Salmonella contamination of recycled effluent of treated sewage and urban waste water

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Abstract

This study was conducted to determine the effectiveness of the removal of enteric pathogens such as *Salmonella* in recycled sewage effluent and urban waste water after the treatment process at the Harare Firle Sewage Works. A total of 633 samples were collected from raw sewage, tank effluent, filter effluent, humus, sludge, maturation pond, final effluent, river upstream and river downstream at the point of effluent discharge. These samples were processed and colonised on nitrocellulose filters to form dot-blots. The colonised filters were prepared for DNA-DNA hybridisation, using a *Salmonella*-specific DNA probe. When the results were analysed, an overall *Salmonella* contamination frequency of 23.7% was obtained. Further analysis at each stage in the sewage treatment process showed that in raw sewage 65% of the samples were positive for *Salmonella* while 52% of tank effluent and 4% of maturation pond-sample were positive respectively. In filter effluent 7.3% of the samples were positive, while the humus effluent and the sludge samples were 2% and 3.12% positive respectively. In the final effluent discharge 1.8% of the samples were positive for *Salmonella*. Thirty four percent (34%) of samples from the river upstream of effluent discharge and 36% of samples from the river downstream of effluent discharge were positive for *Salmonella*. The bacterial concentrations in the positive samples varied from greater than 1×10^8 cells (++++) per tested sample volume in raw sewage which is higher than the clinically infective dose of 1×10^5 cells, to less than 1×10^2 (+) cells per tested sample volume in the final effluent. This indicated that the sewage treatment process at Firle Sewage Works does not effect total removal of *Salmonella*. Other pathogens, which were not tested, could be higher. The final effluent, which is recycled from the Harare Firle Sewage Works for potable water supply and also used directly in agriculture for irrigation, probably supplies *Salmonella* and other pathogens at a contamination rate of 1.8%. The methods employed in this study provide a rapid, sensitive, specific and cost-effective routine detection procedure for the monitoring of *Salmonella* in environmental samples and waste-water treatment works.

Introduction

In recent years, world-wide changes have led to consideration being given to conservation and recycling of many materials (Carrington, 1980; Hillman, 1988). Not least among these considerations is the possibility of greater reuse of sewage and urban waste water in agriculture, industry, recreation and for potable water supply (Pescod, 1985; Diamant, 1985; Cowen and Johnson, 1985). The strategy of planned reuse of waste water in many tropical developing countries is essential in order to recover water as a valuable and limited resource, but the recycling of sewage and urban waste water has been aggravated by droughts which resulted in a shortage of urban potable water supply. The important question to be raised on the reuse (indirect or direct) of sewage and urban waste water is: how efficient are the treatment processes in removing bacterial pathogens? Domestic sewage is known to carry a full spectrum of mainly enteric pathogenic micro-organisms that cause infections such as enteritis, salmonellosis, shigellosis, cholera and yersiniosis (Cowen and Johnson, 1985). Most of these water- and excreta-related diseases are prevalent in developing countries because of poor sanitation and poor treatment of urban waste water (Lewis-Jones and Winkler, 1991).

In a number of studies done on the status of *Salmonella* in conventional sewage and urban waste-water treatment plants, the incidence of *Salmonella* was found to be reduced by 90% or greater using various treatment methods (Feachem et al., 1983). Treatment by pre-screening had no effect on the pathogen content of sewage influent. Primary sedimentation reduced *Salmonella* by 50 to 90% in 3 to 6 h (Feachem et al., 1983). Trickling filter methods of sewage treatment showed reductions of *Salmonella* in the effluent to be in the range of 71 to 99%, if secondary sedimentation was included. The activated sludge method had between 60 and 99% removal capacity at normal aeration times of 6 to 12 h, but this could be as high as 99.9% following extended aeration for more than 24 h (Feachem et al., 1983). Notwithstanding the above, several studies have shown that sewage effluent treated by various methods may still contain 2% of the pathogenic bacteria originally in the incoming sewage (Lewis-Jones and Winkler, 1991). In developed countries, this percentage of pathogens can be eliminated by tertiary treatment. However, tertiary treatment in conventional sewage treatment is normally absent in most developing countries.

Waite and Finch (1986) found that viable salmonellae were reduced by 99.9% in 1 h at 55°C and by 99.9999% in 11 min at 60°C. Thus, even thermophilic sludge digestion produces sludge that may still retain a considerable population of *Salmonella*. Temperature and retention time of sludge in digesters are the main factors that determine survival of *Salmonella*. It is important to note that 100% elimination of *Salmonella* is unachievable by sludge treatment methods (Lewis-Jones and Winkler, 1991).

In Zimbabwe, and in other developing tropical countries, the reuse of treated sewage and waste water has been recognised as a strategy to boost the productivity of available water supplies (Marks and Lock, 1988). Modern sewage works operating throughout Zimbabwe are based on the modified activated sludge (MAS) sewage treatment process. The cities and towns are able to recycle most of their sewage and urban waste water. Greater

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FIGURE 1

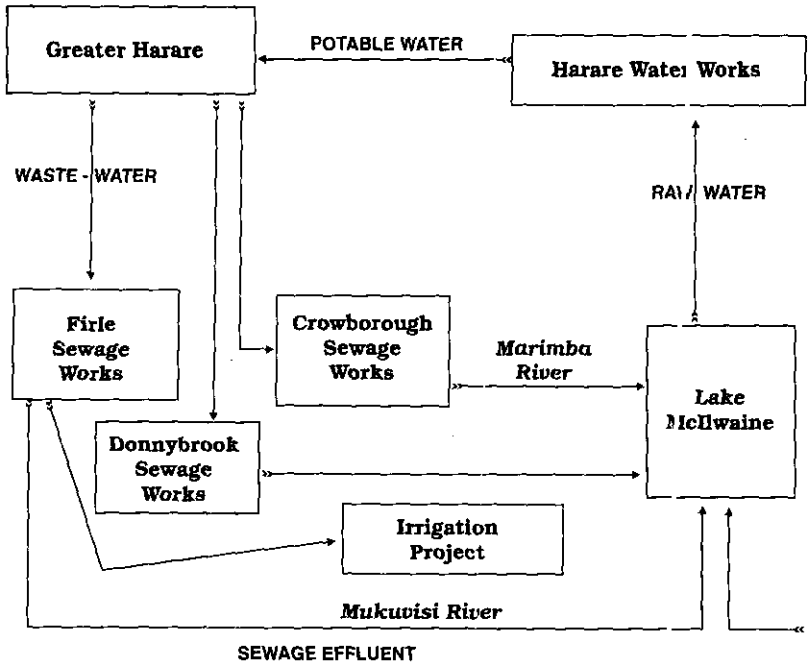

Figure 1

The recycling of treated sewage and urban waste water in Greater Harare. Discharged treated sewage and Urban Wastewater effluents go into Lake McIlwaine, the main supplier of potable water for the City of Harare and its satellite Chitungwiza.

Harare can recycle most of its waste waters directly and indirectly (Fig. 1). Some of the treated sewage effluents from its works are discharged into the Marimba and Mukuvisi Rivers which are the main tributaries of Lake McIlwaine (Chibero), the major potable water supplier to the city (Fig. 1). Investigations made on the incidence of *Salmonella* in raw water of Lake McIlwaine and Darwendale Dam by Gopo et al. (1991), showed 54.35% positive samples for *Salmonella*. The present study was undertaken, using a *Salmonella*-specific DNA probe, to determine the level of *Salmonella* contamination in the recycled water for the city of Harare. The results of this study will be used to decide whether or not the recycled water constitutes a danger to the community as a major source of waterborne diseases such as human salmonellosis. The study was also conducted to determine the effectiveness of the sewage and urban-industrial waste-water treatment process in the elimination of pathogens such as *Salmonella*.

Materials and methods

A *Salmonella*-specific DNA probe (Gopo et al., 1988) was used in this study. Restriction endonuclease HpaI, chemicals and radio isotopes ^{32}p-d-ATP and ^{32}p-d-CTP, were obtained from Amersham, UK, and used according to manufacturer's instructions.

Sample collection

A total of 633 samples were collected between 08:00 and 10:00; and 16:00 to 18:00 according to the method of Ormerod et al., (1982). Samples from raw sewage, tank effluent, filter effluent, humus effluent, sludge and from the maturation pond were collected in 10 mℓ volumes. Samples from the final effluent at the point of effluent discharge into the Mukuvisi River and those from the river upstream above the influent point to the sewage treatment plant, as well as samples from the river downstream beyond the point of effluent discharge, were collected in 100 mℓ volumes.

Sample processing

The 10 mℓ volume samples were filtered through two layers of cheesecloth to remove debris before each whole 10 mℓ filtered sample was used to inoculate 90 mℓ of sterile selenite broth single strength cultures (5.0 g tryptone, 4.0 g lactose, 10.0 g disodium phosphate, 4.0 g of sodium selenite, 0.01 g cystine and 1 000 mℓ distilled water, pH 7.0). The 100 mℓ volume samples were vacuum-filtered through 0.45 µM Sartorium cellulose filters to trap the bacteria. The filter-trapped bacteria were resuspended by vigorously shaking the filters in 10 mℓ of distilled water in centrifuge tubes. The bacteria-free cellulose filters were removed from the centrifuge tubes and the 10 mℓ bacterial suspensions from each sample were used to inoculate 90 mℓ of sterile selenite broth culture. All the inoculated selenite broth cultures were incubated at +37°C for 6 h before further processing. The bacteria in each of the cultures were pelleted by centrifugation at 6 000 r/min in a Beckman bench centrifuge for 30 min. The supernatants were carefully discarded and each resulting bacterial pellet was resuspended in 2 mℓ of distilled water, and transferred to sterile labelled microfuge tubes and centrifuged at 12 000 r/min for 15 min. After carefully discarding the supernatants from each sample, the bacterial pellets were resuspended in 20 µℓ of distilled water. Each whole 20µℓ bacterial suspension, was dot-spotted onto nitrocellulose filter (High Bond C-Amersham), to form dot-blotts for filter colony hybridisation. For bacterial lysis, the colonised filters were transferred, colony side up, onto Whatman 3MM filter papers presoaked in denaturation solution (0.5 N NaOH, 1.5 M NaCl and 0.1% sodium dodecyl sulfate (SDS)) to denature the bacterial chromosomal DNAs. The colonised filters were incubated at room temperature for 30 min before being transferred,

colony side up, to Whatman 3MM paper presoaked in neutralising solution (1.0 M tris-HCl, pH 7.5:, 1.5 M NaCl) and further incubated for 15 min. The filters were air-dried before baking at +80°C for 2 h to filter-bind the denatured bacterial chromosomal DNAs.

Materials

Nick translation experiments

The *Salmonella*-specific DNA probe was prepared for use in DNA-DNA colony hybridisation experiments by labelling it with radio isotopes ^{32}P-d-ATP and ^{32}P-d-CTP, using the Nick translation procedure, with slight modification (Gopo et al. (1991). The reaction mixtures contained 1.0 µg of *Salmonella* DNA probe, 2.5 µℓ of 10x Nick translation buffer (50 mM, $MgCl_2$, 1 mM 2-mercaptoethanol, 500 µg/mℓ bovine serum albumin (BSA), 0.5M tris-HCl, pH 7.5), 2 µℓ 0.2 mM dGTP, 2 µℓ 0.2 mM dTTP, 4 µℓ $^{0.1mM}$P-d-CTP (400 to 2 000 mCi/mMol; 4 µℓ $^{0.1mM}$P-d-ATP (400 to 2 000 mCi/mMol); 1.0 µℓ DNase I (2 mg/mℓ stock solution) stored at -20°C and 1.0 µℓ of DNA polymerase I (5 units) were incubated at +16°C for 2 h. The reactions were terminated by the addition of 25 µℓ of 0.5M EDTA. The unincorporated radio-nucleotides were separated from the radioactively labelled probe DNA by chromatography using G-50 Sephadex mini columns. The columns were prepared by plugging pasteur pipettes with siliconised glass wool and pouring in Sephadex G-50. The columns were equilibrated with 5 bed-volumes of TE buffer (10 mM tris I mM EDTA, pH 8.0). The probe DNA, which is excluded from the Sephadex matrix, eluting ahead of the unincorporated deoxyribonucleotides, was collected in 200 µℓ fractions of T.E buffer. The level of radioactivity was determined using a Cerenchov radioisotope counter. The fractions containing the DNA were pooled and stored at +4°C for DNA-DNA hybridisation.

Hybridisation procedure

Hybridisation experiments were carried out following the methods of Maniatis et al. (1982) with some modification. The baked nitrocellulose filters were prepared for hybridisation by presoaking them in 3 x SSC (0.045 M NaCl, 0.045 M trisodium citrate buffer) for 10 min. The filters were then transferred to hybridisation boxes containing prehybridisation solution (3x SSC, 10% SDS, 5x Denhardt's solution from 50x stock solution [5.0 g Ficol, 5.0 g polyvinylpyrrolidone, 5.0 g bovine serum albumin (pentox Fraction v), and 500 mℓ of distilled water]. The solutions were incubated at +65°C for 4 h. The prehybridisation solutions were removed after the 4 h incubation period and replaced with hybridisation solutions (3x SSC, 0.01M EDTA, and the ^{32}P labelled *Salmonella* DNA probe, which had been denatured by incubation at 100°C for 10 min). The hybridisation mixes in the boxes were incubated at +65°C for 8 h, with gentle shaking in a shaker water-bath. At the end of the hybridisation period, the solutions were removed and stored for further use. The filters were washed three times with low stringency buffer (3x SSC, 0.1% SDS). The levels of radioactivity on the filters, were checked at the end of each wash. A higher stringency wash was carried out in 3x SSC, and 1% SOS, only if the radioactivities were found to be high, reflecting high background radioactivity on the filters. The filters were removed from the hybridisation boxes and air-dried. In order to ensure complete dryness, the air-dried filters were placed between two 3MM filter papers and incubated at +37°C for 1 h. The filters were auto-radiographed by exposing them to an X-ray film in autoradiographic cassettes for 6 to 12 h depending on the strength of the signal. The exposed films were developed, washed and processed for hybridisation analysis.

Probe sensitivity test

In order to estimate the approximate concentration of *Salmonella* bacterial cells on each positive sample, a probe sensitivity test was carried out. *Salmonella typhimurium* was cultured by stab inoculating 10 mℓ of LB broth (10.0 g Bacto-tryptone, 5.0 g Bacto-yeast 10.0 g NaCl and 1 000 mℓ distilled water, pH 7.5) and incubating at +37°C overnight. A maxi-culture was made by transferring 2 mℓ from the mini-culture to 500 mℓ of LB broth. Cell growth was monitored by reading the optical density (OD) at 650 nM hourly until an OD of 0.8 was reached. One mℓ of this culture was removed and a cell count was made using a haemocytometer method, which established the number of cells per mℓ at an OD of 0.8 to be 1 x 10^8. Cell concentrations of 1 x 10^8, 1 x 10^7, 1 x 10^6, 1 x 10^5, 1 x 10^4, 1 x 10^3, 1 x 10^2, and 1 x 10^1 per mℓ were prepared and the OD of each preparation was determined (Fig. 2A and B). Each of these cell preparations was centrifuged in a microfuge at 12 000 r/min for 5 min. The supernatants were discarded and the bacterial pellets were resuspended in 20 µℓ of distilled water. Each whole 20 µℓ bacterial suspension was dot-spotted onto a nitrocellulose filter. The bacterial cells were lysed as above, and processed for DNA-DNA hybridisation as outlined above. These bacterial preparations were also used as positive controls in the experiments.

Results

When 633 samples collected from raw sewage, tank effluent, filter effluent, humus effluent, sludge, maturation pond, final effluent, river downstream and river upstream, were cultured in selenite broth and tested for *Salmonella* presence using a *Salmonella*-specific DNA probe, the results showed an overall contamination rate of 23.7% (Table 1). The following results were obtained for the different stages of the sewage treatment process. In raw sewage 65% of the samples tested, were positive for *Salmonella*, and in tank effluent 52% were positive but only 3.57% of the maturation pond samples were positive. Among the samples collected from

TABLE 1
STATUS OF *SALMONELLA* AT THE VARIOUS STAGES OF THE SEWAGE AND WASTE-WATER TREATMENT PROCESS AT THE HARARE FIRLE SEWAGE TREATMENT PLANT

Sample type	Number tested	Number positive	Per cent positive
1. Raw sewage	91	59	65
2. Tank effluent	78	41	52
3. Filter effluent	75	5	7.3
4. Humus effluent	50	1	2.0
5. Maturation pond	56	2	3.6
6. Sludge	64	2	3.1
7. Final effluent	110	2	1.8
8. River upstream	48	16	34
9. River downstream	61	22	36
Total	633	150	23.69

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