

Genetic characterization of *Salmonella* and *Shigella* spp. isolates recovered from water and riverbed sediment of the Apies River, South Africa

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ABSTRACT

Riverbed sediment is a vital component of river ecosystems and plays an important role in many geomorphological and ecological processes. However, when re-suspension occurs, pathogenic bacteria associated with sediment particles may be released into the water column, thus creating a health risk to those who use such water for drinking, household and recreational purposes. The aim of this study was to investigate the presence of bacterial pathogens *Salmonella* spp. and *Shigella* spp. in the Apies River and to ascertain whether there was any level of genetic relatedness between river water and riverbed sediment isolates of these pathogenic bacteria. A total of 124 water and sediment samples were collected from a site located on the Apies Rivers upstream of the Daspoort Wastewater Treatment Works, Pretoria, Gauteng, South Africa, between August and November 2014. In order to detect and identify the target bacteria, samples were analysed by culture-dependent and culture-independent techniques (quantitative real-time PCR). Genetic relatedness was established using Sanger sequencing of the *invA* gene of *Salmonella* spp. and *ipaH* of *Shigella* spp. Results of this study displayed the presence of the target bacteria both in the water and sediment of the river. The phylogenetic tree of *Salmonella* spp. revealed a $\geq 99\%$ and 99% genetic relatedness between river water and riverbed sediment isolates for *Salmonella* spp. and *Shigella* spp., respectively. The degree of genetic relatedness between sediment and water pathogen isolates suggests that these organisms could possibly have a common origin and that there could be possible movement of microorganisms between the water column and the sediments.

Keywords: *Salmonella* spp., *Shigella* spp., river water, riverbed sediment, genetic relatedness

INTRODUCTION

Many waterborne pandemics are associated with *Shigella* spp. and *Salmonella* spp. (Threlfall, 2002). The genus *Shigella* contains four species, namely *S. flexneri*, *S. sonnei*, *S. boydii* and *S. dysenteriae*. All four species are responsible for shigellosis or bacillary dysentery, a disease that causes high fever, neurological disturbances and mucus-pyo-hemorrhagic dysentery (Sansone, 2001). The global burden of shigellosis has been estimated to be 150 million cases, with 1 million deaths per year recorded in developing countries (Parsot, 2005). *Shigella* spp. are normally found in water polluted with human excrement (Saha et al., 2009). The presence of *Shigella* spp. in drinking water indicates human faecal contamination. This bacterium is of fundamental public health significance because of its great pathogenicity. Outbreaks of shigellosis have been associated with water treatment failures (at times inefficient treatment) in water supply systems (Karanis et al., 2007). Such waterborne outbreaks often lead to a considerable number of individuals being simultaneously affected, and in most cases the outbreak subsides when the water supply is adequately treated (Pillsbury, 2010).

The genus *Salmonella* consists of two species, namely *S. enterica* and *S. bongori*, each of which contains multiple serotypes. Most of the disease-causing serovars are from Subspecies I, with the most important serovars in human

health being Typhimurium and Typhi (Lan et al., 2009).

Typhoid fever is recognised as a devastating disease in several regions in Asia, Africa and South America, while the disease is rare in developed countries. In 2000, the global burden was estimated to be more than 21 million cases, with up to 200 000 deaths (Crump et al., 2004). Enteric fever is widespread in poor nations, affecting around 12.5 million people yearly (CDC, 2005). Waterborne illnesses associated with *Salmonella* spp. are more commonly due to increased faecal pollution of water bodies (Levantesi et al., 2012). In South Africa, the MDG (Millennium Development Goals) 2013 report pointed out that some 3–5 million people still depend on untreated water from rivers and springs (UN, 2013). An epidemiological study by Niehaus et al. (2011), following an outbreak of food-borne salmonellosis after a school function in Durban, KwaZulu-Natal, reported that *Salmonella enteritidis* isolated from patients and food samples could not be distinguished phenotypically and genotypically. The authors suggested a point-source as the origin of the outbreak, with a possibility of continued transmission through the water supply.

Several studies have reported on the occurrence of *Salmonella* spp. (Tobias and Heinemeyer, 1994; Touron et al., 2005) and *Shigella* spp. (Baums et al., 2007; Skariyachan et al., 2015; Xiong et al., 2015) in different aquatic sediments, even when these were not isolated in the overlying water. A study by

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Baudart et al. (2000) showed that *Salmonella* trapped in sediment particles accumulated in the riverbed during low water levels downstream of a river course and were re-suspended during storm events. In another study, the loads of *Salmonella* spp. were found to be high in water resources during severe or frequent disturbance events (Walters et al., 2007). The presence of these pathogenic bacteria of faecal origin poses severe threats to environmental and human health, particularly when sediments go through natural or human-induced re-suspension, resulting in them being transported toward downstream areas (Chapman, 2013). The presence of pathogenic bacteria in riverbed sediments highlights the need to investigate the similarity between river sediment and water isolates at genetic level to better understand the microbial dynamics between these two matrices.

Real-time quantitative PCR (qPCR) has been used in monitoring studies to compare the data acquired by culture-based techniques, which have been generally used for evaluation of bacterial pathogens in environmental samples (Mackay, 2004; Robertson and Nicholson, 2005; Kaushik and Balasubramanian, 2012; Yamahara et al., 2012; Garrido et al., 2013). These molecular methods, when applied to DNA purified from environmental samples, permit the enumeration of pathogenic microorganisms even when dead or in a viable, but not yet cultivable, state (Noble and Weisberg, 2005; Lee et al., 2006; Rantsiou et al., 2013). Genetic approaches also provide additional information such as pathogenicity, especially if the genes targeted are those associated with the disease-causing abilities of the organism (Pathak et al., 2012). As a result, qPCR has been used to enumerate bacteria in different sample types, including food (Elizakvíel et al., 2012), soil (Prévost-Bouré et al., 2011), wastewater (Sidhu et al., 2013) and marine sediments (Luna et al., 2012; Schippers et al., 2012). Despite these numerous applications, the use of qPCR in riverbed sediment studies is still in its early stages. Sediments may be problematic when it comes to the effective utilization of the qPCR methods due to the presence of inhibitory substances co-extracted with DNA, which can hinder the polymerase chain response, hence reducing the capacity to amplify target DNA (Vezzulli et al., 2009; Sidstedt et al., 2015).

The aim of the present study was to establish the genetic relatedness between *Salmonella* spp. and *Shigella* spp. isolated from water and those isolated from riverbed sediments of the Apies River using culture-based and culture-independent methods in a bid to understand genetic similarity among isolates of water and sediments.

MATERIALS AND METHODS

Study area

The Apies River and its tributaries have previously been described by Abia et al. (2015b). Briefly, this river is situated in the Gauteng Province of South Africa, and flows through the city of Pretoria. It falls within the Crocodile (West) Marico Water Management Area, within the Apies River basin. The Apies rises in the Fountains Valley in Pretoria and flows through Gauteng, North-West and Limpopo, where it ultimately joins the Pienaars River to the north of Hammanskraal (Tuwani, 2011). The Apies River has a total flow of approximately 500 m³/year, about 12% of which originates from wastewater treatment works around the river, such as Daspoort, Rooiwal and Mogwase Wastewater Treatment Plants (WWTPs) (Venter, 2007). For the purpose of this study, the sampling point was upstream of the Daspoort Wastewater

Treatment Works (WWTW) effluent discharge point where the river is channelled through Pretoria Central and the National Zoological Garden of Pretoria.

Sample collection

Water and riverbed sediment samples were concurrently collected on a weekly basis from August to November, 2014 from the sampling point located in the Apies River (immediately upstream of the Daspoort WWTP discharge point), resulting in a total of 62 water and 62 sediment samples. Water and sediment samples were collected using 1 L sterile containers as previously described by Abia et al. (2015b). Samples were transported to the laboratory in cooler boxes containing ice and analyses were performed within 3 h of the time of collection.

Culture-based enumeration of *Salmonella* spp. and *Shigella* spp.

For the culture-based isolation and enumeration of *Salmonella* spp. and *Shigella* spp., collected sediment samples were tested using the membrane filtration (MF) methods according to the procedure described in APHA (2001). Prior to the isolation and enumeration of these target pathogens, attached bacteria were dislodged from the sediments using the method developed by Abia et al. (2015a). Briefly, sediment samples were gradually transferred to a graduated 1 L Durham bottle containing 900 mL of 1 × PBS until the 1 000 mL mark was reached, to obtain a 10% dilution (v/v). Thereafter the suspension was vigorously shaken manually for 2 min as described by Abia et al. (2015a), who point out that the water-displacement method is important to dislodge attached microorganisms from the sediment matrix. In addition, use of this method does not influence the growth of target bacteria in the subsequent membrane-filtration step. 100 mL aliquots, along with 10-fold serial dilutions of the resulting phosphate buffer solution, were then analysed using the membrane filtration technique according to standard methods (US EPA, 2002). Two 0.45 µm filters were used; one was placed onto xylose lysine deoxycholate agar (XLD) (Biorad, South Africa) and/or *Salmonella-Shigella* agar (SS) (Merck, South Africa) and incubated at a temperature of 37°C for 24 h under aerobic conditions, and the other was preserved in 1 mL of 15% glycerol at 0°C for genetic analysis. Since the desired counts required were of the actual water and sediment samples, no enrichment step was needed to enhance and multiply the initial bacterial counts, as also performed by Wolffs et al. (2006) and Marathe et al. (2012). The abundance of cultured *Salmonella* spp. and *Shigella* spp. was recorded as colony-forming units (CFU) per 100 mL of water or sediment. Colonies were counted based on their morphological features. The culture-based method was used for presumptive enumeration only. No isolate from this method was used for genetic analysis.

Total bacterial DNA extraction

The preserved filters were thawed and centrifuged for 1 min at 12 000 r/min; then total bacterial DNA from pellets was extracted using InstaGene matrix (BioRad, South Africa) for water samples and ZR Soil Microbe DNA MicroPrep (Zymo Research, USA) for riverbed sediment samples using their respective manufacturer's instructions. The quality and

quantity of the isolated DNA was determined by means of the NanoDrop 2000 spectrophotometer (Thermo Scientific, South Africa).

Standard curve

Standard curves converting cycle threshold (C_t) values to bacterial *invA* and *ipaH* gene copy numbers were generated as follows: a double-stranded DNA oligomer gBLOCKS was synthesized (Integrated DNA Technologies, Inc., USA) to span the region of the *invA* and *ipaH* gene covered by the forward and reverse qPCR primers (Table 2) as listed in Table 1. The lyophilized gBLOCKS were reconstituted to prepare a 20 nM gBLOCKS stock solution that was 10-fold serially diluted and used in the range of 1 ng to 10 fg per reaction (Gunawardana et al., 2014) to produce 10 standards spanning the $20-2.0 \times 10^{-8}$ nM concentration range ($6.8 \times 10^{10}-68$ for *Salmonella* spp. and $7.4 \times 10^{11}-74$ *Shigella* spp. copy numbers). The standard curves were always performed in duplicate. A graph of C_t values versus \log_{10} (copy number) afforded linear calibration curves with typical R^2 values of 0.981 for *Salmonella* spp. and 0.998 for *Shigella* spp.

Enumeration of total *Salmonella* spp. and *Shigella* spp. by qPCR

The qPCR assays were applied for the determination of the total abundance of *Shigella* spp. and *Salmonella* spp. in both river water and riverbed sediment samples. For each type of sample, a volume of 100 mL was filtered through 0.45 μ m nitrocellulose

membrane filters. The membrane filters were then placed in a cuvette containing 1.5 mL of phosphate buffer. The cuvette was then vortexed for 10 min to dislodge bacteria from the membrane for DNA extraction. Bacterial DNA from water and riverbed sediments was extracted using commercial kits InstaGene Matrix (Bio-Rad, South Africa) and ZR Soil Microbe DNA MiniPrep kit (Zymo Research, USA), respectively. The extracted DNA was quantified using the NanoDrop 2000 spectrophotometer (Thermo Scientific, South Africa). For the purpose of qPCR, primers (synthesized by Inqaba Biotec, South Africa), listed in Table 2, were used where *ipaH* targeted the invasive plasmid antigen of *Shigella* spp. and *invA* targeted the invasive gene of *Salmonella* spp. The *ipaH* target gene is carried by all *Shigella* spp. as well as by enteroinvasive *Escherichia coli* (*E. coli*) (EIEC); thus it is used for the diagnosis of dysentery (Sethabutr, 1993). The *invA* target gene is located on the *Salmonella* pathogenicity island 1 (SPI1), which encodes proteins of a type III secretion system (Malorny et al., 2003). Reactions were run using SsoFast EvaGreen Supermix on the CFX96 Touch real-time PCR detection system (Bio-Rad, South Africa). All reactions were run in a total volume of 20 μ L, containing 10 μ L of Supermix, 1 μ L of each primer (final concentration 1 μ M), 5 μ L of template DNA and 3 μ L nuclease-free (NF) water (Fermentas, Germany). The qPCR conditions were optimised in terms of the following thermal cycling parameters: 98°C for 2 min for the enzyme activation, followed by 40 amplification cycles of denaturation at 98°C for 5 s, annealing of primers with the genomic DNA (gDNA) template at 60°C for *Salmonella* spp. and 59°C for *Shigella* spp., and a primer extension at 72°C for 5 s followed by melt curve

TABLE 1
gBLOCKS oligomer for internal control and standard curve generation

Gene	Sequence	Length	GC content	fmol/ng
<i>invA</i>	GGTGAAATTATCGCCACGTTTCGGGCAATTCGTT ATTGGCGATAGCCTGGCGGTGGGTTTTGTGTGTC TTCTCTATTGTCACCGTGGTCCAGTTTATCGTTAT TACCAAAGGTTTCAGAACGTGTCGCGGAAGTCG CGGCCCGATTTTCTCTGGATGGTATGCCCGGTA AACAGATGAGTATTGATGCCGATTTGAAGCCG GTATTATTGATGCGGATGCCGCGCGGAACGGC GAAGCGTACTGGAAAGGGAAAGCCAGCTTTAC GGTTCCTTTGACGGTGCGATGAAG	288	51.39%	5.62
<i>ipaH</i>	GGCCCGCAGATTTACTTCTCCATGAGTGACGGA CAACAGAATACACTCCATCGCCCCCTGGCTGAT GCCGTGACAGCATGGTTCCCGGAAAACAAACA ATCTGATGTATCACAGATATGGCATGCTTTTGAA CATGAAGAGCATGCCAACACCTTTTCCGC	161	49.69%	10.06

TABLE 2
Oligonucleotide primers used in qPCR assay

Designation	Sequence	T_m (°C)	Product size	Reference
invA139F ^a	GTGAAATTATCGCCACGTTTCGGGCAA	61.8	284	Rahn et al. (1992)
invA141R ^a	TCATCGCACCGTCAAAGGAACC	59.9		
ipaH F ^b	CGCGACGGACAACAGAATACACTCCATC	62.4	108	Barletta et al. (2013)
ipaH R ^b	ATGTTCAAAGCATGCCATATCTGTG	56.4		

^a: *Salmonella* spp. primer

^b: *Shigella* spp. primer

analysis steps from 65 to 95°C in 0.5 s increments for 5 s. Cycle threshold (C_t) values were automatically calculated by the Bio-Rad CFX Manager software (Ver. 3.0). Reactions were run in duplicate, using the isolated DNA extracted from both water and riverbed sediment. Purified DNA of *Shigella dysenteriae* (ATCC 11835) and *Salmonella* subsp. enterica serovar Typhimurium (ATCC 14028) obtained from the TUT (Tshwane University of Technology) Water Research Group bacteria stock collections were used as positive control. For each reaction and primer set, negative controls were run in duplicate, consisting of primers, PCR Supermix and nuclease-free water instead of gDNA template. The specificity of the assay was assessed by the analysis of the melting curve (Varga and James, 2005; D'Souza et al., 2009). Melting was performed from 54°C to 95°C and 60°C to 95°C for *Shigella* spp. and *Salmonella* spp., respectively, at increments of 0.5°C/10 s. The melting temperature (T_m) was defined as the peak of fluorescence in the generated melting curve.

Genetic analysis of *Salmonella* spp. and *Shigella* spp. isolated from riverbed sediment and river water samples

Amplification DNA product for Sanger sequencing

For the amplification process, the following primer sets, as listed in Table 3, were generated using the PrimerQuest Tool software available at www.idtdna.com (IDT, USA) targeting the *invA* of *Salmonella* spp. and *ipaH* gene of *Shigella* spp.

Gel electrophoresis

For quality control purposes only, amplicons were run through gel electrophoresis of 1% (w/v) agarose gel stained with ethidium bromide, followed by imaging under ultraviolet light. The FastRulerlow range DNA ladder (Fermentas, Germany) was included in all gels as a size marker as well as positive control (DNA of ATCC cultures). These results were captured using a gel documentation system (Syngene, Cambridge, U.K.)

Sanger sequencing of the *invA* and *ipaH* gene

Following the gel image, all amplicons showing the desired size of *Salmonella* spp. and *Shigella* spp. were sent for sequencing at Inqaba Biotech (South Africa). The dideoxy Sanger sequencing in forward directions only was used with the primer sets listed in Table 3. For this procedure, Big Dye Terminator Cycle Sequencing Kit for ABI3130XL was used according to the manufacturer's instructions and the gel was run on a 3130XL sequencer. Sequences were analysed by comparing them with known *invA* and *ipaH* sequences using the BLASTn (Basic Location Alignment Search Tool for nucleotide) algorithm (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) to find the closest

match in GenBank, EMBL, DDBJ (DNA Data Bank of Japan) and PDB sequence data. Similar type species with 97% resemblance (<3% diversity) to the sequences of isolates were selected as matching species. The *invA* of *Salmonella* spp. and *ipaH* of *Shigella* spp. sequences were aligned with Clustal X2 (Larkin et al., 2007) and then were edited using BioEdit v.7.2.5 software (Hall, 1999). The distances of relatedness for each *invA* and *ipaH* gene were calculated by the neighbour-joining method and phylogenetic trees were created by using MEGA (Tamura et al., 2004). Nucleotide distance matrices of *invA* and *ipaH* were calculated by the neighbour-joining method with the Kimura 2-parameter model of substitution on datasets of 500 bootstrap replicates (Tamura et al., 2004). All locations having gaps and missing data were removed from the dataset using the complete deletion option.

All of the newly-sequenced bacteria were deposited in the DNA Data Bank of Japan (DDBJ) and the accession numbers are listed in the supplementary material.

Statistical analysis

The data were statistically analysed using Microsoft Excel 2010. Mean bacterial counts between the river water and riverbed sediments were assessed by two-way ANOVA to test for differences in the abundance of *Salmonella* spp. and *Shigella* spp., followed by Tukey's test when significant differences were encountered ($p < 0.05$).

RESULTS

Salmonella and *Shigella* culture counts

A total of 124 samples (62 water and 62 sediment samples) were collected from the described sampling site on the Apies River for analysis of the abundance of *Salmonella* spp. and *Shigella* spp. The mean counts of *Salmonella* spp. in water and sediment were, respectively, 2.60 log₁₀ and 4.82 log₁₀ CFU/100 mL. The mean counts of *Shigella* spp. in water and sediment were, respectively, 3.05 log₁₀ and 4.87 log₁₀ CFU/100 mL. Culture counts of *Salmonella* spp. and *Shigella* spp. in water were respectively in the range of zero (9.6%) to 2.98 log₁₀ CFU/100 mL and 2.93 to 3.16 log₁₀ CFU/100 mL, while in the sediment samples the counts were in the range of 4.70 to 4.91 log₁₀ CFU/100 mL for *Salmonella* spp. and 4.78 to 4.92 log₁₀ CFU/100 mL for *Shigella* spp.

For the qPCR counts, the mean counts of *Salmonella* spp. *invA* gene copies in water and sediment were respectively 3.52 log₁₀ and 5.81 log₁₀. The mean counts of *Shigella* spp. *ipaH* gene copies in water and sediment were respectively 3.55 log₁₀ and 5.59 log₁₀. The number of *invA* gene copies of *Salmonella* spp. and *ipaH* gene copies of *Shigella* spp. varied from zero (3.2%) to 1.52 log₁₀ CFU/100 mL and zero (9.6%) to 1.55 log₁₀ CFU/100 mL, respectively, in the water samples, while the

TABLE 3
Oligonucleotide primers for Sanger sequencing

Designation	Sequence	T_m (°C)	Product size
Sal445F ^a	GTCACCGTGGTCCAGTTTATC	55.5	853
Sal1276R ^a	CCTCGCCATCTCGTAAACAATAC	55.3	
Shig937F ^b	GAGTGACGGACAACAGAATACA	54.5	977
Shig1894 ^b	CCGTAATCGGGTCACAGTTT	54.9	

^a: *Salmonella* spp. primer

^b: *Shigella* spp. primer

number of *invA* gene copies of *Salmonella* spp. and *ipaH* gene copies of *Shigella* spp. was 'not detected' (3.2%) to 5.82 log₁₀ CFU/100 mL and zero (22.6%) to 5.86 log₁₀ CFU/100 mL, respectively, in the sediment samples of the Apies River.

The contrast between qPCR and culture-based methods revealed that the abundances of *Salmonella* spp. (5.81 log₁₀) and *Shigella* spp. (5.59 log₁₀) in the riverbed sediment samples obtained by quantification of gene copies were consistently and significantly higher than those obtained using the culture-based approach (*Salmonella* – 4.82 log₁₀ CFU/100 mL; *Shigella* – 4.87 log₁₀ CFU/100 mL) ($p < 0.05$; Fig. 1). In particular, loads determined by qPCR of *Salmonella* spp. and *Shigella* spp. were between 10 and 30 times higher than the loads obtained using culture-based techniques.

Phylogenetic analysis of isolates

The evolutionary history was gathered using the neighbour-joining system (Saitou and Nei, 1987). The phylogenetic trees with the sum of branch lengths = 1.12364635 (*Salmonella* spp.) and 2.25308283 (*Shigella* spp.) are shown in Fig. 2 and 3. The values of replicate trees in which the related taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches (Felsenstein, 1985). The evolutionary distances were processed using the Kimura-2 parameter method (Kimura, 1980) and are in the units of the number of base replacements per location. The analysis involved 28 *Salmonella* spp. and 28 *Shigella* spp. nucleotide sequences and first, second and third codon positions were included. All positions containing gaps and missing information were eliminated. There was a sum of 2 072 (*Salmonella* spp.) and 717 (*Shigella* spp.) positions in the last dataset. Evolutionary analyses were performed using MEGA6 (Tamura et al., 2013).

We analysed the *invA* gene sequences in *Salmonella* spp.; all fragments belonged to *Salmonella enterica* with diverse serovars. In water, the most abundant strains were *Salmonella enterica* subsp. *enterica* serovar Typhimurium at 62% of all the water isolate fragments, followed by 8% of the other isolate fragments, as shown in Fig. 4. The sediment isolates exhibited a more diverse *Salmonella* spp. community than the water, with both *Salmonella enterica* subsp. *enterica* serovar Typhimurium and *Salmonella enterica* subsp. *enterica* serovar Enteritidis having 21.4% of all the isolated sediment fragments, followed by *Salmonella enterica* subsp. *enterica* serovar Newport with 14% and the other serovars with 7% each, as shown in Fig. 4. The only other strains found in water samples were *Salmonella enterica* subsp. *enterica* serovar Senftenberg and Abaetetuba, while the sediment samples were found to contain *Salmonella enterica* subsp. *enterica* serovar Agona, Abony, Cubana, Tennessee and Enteritidis.

As can be seen in Fig. 2, both sediment and water isolates are in a phylogenetic tree with two primary clades. The first clade contains many fragments from water and sediment at 99% gene sequence similarity. The second clade has three fragments, one water isolate and two sediment isolates at 91% genetic similarity. Both water and sediment isolates are clustered together. Although the other observed similarity levels were secondary, no less than 53% sequence similarity was observed.

The *ipaH* gene sequences of *Shigella* spp. were analysed, and *Shigella flexneri* was found to be most abundant both in water and sediment, at 79% and 61%, respectively, of all sequences (Fig. 3). In water, two strains were identified as *Shigella sonnei* and *Shigella dysenteriae* with sequence similarities of 7% and 14%, respectively, while in the sediments three other strains were isolated, namely, *Shigella sonnei* (23%), *Shigella boydii* (8%) and *Shigella dysenteriae* (8%) as shown in Fig. 5.

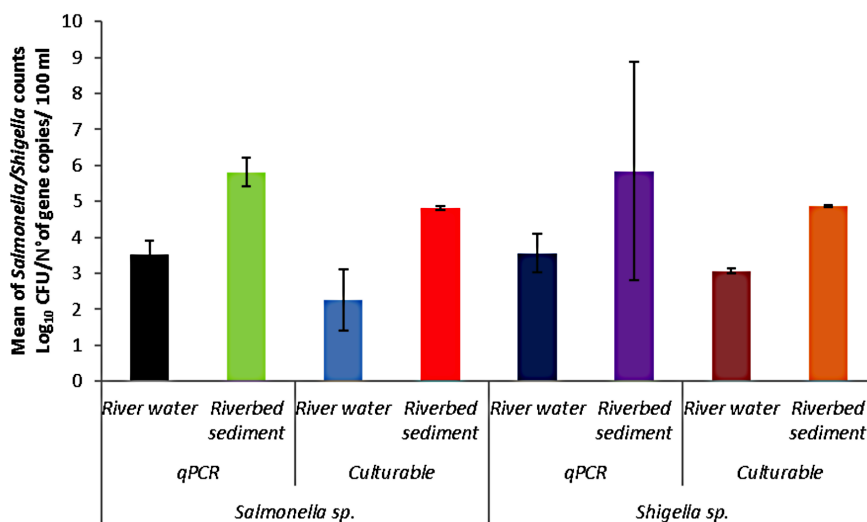


Figure 1
Contrast between water and sediment mean *Salmonella* spp. and *Shigella* spp. counts using qPCR and culture-based approach

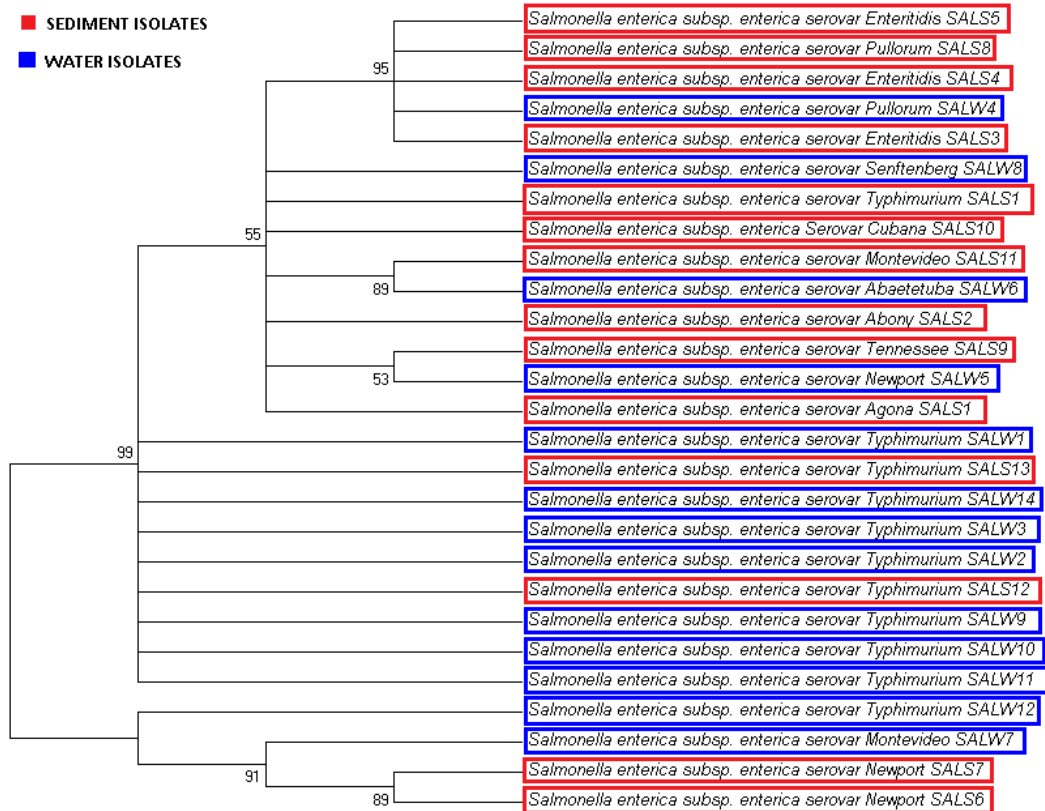


Figure 2
Phylogenetic tree for *invA* gene of *Salmonella* spp. isolates obtained from water (blue) and sediment (red)

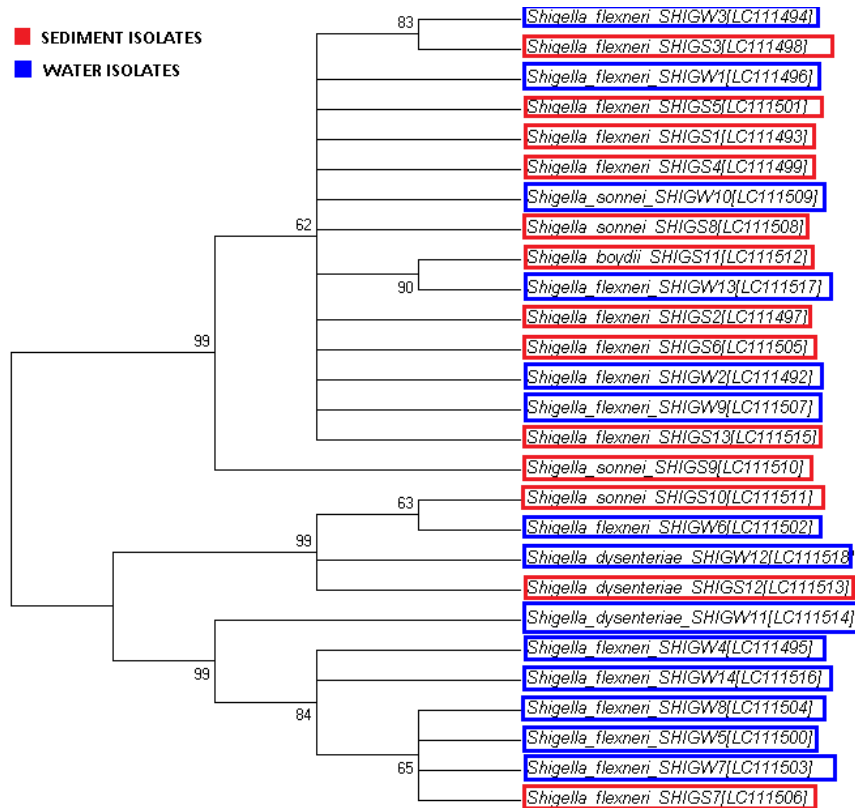


Figure 3
Phylogenetic tree for *ipaH* gene of *Shigella* spp. isolates obtained from water (blue) and sediment (red)

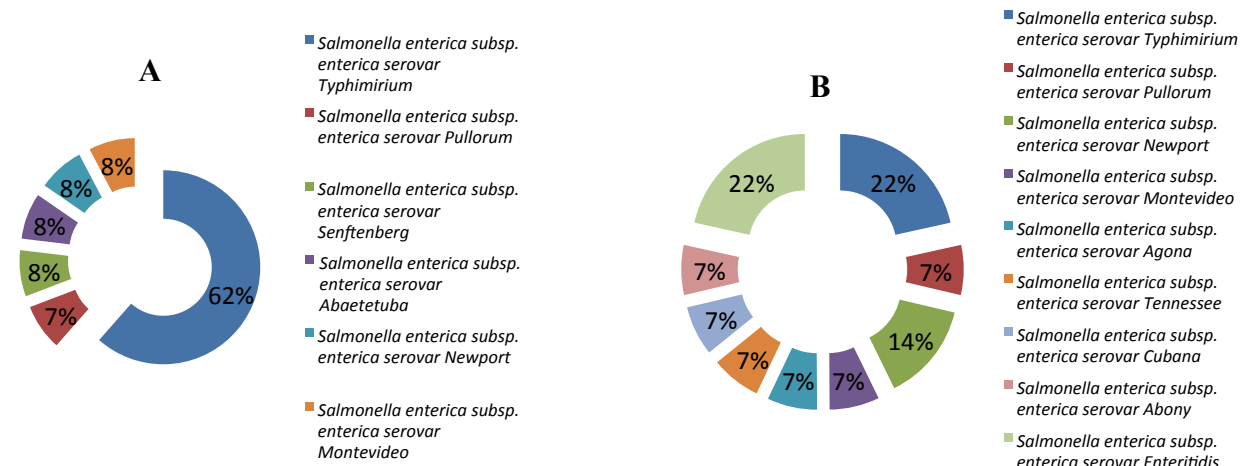


Figure 4
Salmonella spp. diversity in water (A) and sediment (B)

In Fig. 3, three primary clades can be observed. All three clades have 99% similarity among the DNA sequences analysed; DNA sequence clustering was observed in both water and sediment samples at no less than 63% of genetic similarity.

DISCUSSION

Pathogen concentrations

Salmonellosis and shigellosis continue to be a major public health problem worldwide, with 93.8 million and 163.2 million cases, respectively, reported annually (Majowicz et al., 2010; Kotloff et al., 2013). Many interrelated factors contributing to this incidence include increased urbanization, inadequate supplies of clean water, antibiotic resistance and increased regional movement (Boehmer et al., 2009). Real-time PCR is a generally used procedure that permits the precise quantification of a particular gene in ecosystem samples (Luna et al., 2012). This method can likewise be used for the determination of the bacterial loads, including microorganisms of faecal source (Noble and Weisberg, 2005). On the other hand, while this technique has been efficiently used for the quantification of pathogenic bacteria in seawater, soil, food and wastewater specimens (Ibekwe and Grieve, 2003; Haugland et al., 2005; Fukushima et al., 2007; Shannon et al., 2007), its application in riverbed sediment tests has still not been extensively researched (Cébron et al., 2008; Vezzulli et al., 2009). For the effective use of qPCR-based examinations on riverbed sediment samples, numerous scientific steps are required, particularly those required for minimizing the presence of inhibitory substances which are associated with nucleic acids (Vezzulli et al., 2009). Inhibitors should be removed from the sediment samples as these substances interfere with the extraction of DNA and prevent the amplification of the nucleic acids through the PCR. Therefore, extraction and refinement steps are of vital importance in sediment sample preparation, as high levels of organic matter, biological pollutants and trace metals act as PCR inhibitors through interaction with DNA or interference with the DNA polymerase enzyme (Fortin et al., 2004).

When contrasting quantification by culture methods versus qPCR, results of this study revealed that qPCR produced higher values compared to the culture-based method. The real-time

PCR examinations gave much higher (up to 3 logs) estimations of pathogenic bacteria loads than those obtained using culture-based techniques. Similar results were also observed by Luna et al. (2012) when they conducted qPCR techniques coupled with cultivable enumeration. The authors quantified *E. coli*, *Enterococcus* spp. and *Salmonella* spp. from harbour marine sediments using both culture-based and qPCR techniques. The qPCR technique revealed a higher concentration than the culture-based methods. They found that total *E. coli* and *Enterococcus* spp. (qPCR) counts were respectively 65 to 1 571 and 11 to 51 times higher than that from cultured-based quantification. In a study by Su et al. (2013), the high bacterial counts were attributed to viable but non-cultivable bacteria that are found in the environment. In another study, it was found that high qPCR counts may also result from DNA of dead cells in the environment (Wolffs et al., 2005). Culture-based methods are known to seriously underestimate the bacterial pathogen counts in seawater and marine sediment (Shannon et al., 2007; Luna et al., 2010). A similar higher sensitivity of the qPCR method when compared to culture-based approaches has previously been reported in harbour sediment (Luna et al., 2012) and beach sands (Yamahara et al., 2009). These data are vital for an understanding of the potential dangers related to the presence of pathogenic bacteria in sediments. Since the presence of pathogenic bacteria can bring about human diseases with counts of as low as 1.7×10^1 CFU/mL for *Salmonella typhimurium* (Srinivasan et al., 1982) and 1×10^4 CFU/mL for *Shigella* spp. (Kothary and Babu, 2001), such a low identification breaking point makes the real-time PCR assay particularly valuable for surveying the microbiological nature of riverbed sediments. Although qPCR does not necessarily produce results comparable to culture-based methods because qPCR measures a genetic, rather than a growth, endpoint, it remains clear that the sensitivity of qPCR is needed in analysing bacteria of public and environmental interest.

In a study conducted by Lindsay et al. (2013), the authors observed that at $4.38 \log_{10}$ copies/mL of *ipaH* gene present in the stools of infants, a moderate to severe diarrheal infection occurred. Results in our study are substantially above this value; this may suggest that if Apies River water is consumed, this can lead to diarrhoeal infections in infants. Pathogenic

bacteria in sediments have been reported by Atiribom et al. (2007); these authors have reported the isolation of pathogenic organisms such as *Aeromonas hydrophila*, pathogenic *E. coli*, *S. typhi* and *Vibrio* from sediment and the surrounding areas of the Kainji Lake, Nigeria.

Phylogenetic analysis

The *Salmonella* spp. isolated during this study included all *Salmonella enterica* with serovars including Typhimurium, Enteritidis, Pullorum, Senftenberg, Cubana, Montevideo, Abaetetuba, Abony, Tennessee, Newport and Agona. *Salmonella enterica* serovar Typhimurium was the most frequently detected in both water and sediment samples followed by *Salmonella enterica* serovar Enteritidis. The only serovar isolated in water was *Salmonella enterica* serovar Senftenberg, while many other serovars were present only in sediment; this includes: *Salmonella enterica* serovar Cubana, Abaetetuba, Abony, Tennessee and Agona. These results may suggest that sediments of the Apies River harboured more diversity than the water. All these pathogenic bacteria are linked to gastro-intestinal infections worldwide (Weinberger and Keller, 2005) and are reported to be multidrug resistant (Gordon et al., 2008). The *Salmonella enterica* isolated exhibited a 99% genetic relatedness which may suggest a common ancestry and origin between isolates from river water and riverbed sediment, as suggested by Grant et al. (2001); Boehm et al. (2002); Kim et al. (2004); Noble and Xu (2004) and Ekwanzala et al. (2017) – i.e., that faecal bacteria from these environments have a high level of similarity. In a recent study by Njage and Buys (2015) on genetic relatedness between commensal and pathogenic strains of *E. coli* from lettuce and irrigation water using the phylogenetic tree, they found high genetic relatedness despite the sites being 246 km apart.

The *Shigella* spp. phylogenetic tree analysis shown in Fig. 5 revealed that four *Shigella* strains were isolated from water and sediments of the Apies River. These include: *Shigella flexneri*,

Shigella sonnei, *Shigella boydii* and *Shigella dysenteriae*. *Shigella flexneri* was the most predominant species found in both river water and riverbed sediments. With a bootstrap value of 99%, this may suggest that *Shigella* spp. isolated from river water and riverbed sediment are also strongly related at a genetic level. Skariyachan et al. (2015) found that where *Shigella sonnei* is isolated from sediment this is suggestive of natural or environmental sources of contamination in the overlying water.

CONCLUSIONS

Overall, results from this study indicate that the qPCR technique, being highly specific and sensitive, may present a powerful tool which can be routinely used for a true assessment of the pathogenic contamination of water and riverbed sediments. The phylogenetic typing characteristics of isolate sequencing show that pathogenic bacteria isolated from water and sediment samples were closely related (99%). This study recommends future studies to be conducted on multi-locus sequencing or whole genome sequencing techniques in order to emphasize the findings of this study. The presence of pathogenic *Salmonella* spp. and *Shigella* spp. is a matter of concern for the communities along the Apies River, who currently depend on this water sources for multiple purposes, including drinking, bathing, recreational and agricultural purposes. An urgent intervention is required by the local Water Service Authority to supply safe drinking water in order to prevent a severe outbreak of waterborne diseases within these communities.

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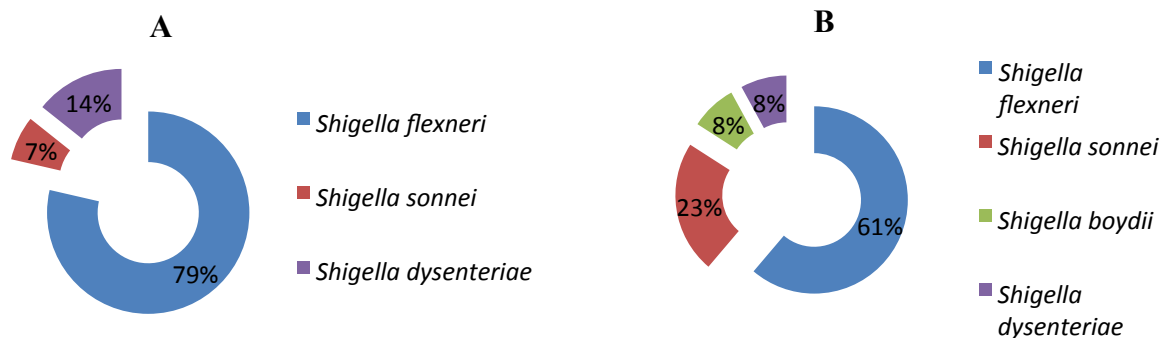


Figure 5
Shigella spp. diversity in water (A) and sediment (B)

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APPENDIX

All the newly-sequenced bacteria were deposited in the DNA Data Bank of Japan (DDBJ) with the following accession numbers: LC111465 (SALW1), LC111466 (SALW2), LC111467 (SALW3), LC111468 (SALW13), LC111469 (SALW14), LC111470 (SALW12), LC111471 (SALW11), LC111472 (SALW10), LC111473 (SALW9), LC111474 (SALS12), LC111475 (SALW01), LC111476 (SALW8), LC111477 (SALS1), LC111478 (SALS2), LC111479 (SALW7), LC111480 (SALS11), LC111481 (SALS10), LC111482 (SALW6), LC111483 (SALS9), LC111484 (SALS8), LC111485 (SALS7), LC111486 (SALS6), LC111487 (SALS5), LC111488 (SALS4), LC111489 (SALW5), LC111490 (SALS3) LC111491 (SALW4), LC111492 (SHIGW2), LC111493 (SHIGS1), LC111494 (SHIGW3), LC111495 (SHIGW4), LC111496 (SHIGW1), LC111497 (SHIGS2), LC111498 (SHIGS3), LC111499 (SHIGS4), LC111500 (SHIGW5), LC111501 (SHIGS5), LC111502 (SHIGW6), LC111503 (SHIGW7), LC111504 (SHIGW8), LC111505 (SHIGS6), LC111506 (SHIGS7), LC111507 (SHIGW9), LC111508 (SHIGS8), LC111509 (SHIGW10), LC111510 (SHIGS9), LC111511 (SHIGS10), LC111512 (SHIGS11), LC111513 (SHIGS12), LC111514 (SHIGW11), LC111515 (SHIGS13), LC111516 (SHIGW14), LC111517 (SHIGW13) and LC11151 (SHIGW12)