Characterisation of *Shigella* species isolated from river catchments in the North West province of South Africa

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ABSTRACT

The occurrence and distribution of *Shigella* species in water from the five river catchments in the North West province of South Africa were investigated. *Shigella* is a Gram-negative, non-motile, facultative anaerobic bacillus that causes shigellosis, an important cause of morbidity and mortality in high-risk populations (such as children, the elderly and immuno-compromised individuals) that depend on river water. A total of 54 water samples collected in winter (April 2007 to July 2007) and summer (December 2007 to March 2008) were cultured on *Salmonella-Shigella* agar by the spread-plate method. Suspected *Shigella* isolates obtained were characterised by primary biochemical (Triple Sugar Iron agar and agglutination) and molecular (polymerase chain reactions, PCR) tests. Amplification of the invasion plasmid gene (*ipaH*) by PCR was done to confirm the presence of *Shigella* spp. in water. In total, 214 *Shigella boydii*, 15 *Shigella dysenteriae*, 11 *Shigella flexneri* and 2 *Shigella sonnei* were confirmed by serotyping in both winter and summer samples. The *ipaH* gene (606 bp) was present in 176 and 49 of the winter and summer isolates, respectively. The presence of *Shigella* spp. in water was confirmed with over 90% specificity. The need for more effective management of these river catchments and the provision of potable water and sanitation facilities is needed to minimise the occurrence and transmission of water-borne diseases caused by these and other pathogenic bacteria.

INTRODUCTION

Shigella species are Gram-negative, non-motile, non-encapsulated, non-lactose fermenting, facultative anaerobes that are pathogenic to humans.[1] Their mode of transmission is usually through the ingestion of contaminated food and water, as well as person-to-person contact.[2] The genus *Shigella* consists of four species and at least 47 serotypes, which include *Shigella dysenteriae, Shigella flexneri, Shigella boydii* and *Shigella sonnei*.[2,3] They are the major cause of shigellosis or bacillary dysentery affecting, in particular, immuno-compromised individuals, children and the elderly.[1] Worldwide, the disease burden is estimated to be 150 million cases, with 1 million deaths per year in the developing world.[4] In 2009, the disease burden in South Africa was 1812 cases for both invasive and non-invasive shigellosis, predominantly in children below the age of 5 years.[4]

Shigellosis is characterised by destruction of the colonic epithelium as a result of an inflammatory response induced upon bacterial invasion of the mucosa.[5] Differentiation of *Shigella* isolates is classically based on serotyping and biochemical assays.[1] Antigenic characterisation relies exclusively on their somatic (O) antigens because they produce neither flagellins nor capsular antigens.[5] Biosynthesis of the O antigen is carried out by genes located in the *galF* and *gnd* clusters. These genes are classified into three different groups, (1) genes for the synthesis of nucleotide sugars specially used as O antigen residues, (2) genes encoding sugar transferases and (3) O unit processing genes normally consisting of O unit flippase gene (*wzx*) and polymerase gene (*wzy*).[6] Based on their antigenic properties, 46 *Shigella* serotypes have been recognised but, with the exception of *S. boydii*, all *Shigella* serotypes show more than 73% DNA relatedness to *Escherichia coli* K-12.[6,7]

Molecular techniques, such as restriction of amplified O-antigen gene cluster loci,[7] pulsed field gel electrophoresis, PCR, DNA hybridisation, ribotyping, and multilocus enzyme electrophoresis-*mdh* gene sequencing and their combinations, have been used to characterise *Shigella* species.[8,9,10,11,12] Several PCR protocols using different molecular markers have been used for the detection of *Shigella* species and other related organisms in environmental, faecal and food samples.[1,2,13,14] The invasion plasmid antigen H (*ipaH*) gene, a virulence gene whose protein product is necessary for invasion of colonic epithelial cells and also for the detection of *Shigella* in the environment, was used because it is carried by all four *Shigella* species as well as enteroinvasive *E. coli* (EIEC).[15]

Incidences of shigellosis outbreaks as a result of poor water quality have been reported throughout the world.[16] In Sierra Leone, a dysentery outbreak caused by *S. dysenteriae* type 1 and *S. flexneri* was reported in the Moyamba and Koinadugu districts. Reports provided by the Ministry of Health gave a total of 3094 cases of shigellosis with 132 deaths from 6 December 1999 to 16 January 2000.[16] In 2003, 379 cases of shigellosis and 23 deaths were reported in Paoua and Bazoum, north-west of Bangui in the Central African Republic.[17] In that same year, a cholera outbreak was also reported in Monrovia, Liberia, affecting 1857 people.[17] A year later, North Darfur (Sudan) reported a shigellosis outbreak caused by *S. dysenteriae* type 1, with 1340 cases of bloody diarrhoea and 11 deaths.[18] South Africa is no exception, with incidences of diarrhoea outbreaks caused by *S. flexneri* and *S. sonnei* reported in the Mpumalanga, Limpopo and Northern Cape provinces.[19] While shigellosis outbreaks have not been reported in the North West province, the possibility of such an occurrence cannot be underestimated as other water-borne diseases such as cholera and typhoid have been previously reported.[16,20,21] Therefore with no available information of shigellosis outbreaks in the North West province, this study aims to create awareness of the presence of *Shigella* spp. in river water and preparedness for any potential full-blown disease outbreak.

MATERIALS AND METHODS

A total of 54 water samples were collected in 500-mL sterile Schott Duran bottles from the major rivers in the Crocodile and Elands, Marico and Molopo, Marico and Hex, Mooi and Vaal, and Harts river catchments in the North West province (Table 1). Water samples were collected from three sites from

TABLE 1
Details of sampling sites

River catchments	Major rivers	Sampled sites	No. of samples
Crocodile and Elands	Crocodile	Hartebeespoort Dam, Brits and Rooikoppies Dam	3
	Elands	Sun City, Swartruggens and Lindleyspoort Dams	3
Marico and Molopo	Groot Marico	Groot Marico, Marico Bosveld Dam and Derdepoort	3
	Molopo	Modimola Dam, Cookes' Lake and Molopo Oog	3
Marico and Hex	Groot Marico	Groot Marico, Marico Bosveld Dam and Derdepoort	3
	Hex	Lesung, Bospoort Dam and Vaalkop Dam	3
Mooi and Vaal	Mooi	Potchefstroom, before Potch Dam and Potch Dam	3
	Vaal	Christiana, after Bloemhof Dam and Bloemhof Dam	3
	Skoonspruit	Orkney, Klerksdorp and Ventersdorp	3
Harts	Harts	Taung, Schweizer-Reneke (Wentzel Dam) and Barberspan	3

each river during the months of April 2007 to July 2007 (winter) and December 2007 to March 2008 (summer). Samples were transported to the Microbiology Research Laboratory of the North-West University.

Isolation of *Shigella*

Ten-fold serial dilutions of water samples were performed using 2% Buffered Peptone water (Biolab, Merck Diagnostics, South Africa). Aliquots of 1 mL of each dilution were cultured on *Salmonella-Shigella* agar by the spread-plate method.[22] Plates were incubated at 37 °C for 24 h.[13] Suspected isolates were picked from culture plates and subjected to further analysis for the identification and isolation of possible *Shigella* isolates.

Bacterial strains

Shigella boydii strain ATCC® 9207, *Shigella sonnei* strain ATCC® 25931, *Shigella flexneri* strain ATCC® 12022 and *Shigella dysenteriae* strain ATCC® 49345 were used as positive controls.[23] Because the *ipaH* gene is also present in EIEC, *E. coli* strain ATCC® 25922 was used as a negative control.[24]

Biochemical characterisation

Triple Sugar Iron agar test

All isolates were characterised using Triple Sugar Iron (TSI) agar to test for glucose, sucrose and lactose fermentation. Bergey's manual of systematic bacteriology[25] was used as an identification aid.

Agglutination test

A total of 235 and 287 isolates, for winter and summer respectively, were presumptively identified as *Shigella* spp. by the TSI agar test and then subjected to serotyping by slide agglutination. Serotyping was done using *S. boydii* Poly C, C1, C2 and C3; *S. dysenteriae* Poly A Types 1, 2, 3, 4, 5, 6 and 7; *S. sonnei* Poly D Phase I and II and *S. flexneri* Poly B Types I, II, III, IV, V and VI antisera according to the manufacturer's instructions (Davies Diagnostics, Johannesburg, South Africa).

Molecular characterisation

Genomic DNA extraction

Bacterial genomic DNA was extracted as described previously.[26] The concentration of the extracted DNA in solution was determined spectrophotometrically (UV Visible spectrophotometer model S-22, Boeco, Germany) at a wavelength of 260 nm and the purity was measured at 280 nm as previously described.[27] The integrity of the purified template DNA was assessed by conventional 0.8% (w/v) agarose gel.

Polymerase chain reactions (PCR)

PCRs were performed in final volumes of 25 µL, with a reaction mixture containing 0.2 µg/µL of the prepared template DNA, 50 pmol of each oligonucleotide primer, 1X PCR master mix and Dnase-Rnase free distilled water (Inqaba Biotechnological Industries Pty Ltd, Pretoria, South Africa). The primers IpaH-F 5-'CCTTGACCGCCTTTCCGATA-3' and IpaH-R 5'-CAGCCACCCTCTGAGGTACT-3' (Inqaba Biotechnological Industries Pty Ltd, Pretoria, South Africa) were used. The amplifications were performed using a Peltier Thermal Cycler (model PTC-220 DYAD™ DNA ENGINE; MJ Research Inc., Waltham, MA, USA) under the PCR conditions described previously.[28] PCR-amplified DNA fragments were separated by electrophoresis on 1% agarose. A wide-range molecular weight DNA marker (100-bp ladder, Inqaba Biotechnological Industries Pty Ltd) was used on each gel as the standard. The gels were stained in ethidium bromide (0.1 µg/mL) for 2 h. PCR products were visualised using a GeneSnap Bio-Imaging System (SYNGENE model GBOX CHEMI HR) and the images captured using image acquisition software version 6. 08. 04.

RESULTS

Based on the TSI agar test, 235 and 287 presumptively positive *Shigella* isolates were identified for the winter and summer samples, respectively. Among the 235 positive isolates obtained for winter, serological analysis confirmed a total of 168 *S. boydii*, 11 *S. dysenteriae*, 9 *S. flexneri* and 1 *S. sonnei*. The results indicated the presence of *S. boydii* and *S. dysenteriae* in all five catchments, whereas *S. flexneri* was present in only three catchments and *S. sonnei* in only one catchment (Table 2). Similarly, of the 287 *Shigella* isolates identified by the TSI test in the summer samples, serological analysis confirmed the presence of 44 *S. boydii*, 4 *S. dysenteriae*, 2 *S. flexneri* and 1 *S. sonnei*. The results indicated the presence of *S. boydii* in all five catchments and *S. dysenteriae* in two catchments, *S. flexneri* and *S. sonnei* were both present in the Mooi and Vaal catchment only (Table 3).

The high *S. boydii* contamination levels in the winter samples in all catchments are of concern. Levels were as high as 88% and 79% in the Harts and Crocodile and Elands catchments, respectively, whilst levels in the other three catchments were between 50% and 74%. For the summer samples, *S. boydii* contamination levels were highest (34%) in the Mooi and Vaal catchment followed by the Crocodile and Elands catchment (21%). The level in the Harts catchment was 13% while the Mooi and Vaal catchment and the Marico and Hex catchment recorded less than 10% agglutination each. All 189 and 51 isolates confirmed by serotyping to be *Shigella* spp. for winter and summer seasons, respectively, were subjected to PCR for the detection of the *ipaH* gene. The *ipaH* gene

TABLE 2
Total number of *Shigella* isolates obtained in winter

River catchments	*S. boydii*	*S. dysenteriae*	*S. flexneri*	*S. sonnei*
Crocodile and Elands	63	1	4	0
Marico and Hex	10	4	3	0
Marico and Molopo	26	4	2	0
Mooi and Vaal	55	1	0	1
Harts	14	1	0	0

TABLE 3
Total number of *Shigella* isolates obtained in summer

River catchments	*S. boydii*	*S. dysenteriae*	*S. flexneri*	*S. sonnei*
Crocodile and Elands	11	0	0	0
Marico and Hex	5	1	0	0
Marico and Molopo	2	0	0	0
Mooi and Vaal	19	3	2	1
Harts	7	0	0	0

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(606 bp) was present in 176 of the winter and 49 of the summer isolates. Figure 1 presents the positive results of PCR detection of the *ipaH* gene for a few of the isolates collected in summer. The PCR confirmed the presence of *Shigella* in river water and showed 93% and 96% specificity for winter and summer *Shigella* isolates, respectively.

DISCUSSION

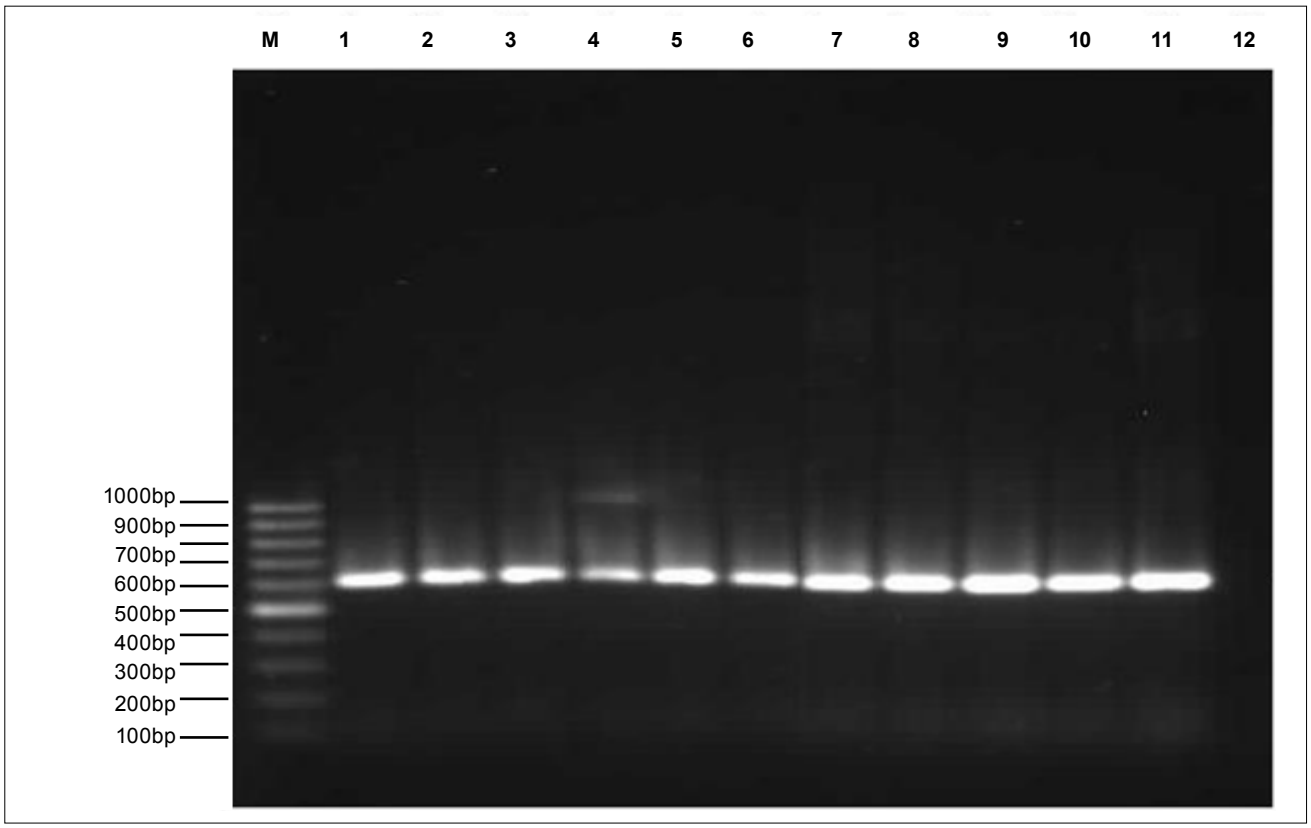
Culture methods have traditionally been used to identify *Shigella* species; however these methods have proved less effective because they only detect a small fraction of the actual number of shigellosis cases, probably because of a low number of causative organisms, competition from other commensal organisms and changes in the ambient temperature during sample collection.[15,29] To optimise the detection rates, we focused on the use of conventional serotyping and PCR typing methods to detect *Shigella* in river waters. In similar studies, the *ipaH* gene was used as an indicator to detect the presence of *Shigella* in environmental waters.[23,28,30,31] Although the PCR assay is rapid and highly specific, limitations such as false positives or negatives also exist because of the presence of impurities.[31] However, DNA extracted from all suspected isolates and control strains used in the study was checked for the presence of any impurity by measuring the optical density at 280 nm and 260 nm. The ratio of the two optical densities was calculated and compared with that of the standard values, the presence of any impurity was thereby eliminated. The PCR assay used was highly sensitive and the presence of *Shigella* spp. using 0.2 µg/µL of DNA was confirmed with over 90% specificity.

Generally, river water harbours a vast majority of enteropathogens derived from municipal sewage discharges, rainfall runoffs from agricultural farms and faecal waste from humans, pets, farm animals and wildlife, because river water is untreated.[32] *Shigella* spp. are sensitive to chlorination at normal levels and can survive for only up to 4 days in river water. In addition, it takes 1 to 2 days to establish a laboratory diagnosis by culture of a patient specimen, making the recovery of *Shigella* from environmental samples almost impossible.[33] However, the findings of this and previous studies[2,13] detected *Shigella* in surface waters and sewage samples, which is an indication that surface waters can possibly transport *Shigella* strains and that there is a possibility of a continuous source of contamination into the rivers. The detection of *Shigella* bacteria in the water samples suggests the likelihood of other pathogenic bacteria and confirms the presence of faecal contamination in the river catchments sampled.

Although *Shigella* bacteria predominantly cause food-borne diseases, shigellosis outbreaks as a result of consumption of contaminated water have also been reported, especially in developing countries with inadequate sanitation facilities.[20,21,23] *Shigella* epidemics spread through contaminated food and water. It is not known what dose of *Shigella* is necessary to cause disease, but the transmission of the pathogen is believed to be facilitated by a very low infectious dose.[34] In the North West province of South Africa, the percentage of households with sustainable access to an improved water source has steadily improved from 78% in 2001 to 97% in 2005.[35] However, an approximate 1 million people are still without access to basic water supply – mostly those in rural villages, farmlands and informal settlements – and still rely on surface water resources, which exposes them to a high risk of contracting water-borne diseases.[36]

CONCLUSIONS AND RECOMMENDATION

The presence of *Shigella* spp. in river water, as demonstrated by this and other studies, is significant and may be of public health concern. The chromosomally located multicopy virulence gene, *ipaH*, which is also known for its role in producing invasive characteristics, was found in all of the *Shigella* strains identified.



Lane M, 100-bp DNA ladder; Lane 1, *S. boydii* strain ATCC 9207; Lane 2, *S. sonnei* strain ATCC 25931; Lane 3, CRU21; Lane 4, HRD55; Lane 5, HRM32; Lane 6, GMM13; Lane 7, H_1RU7; Lane 8, H_1RU11; Lane 9, H_1RU39; Lane 10, MRD34; Lane 11, SRD7; Lane 12, negative control.

CRU, Crocodile River Upstream; HRD, Hex River Downstream; HRM, Hex River Midstream; GMM, Groot Marico River Midstream; H_1RU, Harts River Upstream; MRD, Mooi River Downstream; SRD, Skoonspruit River Downstream.

FIGURE 1
Polymerase chain reaction amplification of the *ipaH* gene from suspected *Shigella* isolates collected in summer

Therefore, PCR screening of environmental samples for the *ipaH* gene should provide a better indicator of the possible presence of *Shigella*. We have previously shown[37] that river water in the North West province is a possible route of human contamination with pathogenic bacteria such as *Shigella*, by direct or indirect consumption of river water through its use for recreation, domestic and agricultural purposes. The need for more effective management of these river catchments and the provision of potable water and sanitation facilities is needed to minimise the occurrence and transmission of water-borne diseases.

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