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Full Length Research Paper

Genetic diversity and *in vitro* antibiotic susceptibility profile of *Salmonella* species isolated from domestic water and wastewater sources in the Eastern Cape Province of South Africa

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We assessed the genetic diversity of forty *Salmonella* isolates obtained from selected domestic water and waste water sources in the Eastern Cape Province of South Africa using DNA fingerprinting and antibiotic susceptibility profile as test indices. Restriction digests and SDS/PAGE as well as the DNA dendograms of the isolates revealed that most of these strains show a high percentage of genetic similarity, and suggests that these methods are valuable tools for evaluating the relatedness of *Salmonella* species. Also, of the seven antibiotics and sulfonamides tested against the *Salmonella* species, five namely, neomycin, chloramphenicol, kanamycin, streptomycin and cotriomoxazole were significantly inhibitory, while the bacteria showed considerable resistance to doxycycline and sulfomethoxazole.

Key words: Genetic diversity, *Salmonella*, water, antibiotic susceptibility.

INTRODUCTION

South Africa is a water-scarce country and water borne diseases have proven to be a prime threat to human health in the country as in other parts of the world (DWAF, 1994) with cholera, dysentery and typhoid as the leading causes of morbidity and mortality (WHO, 2003). *Salmonella* is one of the most important water transmitted bacteria because of the severe diarrhea and typhoid fever they cause with human and animal excreta acting as sources of the pathogen. These species cause a variety of infections in humans and domestic animals, ranging from mild food poisoning such as gastroenteritis caused by *Salmonella* t*yphimurium* to a severe systemic disease known as typhoid caused by *Salmonella typhi* (Baudart et al., 2000). Typhoid fever causes over 20 million cases annually with at least 700, 000 deaths (Cooke and Wain, 2004).

Most studies have focused on the determination of *Salmonella* strain concentrations in some polluted areas (Baudart et al., 2000). However, information on the diversity and occurrence of *Salmonella* strains is scarce and as a consequence, the ecology of these species remains poorly understood. This is partly due to the laborious methods required for the detection, isolation and identification of *Salmonella* strains (Baudart et al., 2000).

Rapid analysis of diversity of complex microbial communities has remained an elusive but important goal in microbial ecology. One of the methods of examining bacterial diversity involves DNA amplification by PCR followed by restriction digestion of amplified samples to identify differences in the genomes of species. More refined approaches reveal differences not only in community composition but also in community organization by measuring the number (richness) and relative abundance (structure or evenness) of species (Dunbar et al., 2000).

The emergence of antibiotic resistant bacterial pathogens has become a major public health concern (Cheng et al., 2004). Conventional antimicrobial agents, such as ampicillin, chloramphenicol and trimethoprim-sulfamethoxazole have been the drugs of choice in the treatment of salmonellosis before 1980 (Cheng et al., 2004). However,

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multidrug resistance with rates of resistance to these antimicrobial agents of more than 50%, has been reported in many areas of the world. Extended-spectrum cephalosporins and fluoroquinolones have been suggested as alternative agents in the treatment of infections caused by multidrug resistant *Salmonella* serotypes. However since 1991, cases of infections caused by *Salmonella* serotypes resistant to extended-spectrum cephalosporins or fluoroquinolones have been increasingly reported (Cheng et al., 2004). In this paper, we report the diversity of *Salmonell*a isolates obtained from selected drinking water and waste water sources in the Eastern Cape Province of South Africa as well as their antibiotic susceptibility pattern.

MATERIALS AND METHODS

Sampling

Waste water samples were collected using sterile 1 L Nalgene bottles from Fort Hare waste water treatment plant in Alice, Amalinda waste water treatment plant in East London and Schornville waste water treatment plant in King Williams Town. Also domestic water samples (1 L) were collected from Gogogo and Tyume rivers. The samples were transported on ice in cooler boxes to the laboratory of the Department of Biochemistry and Microbiology, University of Fort Hare, Alice for further processing.

Isolation and identification of *Salmonella* species

500 ml of water samples were centrifuged in sterile 50 ml centrifuge tubes at 3500 ×g for 25 min using the Beckman type centrifuge (TJ-6 Centrifuge Scotland). The supernatants were discarded and the pellets were resuspended in 20 µl of sterile normal saline. The cell suspensions were used for *Salmonella* species isolation. The suspensions were enriched in tetrathionate broth (Merck, Darmstardt, Germany) in a 1:1 ratio (sample: volume) and incubated for 18 - 24 h at 35°C on an orbital incubator (Stuart Scientific U.K.). At the end of the incubation period, a loopful of the enriched culture was transferred onto XLD agar (Biolab Diagnostics, Saarchem, S.A) and incubated at 35°C for 18 - 24 h. Red colonies with black spots in the center that appeared on the agar were randomly picked, purified and gram stained. The isolates were further confirmed as *Salmonella* species using API 20E test kit (Bio Merieux, Lyon, France). Forty confirmed *Salmonella* isolates were selected for further studies.

Antimicrobial susceptibility test of isolates

The susceptibility of the *Salmonella* isolates to antimicrobial agents was determined using the Kirby-Bauer disk diffusion method (Bauer et al., 1996). A bacterial lawn was prepared by transferring a bacterial colony in 2.5 ml normal saline using a sterile inoculating loop. The suspension was vortexed and 100 µl of it was spread onto the Muller-Hinton agar (Biolab Diagnostics, Saarchem, S.A). The excess inoculum was siphoned with Pasteur pipettes. Plates were allowed to dry at room temperature in a laminar flow. Disks containing predetermined amounts of antibiotics were dispensed onto the bacterial lawn and the plates were incubated at 37°C for 18 - 24 h. After the incubation period, the diameters of the inhibition zones were measured and interpreted as described by NCCLS (1999). The following antibiotics and sulfonamides were used: neomycin (10 µg); chloramphenicol (30 µg); doxycycline (30 µg); sulphonamides (50 µg); kanamycin (30 µg); streptomycin (300 µg); and cotrimoxazole (25 µg). The test was done in triplicates. The recorded zones of inhibition were interpreted in accordance with the description of the United States National Committee for Clinical Laboratory Standards (NCCLS) (Bauer et al., 1996) now known as CLSI (Clinical and Laboratory Standard Institute).

Isolation of DNA and restriction digestion analysis

Genomic DNA from forty *Salmonella* isolates cultured in a nutrient broth was isolated as described by Neumann et al. *(*1992). Cells were harvested by centrifugation at 1000 ×g for 20 min, using a JA20 angle rotor (Beckman centrifuge). The pellets were resuspended in 400 µl Tris-EDTA (TE) buffer, containing 0.01M Tris-HCl pH 7.4, 0.001M EDTA, (Saarchem, Gauteng, S.A) by vortexing. 50 µl of 10% Sodium Dodecyl Sulphate, SDS (Saarchem, Gauteng, S.A) was added to the suspensions. Samples were digested with 50 µl (20 mg/ml stock solution) proteinase K (Merck, Darmtadt, Germany) for 1 h at 37°C. DNA was extracted twice with equal volumes of phenol: chloroform (1:1) (Merck, Darmstadt, Germany) by centrifugation at 1000 ×g for 1 min and re-extracted with equal volume of chloroform followed by centrifugation at 1000 ×g for 10 min. The supernatants were treated with 5 µl RNase A (5 $mgml^{-1}$ in RNaseA buffer containing 0.5M NaCl, 0.01M EDTA) and the samples were incubated at 37°C for 30 min. DNA was precipitated with 5 M ammonium acetate (final concentration of 0.5 M) and 2 volumes of isopropanol by incubation at 4°C overnight. The pellets were centrifuged for 10 min at 10 000 ×g and washed twice with 70% ethanol. The pellets of DNA were dried at room temperature and reconstituted in 50 - 100 µl TE buffer.

2 - 5 µg of the DNA samples were digested with 20 - 50 units of each of the restriction endunucleases Xba1 and Nde1, and Bst Z1 (Promega, Madison. USA) in the presence of buffer D (Promega, Madison, USA) in a final volume of 20 µl. The digestion of the DNA samples was allowed to proceed at 37°C for at least 3 h. The digested DNA samples were separated on 5% Acrylamide/Bisacrylamide (30/0.8% w/v stock solution) (Merck, Darmstadt, Germany) gel containing 0.04 M Tris acetate and 0.002 M EDTA pH 8. The gels were run at 90 mV/30 mA for $5\ ^{1}/_{2}$ h. When the bromophenolblue dye reached the end of the gel, the electrophoresis was stopped. The gels were stained with silver sequence DNA staining kit (Promega, Madison, USA). Images of the gels were taken using Biodoc-IT System with a built-in CCD Camera (Transilluminator, UVP, Upland, CA). Dendograms were constructed using Jackard simple matching coefficient (found in the vegan package) as the input into the UPGMA clustering technique in the R statistical computing environment (RDCT, 2005).

RESULTS

Antibiotic sensitivity test of *Salmonella* species

Antibiotic sensitivity test was performed on 40 purified and confirmed *Salmonella* strains. 90% of the isolates were susceptible to neomycin while 10% were intermediate (Table 1). Also, 72.5% of the isolates were susceptible to chloramphenicol while 22.5% were intermediate and 5% were resistant. Against doxycycline, 57.5% of isolates were resistant. Also 92.5% of the isolates were resistant to sulphonamide while 90 and 85% were susceptible to kanamycin and streptomycin/ cotrimoxazole respectively.

Table 1. The effect of tested antimicrobials on *Salmonella* species.

Antibiotic	Antibiotic concentration on the disc in Mg	Antibiotic sensitivity (percentages)		
		Resistant	Intermediate	Susceptible
Neomycin	0.03	0	10	90
Chloramphenicol	0.01	5	22.5	72.5
Doxycycline	0.03	57.5	37.5	5
Sulfonamides	0.05	92.5	7.5	0
Kanamycin	0.03	5	5	90
Streptomycin	0.3	0	15	85
Cotrimoxazole	0.025	5	10	85

Table 2. Distribution of DNA groups according to the similarity coefficient (S_{sm}) based on restriction digest profile.

Group 1(80 - 93%)	Group 2 (60 - 69%)	Group 3 (42 - 51%)	Group 4 (21 - 35%)
4, 5, 6, 7,8, 9,10, 11,12,13 14, 15, 16, 24,25 26, 27, 30, 31,32 33, 36, 37, 38,39 40	1, 21, 22, 23, 28	18, 20, 29, 34, 35	2, 3, 17, 19

DNA profile of the *Salmonella* strains

Restriction digestion of genomic DNA of the 40 isolates with EcoR1 and Sma1 revealed different band patterns ranging in size from 500 to 12000 bp (Figure 1). The dendogram obtained (Figure 2) revealed four similarity groups of strains (Table 2). Group 1 (Table 2) comprises 65% of all isolates and includes strains 5, 6, 11, 12, 13 and 15 recovered from Amalinda waste water plants' sludge tank; strains 26, 32, 36, 37, 38, 39 and 40 isolated from Amalinda waste water plant's sludge tank; strains 9, 24, 27, 31, 33 isolated from Shornville waste water plat's sludge tank; strains 7, 14, 16 and 30 recovered from Shornville waste water plant's secondary clarifiers; strain 25 recovered from Fort Hare waste water plant; strains 4 and 8 recovered from Gogogo river and strain 10 recovered from Tyume river. All 26 strains clustered as a tight and distinct group with genetic similarity ranging from 80 to 93%. They formed a well defined group, clearly separated from the rest of the isolates.

The second similarity group comprised the following strains 1, 22, and 23 isolated from Shornville waste water plant's sludge tank; strain 21 isolated from Shornville waste water plant's secondary clarifier; and strain 28 isolated from Tyume river. The genetic similarity values of the five strains ranged between 60 and 69%. The third similarity group included strains with similarity values ranging from 42 to 51%. These include strains 29 and 35 recovered from Amalinda waste water plant's secondary clarifier; strain 20 recovered from Amalinda waste water plant's sludge tank; strain 34 recovered from Shornville waste water plant's sludge tank; and strain 18 recovered from Shornville waste water plant's secondary clarifier (Table 3).

The fourth similarity group comprised a tight and distinct cluster with similarity values ranging from 21 to 35% and includes strains 2 and 19 isolated from Amalinda waste water plant's sludge tank; and strains 3 and 17 isolated from Fort Hare waste water plant's sludge tank. They formed a small distinct group (Figures 1 and 2)

DISCUSSION

Our results on antibiotic susceptibility of *Salmonella* strains indicate that out of seven antibiotics and sulfonamides tested on the forty *Salmonella* species, namely: neomycin, chloramphenicol, doxycycline, sullfamethoxazole, kanamycin, streptomycin and cotrimoxazole, five proved to have a substantial effect on these *Salmonella* strains. Comparable observation was reported by Thong and co-workers (2002) who demonstrated the sensitivity of *Salmonella* isolates towards chloramphenicol, neomycin, kanamycin streptomycin and cotrimoxazole. These results agree with that of Obi et al. (2007), who reported similar patterns of sensitivity of *Salmonella* isolates from HIV/AIDS patients in Limpompo, South Africa towards kanamycin. The introduction of antibiotics for the chemotherapy of bacterial infection has been one of the most important medical breakthroughs since their discovery. However, the emergence of bacterial resistance to antibiotics undermines the therapeutic utility of existing agents and thereby necessitating more research in this field. Towards doxycycline and sulphamethoxa-

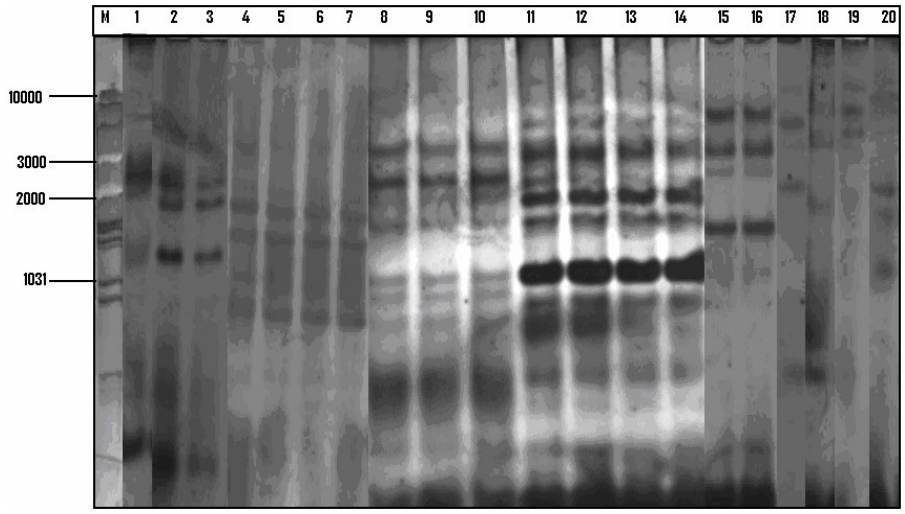

Figure 1. Lanes of digested DNA samples on the gel (Lanes 1-20).

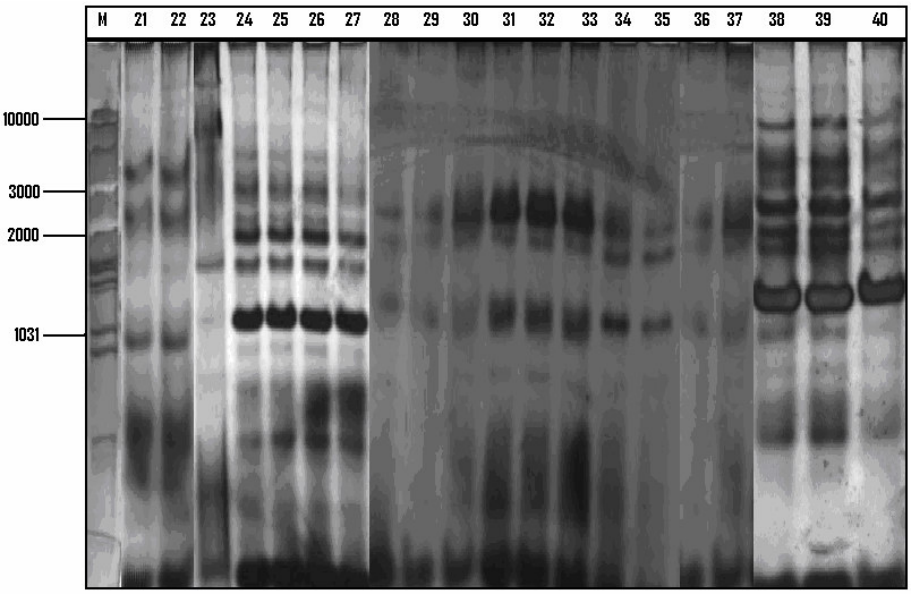

Figure 1 (continuation). Lanes of DNA samples on the gel (Lanes 21-40).

zole, the *Salmonella* strains showed considerable resistance of 57.5 and 92.5% respectively. This indicates that the two drugs can hardly be considered for the treatment of *Salmonella* infections as compared to neomycin, chloramphenicol, kanamycin, streptomycin and cotrimoxazole.

Using restriction digestions of genomic DNA, followed by SDS/PAGE electrophoresis and construction of dendograms, four similarity groups of *Salmonella* strains were identified based on their DNA profiles with different base pair lengths. DNA profiles revealed four similarity groups showing 18 different bands with base pairs length varying from 500 to 12,000 bp. Most of the strains (approximately 65%) recovered from the Amalinda, Shornville and Fort Hare waste water plants, Gogogo and Tyume rivers showed a high percentage of genetic similarity ranging from 80 to 93%. The genetic variability of the strains between group 1 and the rest three groups ranging from 21 to 69% suggests a high degree of genomic rearrangements that could be associated with the acquisition of mobile genetic elements, insertion sequences and mutations, resulting from the different environmental conditions of the water sources from where the strains have been recovered, as well as the environment of the areas whose waste water were collected in the Amalinda, Shornville and Fort Hare waste water

Table 3. *Salmonella* isolates sources representations on SDS-PAGE gels.

Water sources	Lanes on agarose and SDS-PAGE gels
Amalinda waste water plant (sludge tank)	
Site 1	Lane 2
Site 2	Lane 5
Site 3	Lane 6
Site 4	Lane 11
Site 5	Lane 12
Site 6	Lane 13
Site 7	Lane 15
Site 8	Lane 19
Site 9	Lane 20
Amalinda waste water plant (secondary clarifier)	
Site1	Lane 26
Site 2	Lane 29
Site 3	Lane 32
Site 4	Lane 35
Site 5	Lane 36
Site 6	Lane 37
Site 7	Lane 38
Site 8	Lane 39
Site 9	Lane40
Shornville waste water plant (sludge tank)	
Site 1	Lane 9
Site 2	Lane 22
Site 3	Lane 23
Site 4	Lane 24
Site 5	Lane 27
Site 6	Lane 31
Site 7	Lane 33
Site 8	Lane 34
Site 9	Lane 1
Shornville waste water plant (secondary clarifier)	
Site 1	Lane 7
Site 2	Lane 14
Site 3	Lane 16
Site 4	Lane 18
Site 5	Lane 21
Site6	Lane 30
Gogogo river	
Site 1	Lane 8
Site 2	Lane 4
Tyume river	
Site1	Lane 10
Site 2	Lane 28
Fort Hare waste water plant	
Site 1	Lane 3
Site 2	Lane 17
Site 3	Lane 25

plants. Ribeiro and co-workers (2007) used PFGE for studying genetic diversity of *Salmonella* strains isolated from salami. They observed low genetic similarity amongst strains indicating that the strains could be from

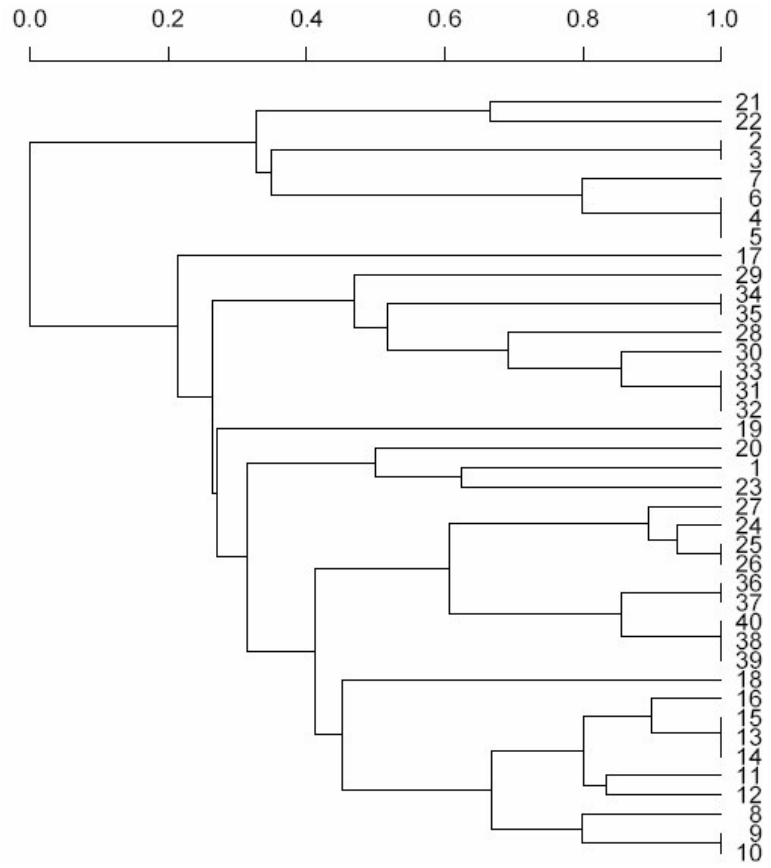

Figure 2. The DNA dendograms obtained for samples 1-40.

different sources. Thong and co-workers (2002) reported that the DNA profiles of most of the *Salmonella* serotype (Weltevreden isolates) from various hospitals varied greatly indicating that the isolates belong to different serogroups.

Conclusion

Antibiotics susceptibility test revealed that the forty *Salmonella* isolates are susceptible to five antibiotics and significantly resistant to 2 antibiotics which is of clinical importance in the treatment of *Salmonella* outbreaks. Also, the restriction digest pattern, SDS/PAGE and dendogram construction shows that there is a high similarity between the forty *Salmonella* strains studied. 65% of the strains based on DNA show similarity co-efficient in the range of 80 – 93% which indicates a high genetic relatedness between the strains investigated.

REFERENCES

Baudart J, Lemarchand K, Brisabois A, Lebaron P (2000). Diversity of *Salmonella* strains isolated from the aquatic environment as determined by serotyping and amplification of the ribosomal DNA spacer regions. Appl. Environ. Microb. 66: 1015-1021.

Bauer AW, Kirby WM, Sherris JC, Turek M (1996). Antibiotic susceptibility testing by a standardized single disk method. Am. J. Clin. Pathol. 45: 493-498.

Cheng S, Zhao S, White DG, Schroeder CM, Lu R, Yang H, McDermott PF, Ayers S, Meng J (2004). Characterization of multiple anti-microbial resistant *Salmonella* serovars isolated from retail meats. Appl. Environ. Microbiol. 70(1): 1-7.

Cooke FJ, Wain J (2004). The emergence of antibiotic resistance in typhoid fever, ScienceDirect-Travel Medicine 2: 67-74.

Dunbar J, Ticknor LO, Kuske RC (2000). Assessment of microbial diversity in four Southwestern United States soils by 16S rRNA gene terminal restriction fragment analysis. Appl. Environ. Microbiol. 66(7): 2943-2950.

DWAF (1994). White paper on water supply and sanitation policy. Department of Water Affairs and Forestry.

National Committee for Clinical Laboratory Standards (NCCLS) (1999). Performance standards for antimicrobial disc susceptibility test 4th ed. Approved document M2-A4 Villanova Pa.

Neumann B, Pospiech A, Shairer HU (1992). Rapid isolation of genomic DNA from gram-negative bacteria, PMID 8(10): 332-333.

Obi CL, Ramalivhana J, Momba MNB, Igumbor J (2007). Scope and frequency of enteric bacterial pathogens isolated from HIV/AIDS patients and their household drinking water in Limpopo Province. Water SA, 33(4): 539-548.

RDCT (2005). (R Development Core Team): a language and environment for statistical computing, version 2.2.0. R Foundation for Statistical Computing.

Ribeiro VB, Andrighet C, Bersot LS, Barcellos V, Reis ER, Destro MT (2007). Serological and genetic diversity amongst *Salmonella* strains isolated in a salami processing line. Braz. J. Microbiol. 38(1): 178-182.

Thong KL, Goh YL, Radu S, Noorzaleha S, Yasin R, Koh YT, Lim VKE,

Rusul G, Puthucheary SD (2002). Genetic diversity of clinical and environmental strains of *Salmonella entrica* serotype Weltevreden isolated in Malyasia. J. Clin. Microbiol. 40: 2498-2503.
WHO (2003). Emerging issues in water and infectious disease. World Health Organization, Geneva, Switzerland.