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Usefulness of faecal Streps as indicator of presence of *Salmonella* sp. and *Vibrio cholerae* in sewage effluents

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ABSTRACT

Enteric pathogens are the most frequent cause of diarrheal illness, which account for an annual mortality rate of three million people and an estimated four billion infection worldwide. One way of preventing this is by ensuring proper sewage treatment. The study was carried out to provide data for level of microbiological contamination as well as baseline data for the future assessment and monitoring of pollution levels of sewage lagoons around Kenyatta university sewage treatment plant. It was also aim to find out the indicator organism that is suitable for the assessment and monitoring of faecal pollution. This paper contains the results of isolation, identification and quantification of faecal coliforms, streps, S*almonella* sp. and *Vibrio cholerae* from Kenyatta university sewage treatments ponds. For the faecal coliforms, detection and quantification was done using the Most Probable Number (MPN) technique. The isolation and enumeration of faecal streps, *Salmonella* sp. and *V. cholerae* was done using standard methods. Correlation of faecal coliforms with *Salmonella* sp. and *V. cholerae* was 85% and 2% respectively. For the faecal streps, correlation with *Salmonella* sp. and *V. cholerae* was 78% and 12% respectively. This indicates that faecal streps should be included as indicator organisms of the potential health hazards of polluted water. Most international drinking water quality guidelines and standards include bacterial indicators as a measure of microbial water quality, and for compliance reporting. The results from the study support the idea of using both the faecal streps and coliforms as indicators of faecal pollution.

Keywords: faecal coliforms, faecal streps, *Salmonella* sp.*, Vibrio cholerae.*

INTRODUCTION

Waterborne diseases whose burden is most felt in all African countries especially in the tropical areas of the region, such as Kenya, are among the most emerging and re-emerging infectious diseases in the world (AMREF, 1999, WHO, 1999, Edwards, 1999). They include cholera and typhoid (Talaro and Talaro, 2002).

Lack of potable water and poor sewage effluent are responsible for billions of such cases of diarrhoeal illness which kill two million children each year (Talaro and Talaro, 2002).This is primarily because of pathogenic bacteria such as *Vibrio cholerae* and *Salmonella* sp. that are present in the effluent (CJM, 1994, Chabalala and Mamo, 2001).

The possible sewage contamination indicators have their advantages and disadvantages. Studies have demonstrated a number of deficiencies in the use of faecal coliforms as indicator organisms in marine waters (APHA, 1998). The validity and usefulness of the indicator concept depend on the existence of a constant quantitative relationship between the indicator organisms and the pathogens they monitor (Warrington, 2001). Faecal coliforms (FC) rather than faecal streps (FS) are usually used as indicators of faecal pollution (Harrigan, 1976). Their presence in water is a confirmatory test of recent faecal contamination (Warrington, 2001).

The aim of this study was to assess faecal contamination using indicator organisms, faecal coliforms and streps as surrogates for actual pathogens of concern and find out which one gave the most desired results. Faecal streptococci bacteria are more closely associated with human sewage and their presence in a sample is believed to be a better indicator of sewage contamination than coliforms because they have low die- off rates than the FS (Warrington, 2001).

It has been showed (Gabutti, 2004) that the presence of *Salmonella* sp. is not necessarily correlated with great concentration of the faecal pollution indicators, and therefore it would be advisable to always perform the detection of *Salmonella* sp. beside the traditional indicators. They are therefore being substituted by more specific indicators, which include the *Escherichia coli* (Warrington, 2001). A number of studies have shown that faecal streps may be ideal indicators.

The Annapolis protocol identified faecal streptococci (for temperate marine and fresh water), *E. coli* (for temperate fresh waters) and sulphite-reducing *Clostridium perfringens* (for temperate, tropical marine and fresh waters) as primary microbial indicators (WHO, 1999).

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Routine monitoring experiments were carried out in New York which gave guidelines based on geometric mean of at least five samples per 30-day period during swimming season (Crossan, 2005). It was proposed that faecal streptococci be used in determining the extent of faecal contamination in recreational fresh waters and marine waters. The guideline given was 33 FS/100 mL for recreational water and 35 FS/100 mL for marine waters. In another study on microbial pollution of river catchments (Allen *et al.,* 2003), it was found that 'excellent' classification was attained where faecal streptococci were used as indicators. When looking for contamination with *Salmonella*, nearly all the time, the organism was isolated 67% from shellfish with faecal coliforms levels <300/100 uL which supported the view that low levels of faecal coliforms do not necessarily indicate the absence of *Salmonella*.

This study investigated the suitability of the faecal coliforms as indicator organisms and the possibility of coupling it with faecal streps to improve the reliability of the indicator system.

MATERIALS AND METHODS

Sample collection and preparation

Water samples were collected in sterile 250 mL glass bottles from seven sampling points at the sewage treatment plant (Figure 1). They were transported to the laboratory, in ice cooler box and analyzed following standard procedures as outlined by APHA (1998).

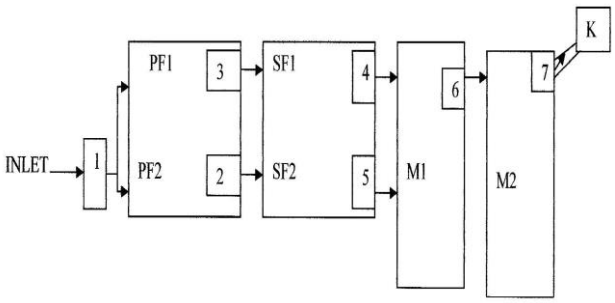

Figure 1: Sampling sites of the various ponds of the treatment plant

PF 1 and PF 2- First and second primary facultative ponds; SF 1 and SF 2-First and second secondary facultative ponds; M 1 and M 2- First and second maturation ponds; No. 1 to 7- Sampling sites were seven (N=7) and during the analysis, the experiments were carried out in triplicate before determining the Mean Bacterial Densities.

Bioassays

Screening faecal coliforms (FC)

The analysis of water for the presence of faecal coliforms was carried out using the multiple–tube fermentation technique (APHA, 1998). This was done in three steps; the presumptive, the confirmed and completed tests. Each batch was inoculated with the diluted (all dilutions were done using sterile water blanks) water samples. In the presumptive test a three series of five tubes each containing 10 mL, 1 mL and 0.1 mL portions of the sample were inoculated with lactose broth that was initially sterilized by autoclaving at 121 °C for 15 min. Pure sterile lactose broth was inoculated with sterile distilled water in the same way and used as controls. Inoculated tubes were then placed in an incubator at 37 °C for 48 h (Cheesbrough, 1985). Sterile loop transfers were made from all tubes showing acid and gas production (of total coliform MPN) to tryptose bile broth (EC Broth) and incubated at 44 °C for 24 h. Gas production in a fermentation tube within 24 h or less was considered a positive reaction. The estimated number of faecal coliforms, present in 100 mL was read from a tabulated probability table using corresponding results of various combinations of positive and negative reactions from each of the three batches (APHA, 1998). For confirmation, samples considered to have a positive reaction from the tryptose broth were streaked on a plate of Eosin Methyl Blue (EMB) agar to give well isolated colonies. Incubation was done at 37 °C for 48 h. Development of the typical colonies on the plates was observed and a Gram staining done.

Completed test was done when two colonies considered to be of faecal coliforms, were picked and transferred to nutrient agar slopes and fermentation tubes containing brilliant green lactose broth. Incubation was again done at 37 °C for 24 to 48 h. From the agar slope, a Gram stain was made to confirm the completed test. Brilliant green lactose broth was also observed for gas production.

Screening for faecal streptococci (FS)

Enterococcus presumptive broth was used for the detection of faecal streptococci in the wastewaters. Inoculation was done of the single double strength samples with 10 mL of the samples. Tubes were then incubated in a water bath at 45 °C and observed after 8 h for appearance of turbidity and production of acid (indicated by the yellow colour change of the Bromthymol blue).

As soon as the positive reaction appeared, a loopful of the material was transferred to a confirmatory slant (enterococcus confirmatory slant). The confirmatory slants were used with the enterococcus confirmatory agar, to confirm the presence of faecal streps (FS) in the wastewater samples. Slants of the confirmatory agar were prepared and a loopful of material from the broth streaked on the surface of the slant. The tubes were then incubated at 37 °C for 12 h and examined for the presence of pinpoint colonies on the slant. A Gram stain reaction was carried out for microscopic evidence of the presence of FS. For confirmation, KF streptococcal agar, a selective media was used, by direct plating (pour plating). 1 mL of diluted (10^4 or 10^5) samples were used in duplicate plates.

Incubation was done for 48 h at 35 °C and all red and pink colonies counted as (using colony counter) faecal streptococci (Carski *et al.,* 1973).

Screening for Salmonella

Detection of *Salmonella* was done in three successive phases. The first phase of selective enrichment was done using the tetrathionate broth base as outlined by APHA (1998). 1 mL of each sample from different sites was mixed well with 10 mL of tetrathionate broth and the mixture incubated for 24 h at 35 °C.

For selective growth, the phase of plating (pour plating method was used) was done using 1 mL of enriched samples. Streaking was done from the same enriched samples on Deoxycholate Citrate (DCA) agar, Salmonella-Shigella (SS) agar and MacConkey agar (Andrews and Hammocks, 2003). An incubation temperature of 37 °C was used for 24 h. Enumeration of typical colonies (typical colonies clear to pale pink on DCA agar, pink on SS agar and white on MacConkey agar) was done using colony counter and Gram staining carried out. Typical colonies were subjected to various standard biochemical procedures.

Screening for V. cholerae

Detection of *V. cholerae* was done in three successive phases.

(i) Enrichment in a non-selective medium.
1 mL of the samples was enriched in sterile alkaline peptone water and dispensed in 10 mL tubes (HPA, 2003). Incubation was done for 18 h at 35 °C.

(ii) Plating out on selective medium
The streaking of the enriched samples was done on Thiosulfate citrate bile salts sucrose (TCBS) agar. For quantitative analysis, pour plating of the samples was done. The agars were incubated at 35 °C for 24 h for presumptive tests. To differentiate Vibrio cholerae, streaking of sucrose positive (yellow) colonies was done on nutrient agar (Cheesbrough, 1985).

(iii) Biochemical reactions
Gram staining was carried out and observations made before carrying out the biochemical tests. First the presumptive colonies on nutrient agar were inoculated onto the TSI agar slants and incubated at 37 °C for 24 h. Simmons citrate agar slants were also inoculated and incubated at 36 °C for 72 h. The media was observed for growth and colour changes. The cytochrome oxidase test was carried out by placing a piece of filter paper in a clean Petri dish and adding 3 drops of freshly prepared Oxidase reagent. Using a glass rod, a colony of the test organism was applied on the filter paper, and development of a blue purple colour observed after a few seconds (for the positives).

For motility test, Motility Indole Urease (MIU) media was stabbed through the centre (done in same tubes with those of urease test) to test for motility by observing if there was spreading of growth from the stabline or turbidity throughout the medium. This was also used to test for urease production, by examining if there was any colour change (Cheesbrough, 1985). An uninoculated tube was used as control.

RESULTS

Screening for faecal coliforms indicated fluctuating densities down the treatment process (Table 1). The effluent discharged had a mean density of 7.9×10^5 MPN/100 mL. The correlation between the faecal coliforms and *Salmonella* sp. was 85% whereas the correlation with *V. cholerae* was 2% (at 0.05 level).

Typical colonies gave growth on nutrient agar slants and gas was formed in broth tubes hence the completed test was positive for faecal coliforms. The density of faecal streptococci was far below that of the faecal coliforms (Table 1). The faecal streptococci also showed fluctuation in density from one oxidation point to another (Figure 2), with the mean density of 2.64×10^6 cfu/100 mL. The correlation of faecal streps with *Salmonella* sp. was 78% and 12% with the *V. cholerae.*

Table 1: Mean bacterial densities in 100 mL from the Kenyatta University oxidation ponds

Organism	1	2	3	4	5	6	7
FC	3.5×10^7	9.2×10^6	3.3×10^7	$3.3. \times 10^6$	4.6×10^6	9.2×10^5	7.9×10^5
FS	5.4×10^6	5.3×10^6	5.6×10^6	9.9×10^5	1.0×10^6	1.7×10^5	1.4×10^4
Salmonella sp.	1.64×10^4	7.6×10^3	8.4×10^3	2.4×10^3	2.8×10^3	5.4×10^3	2.4×10^3
Vibrio cholerae	1.1×10^3	9.0×10^2	3.0×10^2	1.6×10^3	3.0×10^2	7.0×10^2	2.0×10^2

FC – Faecal coliforms, density expressed in MPN/100mL (using MPN technique).
FS – Faecal streptococci, density expressed in cfu/100mL (Colony forming unit).
Salmonella sp. – density expressed in cfu/100mL.
V. cholerae – density expressed in cfu/100mL.

Screening indicated that the density of *Salmonella* sp. was far less in magnitude than the faecal indicators (Figure 3). The range was 1.46×10^6 cfu/ 100 mL with a mean density of 6.49×10^3 cfu/ 100 mL. Even the effluent had high counts of *Salmonella sp*. whose mean density was 2.4×10^3 cfu/ 100 mL (Table 1). The density also fluctuated from one oxidation pond to another (Figure 3). The Gram stain reaction of the typical colonies from Salmonella-Shigella agar, Deoxycholate Citrate agar and MacConkey agar was negative.

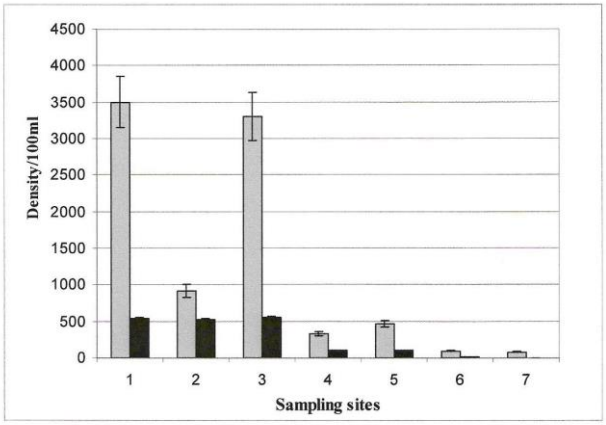

Figure 2: Bar graph showing density of faecal coliforms (FC) and streps (FS).
Seven sites were sampled and analysis of water for the presence of faecal coliforms was carried out using MPN technique at varying temperatures through the three steps. For the FS, pour plate method was used to enumerate at 35 °C for 48 h.

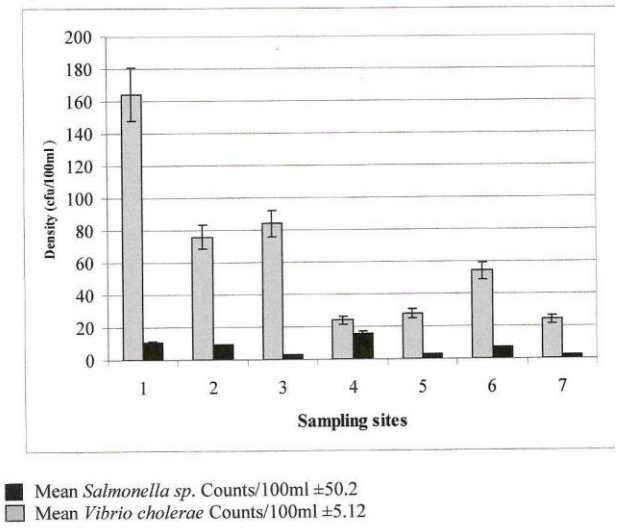

Figure 3: Bar graph showing density of *Salmonella* sp. and *V. cholerae*
Seven sites were sampled and cfu determined at 37 °C and 35 °C respectively.

The biochemical tests gave various results (Table 2). The TSI agar slants inoculated with typical colonies (for hydrogen sulfide and glucose test) had some blackening. There was also formation of gas in peptone water enriched with glucose. Uninoculated controls gave negative results.

There was growth and development of blue colour on the Simmons citrate agar. On the Motility-Indole-Urease (MIU) media, there was the spreading of turbidity from the stabline but no colour change.

Table 2: Biochemical reactions of *Salmonella* isolated from the various ponds of the treatment plant

Test/ Substrate	Results		*Salmonella sp.* reaction
	Positive	Negative	
Glucose(TSI) Peptone water	Yellow butt on TSI	Red butt	+
Enriched with glucose	Gas formed	No gas formation	+
Hydrogen sulfide(TSI)	Blackening	No blackening	+
Urease	No change in colour	Change in colour to pink	-
Simmons citrate	Growth; blue colour	No growth; no colour change	+
Motility	Turbidity	No turbidity	+
Oxidase	Colour change to blue – purple	No colour change	-

The results for biochemical tests of *Salmonella* sp.
Key
+ - positive results
- - negative results

Table 3: Biochemical Reactions of *V. Cholerae* isolated from the various ponds of the treatment plant

TEST/ SUBSTRATE	RESULTS		*Vibrio cholerae*
	Positive	Negative	reaction
Glucose (TSI)	Yellow butt	Red butt	+
Peptone-water enriched with glucose	Gas formation	No gas formation	+
H_2S (TSI)	Blackening	No blackening	-
Urease	Purple-red colour	No colour change	-
Simmons citrate	Growth; blue colour	No growth; no colour change	v
Motility	Turbidity	No turbidity	+
Oxidase test	Blue-purple colour	No colour change	+

Results for biochemical tests of *V. cholerae*
V= variable results got
+ - positive results
- - negative results

For *V. cholerae*, the densities were found to be below those of *Salmonella* sp. and far less in magnitude than the faecal indicators (Table 1). The mean density of *V. cholerae* was 7.29 x 10^2 cfu/100 mL with the effluent

alone having a mean of 2.0x 10^2 cfu/100 mL. The density fluctuated down the treatment process (Figure 3).

The presumptive colonies on the TCBS were yellow, with a diameter of 2-3 mm. Other colonies were green and varied in sizes, just as the yellow ones. The expected characteristic appearance of Gram-negative rods that is comma-shaped was not observed during the Gram reaction.

The subcultured colonies on nutrient agar gave cream colonies. Biochemical tests were carried out on the presumptive colonies (Table 3). On the TSI, the results were negative for H_2S test. The slants were red and the butts yellow. On the Simmons citrate agar, there was growth in all the slants. The presumptive colonies gave positive results for motility test, indicated by the turbidity that spread in the MIU media. Urease test was also negative. For the cytochrome oxidase test, the presumptive colonies were positive as was indicated by the development of blue-purple colour.

DISCUSSION

The aim of the study was to determine the sewage effluent quality and to correlate the two indicators to pathogens and find out which one is best suited to be used as a measure of faecal contamination.

Results showed that the die-off rate of faecal streptococci was low compared to that of the faecal coliforms. The distribution of faecal coliforms appeared to correlate well with *Salmonella* sp. (85%), but with *V. cholerae*, the correlation was 2%. For the faecal streps, the correlation was 75% and 12% with *Salmonella* sp. and *V. cholerae* respectively. The results indicated that both indicator systems can be used to establish the possible presence of *Salmonella* sp. and *V. cholerae.*

The findings are consistent with an earlier finding (Kruize, 1993) whose correlation between faecal streps and *Salmonella* sp. was 57% while it was 51% for the case of the faecal coliforms. Enterococci were found to be better indicators than the faecal coliforms. Considering the results of this study, it is reasonable to conclude that the die-off rate of FC is low compared to the FS and correlate better with the pathogenic microorganisms.

The sewage treatment plant was found not effective. With means of effluent density 7.9 x 10^5 MPN/100 mL, 1.4 x 10^4 cfu/100 mL, 2.4 x 10^3 cfu/100 mL and 2.0 x 10^2 cfu/100 mL for faecal coliforms, fecal streps, *Salmonella* sp. and *Vibrio cholerae* respectively, the effluent cannot even be recommended for general irrigation (WHO, 1998). It is recommended that the FS be ≤ 1000 FS/100 mL (Warrington, 2001), and the FS be ≤ 250/100 mL for the effluent to be used for general irrigation.

This high microbial density may be attributed to poor maintenance. The floating macrophytes such as *Eichhornia crassipes* (water hyacinth) and *Lemna* sp. may be responsible for the poor sewage treatment. These macrophytes have been found to shade out the algae (Mara, 2004), so reducing effluent BOD and suspended solids but reducing disinfection with the result that microbial density will be high. Algal photosynthesis which is supposed to raise the pond pH is minimized because of the floating macrophytes since oxygen is lost directly to the atmosphere and the pond temperature is low because of obstruction. Light mediated-die off is therefore interfered with (Mara *et al.,* 1992).

Of great concern is the presence of *Salmonella* sp. and *V. cholerae* which are easily transmitted through water (Mara *et al.,* 1992). It has to be pointed out that the sewage effluent poses a great risk to farmers using it for irrigation downstream, the farm workers and consumers of vegetables such as cabbages who use the produce to prepare salads.

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