	-	-	-	-	+	+	-	+	Ul *
37	29b	-	-	+	-	-	-	-	-	+	+	-	-	Ul *
38	41a	+	+	+	+	+	+	+	+	+	+	-	+	*Ent.* Spp.
39	41b	+	+	+	+	+	+	-	+	+	+	-	+	*Ent.* Spp.
40	41c	+	+	+	+	+	-	-	+	+	+	-	+	Ul *
41	47a	-	+	+	+	+	+	+	+	+	+	+	+	*E. faecuim*
42	47b	-	+	+	+	+	+	+	+	+	+	+	+	*E. faecuim*
43	47c	+	+	+	+	+	+	+	+	+	+	+	+	*E. faecuim*
44	29c	-	+	-	-	-	+	+	-	+	+	-	-	Ul *
45	48	-	+	+	+	+	+	-	-	+	+	-	+	Ul *
46	52a	+	+	-	-	+	+	+	+	+	+	+	+	*E. faecalis*
47	52b	+	+	+	+	+	-	+	-	+	+	+	-	Ul *
48	61a	+	+	+	+	+	+	+	+	+	+	+	+	*Ent.* Spp.
49	61b	-	+	+	+	+	+	+	+	+	+	+	+	*E. faecuim*
50	61c	+	+	+	+	+	-	+	-	+	+	+	-	Ul *
51	66	-	+	+	+	+	+	-	-	+	+	+	+	Ul *
52	69a	+	+	-	+	+	+	+	+	+	+	+	+	*E. faecalis*

53	69b	+	+	+	+	+	+	+	-	+	+	+	+	Ul *
54	71	+	+	+	+	+	+	+	+	+	+	+	+ +	*Ent.* Spp.

*Ul, unidentified; so, sorbitol; su, sucrose; m, mannitol; mbm; methylene blue milk.
60°C, survival for 30 min. at 60°C; Arg, arginine; Aes, aesculin.

Table III.- Susceptibility of *Ent. faecium, Ent. faecalis, Ent. avium, Ent. durans,* and some other *Ent.* spp. to various antibiotics.

Enterococci species	No. of isolates	Antibiotics	Resistance	Moderate resistance	Susceptibility
Ent. faecium	17	Penicillin	8	9	-
		Methicillin	17	-	-
		Orbenin	17	-	-
		Augmentin	2	1	14
		Trimethoprim	15	-	2
		Ceftriaxone	16	-	1
		Cefaclore	16	5	2
		Velosef	10	2	2
		Ampiclox	13	1	15
		Tetracycline	1	-	1
		Erythromycin	16	7	5
		Lincomycin	5	-	-
Ent. faecalis	6	Penicillin	5	-	1
		Methicillin	6	-	-
		Orbenin	5	-	1
		Augmentin	-	1	5
		Trimethoprim	4	-	2
		Ceftriaxone	3	2	1
		Cefaclore	5	-	1
		Velosef	3	2	1
		Ampiclox	-	1	5
		Tetracycline	-	5	1
		Erythromycin	1	4	1
		Lincomycin	5	1	-
Ent. avium	3	Penicillin	-	2	1
		Methicillin	1	1	1
		Orbenin	1	1	1
		Augmentin	-	-	3
		Trimethoprim	3	-	-
		Ceftriaxone	1	1	1
		Cefaclore	1	-	2
		Velosef	1	-	2
		Ampiclox	-	-	3
		Tetracycline	-	-	3
		Erythromycin	1	1	1
		Lincomycin	3	-	-
Ent. durans	2	Penicillin	1	1	-
		Methicillin	2	-	-
		Orbenin	1	-	1
		Augmentin	-	-	2
		Trimethoprim	2	-	-
		Ceftriaxone	1	-	1
		Cefaclore	1	-	1
		Velosef	1	-	1
		Ampiclox	-	-	2
		Tetracycline	1	1	-
		Erythromycin	-	-	-
		Lincomycin	2	-	-
Ent. spp.*	11	Penicillin	6	3	2
		Methicillin	9	-	2
		Orbenin	9	-	2
		Augmentin	-	4	7
		Trimethoprim	9	-	2
		Ceftriaxone	9	-	2
		Cefaclore	7	1	3
		Velosef	5	2	4

Ampiclox	-	2	9
Tetracycline	3	-	8
Erythromycin	4	5	2
Lincomycin	10	1	-

*Enterococci that were not characterized to species level.

shows susceptibility to cephalosporin and ceftriaxone. *Ent. faecalis* showed 17% susceptibility to penicillin, orbenin, cephalosporin, erythromycin, tetracycline, ceftriaxone, cefaclor and velosef. ampiclox and augmentin were 82% effective among *Ent. faecium* and 80% in *Ent. faecalis.*

DISCUSSION

Our investigation reveal that 67% samples were positive for FS which is in accordance with the findings of Attar *et al.* (1982) who found 64% of the samples positive in rural drinking water. This means that water from majority of the hand pumps was faecally contaminated and was not suitable for domestic use. French standards according to European regulation provided for the absence of faecal streptococci in 100ml of drinking water (Collin *et al.*, 1988). 62.7% FS (MPN positive) samples formed red or pink colonies on KF streptococcal agar, but 37.3% failed to grow. The second category was Gram stained from azide dextrose broth and it was found Gram positive diplococci. It is probable that *Micrococci* inhibited the growth of few streptococci on the KF Streptococcal agar.

In this report 72% strains of FS were identified as enterococci that belong to Group D streptococci. *Ent faecium* was present in appreciable percentage in this survey as Poucher *et al.* (1991) also indicated that *Ent. faecium* always represented over 25% of streptococci in different types of faecal samples. *Ent. faecalis* and *Ent. faecium* are inherently resistant to multiple antibiotics and hence reduce susceptibility to cell wall active agents such as β-lactams. Our results were also in accordance with this statement as the above two enterococci were found highly resistant to methicillin and showed a varying degree of resitance to penicillin, orbinin, cephalosporins (cefriaxone, cefaclor and velosef). *Ent. faecalis* and *Ent. faecium* are the predominant enterococcal species associated with clinical infection in human. In general *Ent. faecium* strains are less susceptible to β-lactams than *Ent. faecalis* (Herman and Gerding, 1991). Our results showed approximately a similar pattern, except methicillin to which both strains are highly resistant.

Enterococci are opportunistic pathogen (Geldreich 1990; Valerie *et al.*, 2005), cause infections of urogenital tract, endocarditis and wound infections in humans. So their presence is a cause of serious concern. As TS and FS were found in 80% and 67% samples respectively, they may be considered as indicators of pollution and the presence of other intestinal pathogens following the presented here. Enterococci are facultative anaerobe and are able to withstand diverse conditions (Garg and Mital, 1991). Hence their presence in water may cause problems as water is used extensively in daily life and government should device ways to regularly monitor water supplies and provide potable water to general public. Instead of spending a huge amount on importing medicines, if investments are geared towards the safe water supply, the situation will become more acceptable.

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