African Journal of Biotechnology Vol. 9(43), pp. 7295-7301, 25 October, 2010
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB10.321
ISSN 1684–5315 © 2010 Academic Journals

Full Length Research Paper

Prevalence of *Vibrio cholerae* in rivers of Mpumalanga province, South Africa as revealed by polyphasic characterization

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Accepted 31 August, 2010

Cholera is a life-threatening diarrhoeal disease, which mainly affects inhabitants of developing countries due to poor socio-economic conditions and lack of access to potable water and sanitation. Toxigenic *Vibrio cholerae* are the aetiological agents of cholera. These bacteria are autochthonous to aquatic environments, hence water plays a central role both in the epidemiology and transmission of cholera. The aim of this study was to determine the prevalence of *V. cholerae* from 32 sites of major rivers in Mpumalanga province of South Africa using a polyphasic approach. Water samples (594) collected over for 4 months were cultured on thiosulphate-citrate-bile salt-sucrose agar, and oxidase positive (88) isolates were subjected to biochemical tests and duplex polymerase chain reaction targeting the outer membrane protein (*ompW*) and cholera toxin (*ctxAB*) genes. All *ompW* PCR positive *V. cholerae* isolates were subjected to *rfbO1* PCR. Fifteen isolates from Crocodile, Komati and Gutshwa rivers were assigned to *V. cholerae* by both biochemical tests and PCR, of which no isolates were positive for *ctxAB* and *rfbO1* genes. The polyphasic approach was effective at revealing non-O1 and non-toxigenic *V. cholerae* in some rivers. Such information is important for raising awareness regarding the presence of *V. cholerae* so that precautionary measures are taken on time.

Key words: *Vibrio cholerae*, *ompW* gene, *ctx* gene, *rfbO* 1, surveillance.

INTRODUCTION

Worldwide, waterborne diseases such as cholera contribute significantly to mortality and morbidity of human beings if they are not treated (Topps, 2006). Due to the poor socio-economic conditions and lack of access to potable water and sanitation, the African continent is the major contributor of cholera in the world, with a proportion of cases that ranged from 93.6 to 99% from 2004 to 2008 (World Health Organization WHO, 2005, 2006, 2007, 2008, 2009). This has been exemplified by the cholera outbreak that occurred in Zimbabwe from 2008 - 2009, which spread quickly across the entire country causing 60,055 cases and 2,928 fatalities in 2008 alone (Nelson et al., 2009; WHO, 2009). In 2008, South Africa reported 3,907 cases and 22 fatalities to WHO (WHO, 2009). As of March 2009, Mpumalanga province recorded the highest number of cholera cases (6,644) (WHO, 2009a), hence it is important to determine the prevalence of *Vibrio cholerae* in this province.

The aetiological agents of cholera are toxigenic strains of *V. cholerae*, which are native inhabitants of aquatic ecosystems such as riverine, coastal and estuarine environments where they exist in culturable or nonculturable states (Shukla et al., 1995; Huq and Colwell, 1996; Goel et al., 2007). For this reason, the epidemiology and transmission of cholera is greatly influenced by water (Faruque et al., 1998). Indeed, cholera is transmitted through the fecal-oral route after consumption of contaminated water and/or food. It is therefore imperative to systematically monitor these bacteria in aquatic

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Abbreviations: ***ompW,*** Outer membrane protein gene; *ctxAB*, cholera toxin gene; **PCR,** polymerase chain reaction; **COD,** chemical oxygen demand.

environments so as to curb cholera epidemics (Choopun et al., 2002). This is important not only from academic viewpoint, but also for raising awareness and implementing strategies that may protect prone communities from cholera epidemics, especially the young, elderly and individuals with low immunity.

In general, classical and/or molecular-based methods are used for identification of *V. cholerae*. Classical methods involve techniques that are not based on DNA or RNA. For characterization of *V. cholerae*, classical methods involve enrichment of samples in alkaline peptone water (APW), followed by inoculation on suitable culture media such as thiosulphate citrate bile sucrose (TCBS) agar and aerobic incubation at 37°C (Nandi et al., 2000). Subsequently, the morphology, physiology, biochemical and serological reactions are determined to confirm *V. cholerae* (Choopun et al., 2002). Classical methods are significant and offer great value for identification of *V. cholerae* from clinical and environmental samples. However, classical methods may generate ambiguous results, particularly among the closely related *Vibrio* spp. (Nandi et al., 2000). Alternatively, DNA-based techniques with high resolution are advantageous for diagnosis and epidemiological surveillance of *V. cholerae* (Vandamme et al., 1996). These techniques include species-specific PCR targeting the outer membrane protein, which is very sensitive, specific and efficient for *V. cholerae* identification (Nandi et al., 2000). Even so, molecular-based techniques have some inherent limitations that include amplification bias, hence, a polyphasic approach that consists of both classical and molecular-based techniques is preferable for diagnosis of *V. cholerae* (Vandamme et al., 1996).

The prevalence of *V. cholerae* in surface water has been investigated using classical microbiological methods that involved performing a battery of biochemical tests (Choopun et al., 2002). However, classical methods for detecting *V. cholerae* are laborious and time-consuming as these methods require cultivation on selective media (Nandi et al., 2000; Tamrakar et al., 2006; Goel et al., 2007). Classical techniques are mainly useful for screening purposes and they may not be suitable for unequivocal identification of microorganisms. Alternatively, molecular methods that are based on nucleic acids have been shown to be more accurate, fast, sensitive and reproducible for the diagnosis of *V. cholerae* (Nandi et al., 2000; Goel et al., 2005; Goel et al., 2007). Despite the usefulness of the rRNA gene for characterization of various bacterial species, the resolution of this gene for identification of vibrios is limited due to the high similarities among members of this genus (Kita-Tsukamoto et al., 1993; Ruimy et al., 1994). Nevertheless, Nandi et al. (2000) designed primers that are based on the outer membrane protein (*ompW*), which is specifically present in *V. cholerae* and acts as internal controls for these bacteria (Goel et al., 2007). This provided a platform for rapid, sensitive and reliable species-specific characterization of *V. cholerae* (Goel et al., 2007).

Despite their numerous advantages, molecular methods are also prone to inherent limitations, hence, a polyphasic approach, which incorporates both classical microbiological methods and molecular techniques, is usually recommended. Therefore, the aim of this study was to investigate the prevalence of *V. cholerae* in all major rivers in Mpumalanga province, South Africa using a combination of selected biochemical tests and PCR targeting *ompW* genes. Furthermore, the toxigenicity and presence of O1 serogroup were tested by PCR targeting *ctxAB* and *rfbO1* genes. This is important for raising awareness of the presence of these bacteria in rivers in order to control possible cholera epidemics in South Africa.

MATERIALS AND METHODS

Bacterial strains

V. cholerae O1 ATCC 5941 and O139 NICD 12945 standard cultures were obtained from the Centre of Scientific and Industrial Research (CSIR, Pretoria, South Africa). These strains were maintained on tryptic soy (TS) and brain heart infusion (BHI) agar at 4°C and they were used for reference purposes during the entire study period.

Study area

The sites of the study were major rivers in Mpumalanga province, South Africa. Water samples were collected monthly from 32 different sites of the major rivers in Mpumalanga Province (Table 1), South Africa over a 4-month (August to November, 2009) period using 3 different approaches. The criteria used for choosing sampling sites were drafted in conjunction with the Department of Water Affairs (DWAF) in Nelspruit, South Africa. Water samples were collected at sites that are in close proximity to human settlements, farming communities, downstream and upstream of sewage treatment plants and areas that are not prone to human and animal influence.

Collection of water samples from rivers

Water samples (594) were collected aseptically once a month over a 4-month period (August to November 2009) from 32 sampling sites of major rivers in Mpumalanga province of South Africa. Three different approaches were used for collecting samples from above-mentioned rivers. Two of the methods involved planting either sterile Moore gauzes or tampons (tied at the centre to strong twine) in duplicate in flowing water at various river sites (Standard Methods, 1998; South African Bureau of Standards, 2001). Weights were also tied to the gauzes and tampons to ensure submergence in rivers. The gauzes and tampons were left in the various rivers for 5 - 6 days in order to maximize trapping of *V. cholerae*. The third method of sampling involved the collection of 5 L of water in sterile containers, followed by sealing of the containers and appropriate labelling. All samples were transported to the Department of Environmental, Water and Earth Sciences of the Tshwane University of Technology, South Africa, followed by analyses within 24 h of collection.

Microbiological analysis

After 5 - 6 days, the gauzes and tampons were removed from

Table 1. Coordinates and status of rivers during the study period.

Sampling site	Sampling site coordinates	Status of river*	
		Average pH	Average turbidity (NTU)
Crocodile river at Kanyamazane N4 bridge	S25° 29.6 E31° 10.4	6.75	6.08
Crocodile river downstream of Kanyamazane sewage treatment plant (STP)	S25° 29.3 E031 10.4	8.19	8.35
Crocodile river at Karino bridge	S25° 28.1 E31° 6.2	8.39	5.11
Crocodile river at Anglican club	S25° 27.2 E30° 57.3	8.18	71.8
Crocodile river at Schagen	S25°25.9 E30° 47.4	8.10	8.30
Crocodile river at Bambi bridge	S25° 23.7 E30° 35.9	8.76	2.63
Crocodile river at Rivulets	S25° 25.8 E30° 45.4	6.50	6.05
Crocodile river at Montrose	S25° 25.9 E30° 45.2	8.58	3.04
Gladdespruit downstream of Papas quary	S25° 27.4 E30° 57.3	8.58	2.06
Gutshwa stream upstream of Kabokweni STP	S25° 18.1 E31° 10.1	8.2	7.72
Gutshwa stream	S25° 18.2 E31° 10.3	7.88	38.6
Gutshwa stream downstream of Kabokweni STP	S25° 18.9 E31° 10.4	8.86	253
Nsikazi river at Manzini	S25° 10.9 E31° 8.9	7.13	8.91
Luphisi river	S25° 24.0 E31°16.5	7.53	21
Sipelanyane river	S25° 21.5 E31° 16.1	6.38	15
Ngodini river	S25° 21.2 E31° 8.0	6.93	94.5
Elands river at Lindenau Weir	S25° 36.3 E30° 41.6	6.90	3
Elands river downstream of Waterval boven STP	S25° 38.4 E30° 21.3	8.96	6
Elands river upstream of Waterval boven STP	S25° 38.8 E30° 20.3	8.6	5
Elands river upstream of Machadodorp STP	S25° 39.4 E30° 14.5	6.97	9.15
Elands river downstream of Milly's STP	S25° 41.1 E30° 12.5	8.05	5
Elands river at Hemolck	S25° 35.3 E30° 34.3	6.65	5
Leeuspruit downstream of Emthonjeni STP	S25° 41.3 E30° 15.2	7.02	5.08
Leeuspruit downstream of Assmang Chrome	S25° 43.6 E30° 14.1	7.52	10
White river downstream of White river STP	S25° 19.1 E31° 2.6	7.32	12
Sabie river upstream of Hoxani	S25° 1.2 E31° 12.3	6.72	7
Blyde river	S24° 43.2 E30° 50.4	8.41	1.0
Komati river	S25° 41.0 E31° 46.9	8.33	50
Lomati river	S25° 37.2 E31° 40.1	6.79	5
Sand river at Sanibonani lodge	S25° 2.0 E31° 9.3	6.93	7.81
Besterspruit upstream of MMC Delta	S25° 27.5 E30° 58.2	8.97	6.32
Olifants river	S25° 52.8 E29°18.2	7.87	55

*Refers to the status of the various rivers during the 4-month sampling period.

various rivers and enriched in sterile alkaline peptone water (APW; 1% peptone, 1% NaCl, pH 8.5) at a 1:1 (w/v) ratio (Momba et al., 2006) for 6 - 8 h at 30 ± 2°C and 35 ± 2°C. The alkaline pH of APW has been shown to promote the growth of members of the *Vibrio* genus. Regarding 5 L water samples collected directly from the rivers, a litre was transferred to a sterile Schott bottle containing sterile pre-weighed Moore swabs and processed according to Momba et al. (2006), followed by incubation at 30 ± 2°C and 35 ± 2°C. The incubation was carried out at 30 ± 2°C and 35 ± 2°C to enhance the recovery rate as it has been suggested that recovery of environmental *V. cholerae* may be higher at 30°C. Two loopfuls of the top-layer APW culture broths were sub-cultured onto thiosulphate-citrate-bile salts-sucrose (TCBS) agar (Merck, Damstardt, Germany). The TCBS agar plates were incubated aerobically at 35 ± 2°C for 24 h. All flat, smooth and yellow colonies of approximately 3 - 5 mm diameter were sub-cultured three times on TS and BHI agar in order to obtain pure cultures for characterization.

Biochemical analysis

The pure cultures were subjected to the oxidase test to determine the presence of cytochrome oxidase enzymes on all purified presumptive colonies. For this purpose, the bacterial cultures were initially purified on BHI and TS agar, which do not contain carbohydrates, as acidic environments interfere with the activity of the oxidase enzyme (Tamrakar et al., 2006). The purified isolates were then streaked onto filter paper saturated with 1% tetramethyl p-phenylenediamine hydrochloride (PRO-LAB Diagnostics, Texas, U.S.A.) using sterile toothpicks. Oxidase positive cultures showed a dark purple colour within 30 s. Further biochemical tests were performed on all oxidase positive isolates according to Tamrakar et al. (2006). BHI agar plates consisting of 0.1% esculin and 0.05%

Table 2. Sequences of primers used in this study.

Primer	Sequence of primer	Amplicon size (bp)	References
ompW (F)	5'-caccaagaaggtgactttattgtg-3'	588	Nandi et al. (2000).
(R)	5'- gaacttataaccacccgcg-3'		
ctxAB (F)	5'-cgggcagattctagacctcctgatg-3'	1069	Tamrakar et al. (2006).
(R)	5'-gccatactaattgcggcaatcgcatg-3'		
rfbO1 (F)	5'-tctatgtgctgcgattggtg-3'	638	Goel et al. (2007).
(R)	5'-ccccgaaaacctaatgtgag-3'		

ferric chloride were inoculated with the oxidase positive isolates, followed by incubation at 35 ± 2°C for determining the hydrolysis of esculin. In order to determine the production of indole, isolates were grown on tryptone broth (Oxoid, Cape Town, South Africa) for 48 h, after which 4 drops of Kovac's reagent were added and the results were recorded. Fermentation of glucose was determined by inoculating the bacterial isolates in Hugh-Leifson's broth (0.5% NaCl, 0.2% peptone, 0.03% K_2HPO4, 0.3% agar, 0.2% bromothymol blue, 1% sugar, pH 7.1). The inoculated broths were incubated at 35 ± 2°C for 48 h. The string test was performed on all oxidase positive isolates according to Tamrakar et al. (2006).

Molecular characterization

All 88 oxidase positive isolates obtained from all the sampling points over a 4-month period were subjected to molecular characterization. Total genomic DNA from the isolates was extracted using the DNeasy DNA purification kit (QIAGEN) and ZR Fungal/Bacterial DNA kit™ (ZYMO Research, U.S.A.) according to the manufacturer's instructions.

The primers used for polymerase chain reaction (PCR) amplification are listed in Table 2. Initially, *V. cholerae* isolates were confirmed by PCR amplification using *ompW* primers. For this purpose, 25 µl reaction volume was used for PCR amplification of the target DNA and each 200 µl PCR tube contained 3 µl of template DNA (50 ng/µ), 12.5 µl DreamTaq Master mix (2x) (Fermentas), 0.5 µl of each primer(10 pmol) and 8.5 µl of nuclease-free water. All reaction mixtures were placed in an MJ MINI thermal cycler (BIORAD), which was programmed for 35 amplification cycles using the following conditions: denaturation at 94°C for 30 s, annealing of primers with template DNA at 55°C for 30 s and primer extension at 72°C for 30 s. In order to ensure that there is a final extension, the reaction mixtures were subjected to 72°C for 7 min. Prior to the initial cycle, a pre-denaturation step was carried out to ensure total denaturation of DNA. A negative control reaction that contained all other reactions in the master mix and sterile molecular grade water instead of DNA was also subjected to PCR. The template DNA of reference strains was included as positive controls. After PCR amplification, the amplicons were resolved through electrophoresis of 1.5% (w/v) ethidium bromide stained agarose gel followed by visualization under ultraviolet light. The middle range Fast Ruler (Fermentas) was included in all gels. All results were recorded using a gel documentation system (Syngene, Cambridge, U.K.). Subsequently, all the PCR positive *V. cholerae* were subjected to a duplex PCR, whereby primers targeting the *ctxAB* and the *ompW* genes were simultaneously added in one mixture (Nandi et al., 2000; Tamrakar et al., 2006) and the mixture was subjected to the cycling conditions described earlier. All *ompW* positive *V. cholerae* were further subjected to PCR targeting the *rfbO1* gene. This gene could not be included in a duplex PCR with other genes as the amplicons of the *ompW* and *rfbO1* (588 and 638 bp, respectively) cannot be clearly resolved on agarose gel.

RESULTS

Microbiological analyses

Out of the 594 samples collected during the four months, 200 yielded yellow, smooth and small to medium-sized flattened colonies on TCBS agar and these were selected for further characterization (Bolinches et al., 1988). Out of the 200 samples that yielded yellow colonies, 88 colonies were oxidase positive and these were subjected to further biochemical identification.

The results of the selected biochemical tests are summarised in Table 3. Of the 88 oxidase positive isolates, 73 did not hydrolyse esculin as there was no blackening in the media. Only 15 isolates produced indole as evidenced by a deep pink colour after addition of a few drops of Kovac's reagent to the inoculated tryptone broth. The 15 isolates that produced indole also fermented glucose and were positive for the string test. Taken together, 15 isolates were presumptively considered to be *V. cholerae*.

Molecular characterization

The results of PCR targeting *ompW* genes are shown in Figure 1. The PCR amplification of *ompW* gene showed that 15 isolates displayed the expected product of 588 bp. This confirmed the presence of *V. cholerae*, as the *ompW* gene is specific for these bacteria (Nandi et al., 2000). The *V. cholerae* isolates were obtained during the months of September, October and November from Komati river (3 isolates), Crocodile river at Anglican club (9 isolates) and Gutshwa river downstream of Kabokweni sewage treatment plant (3 isolates). No *V. cholerae* were isolated in August. The *V. cholerae* that were confirmed with PCR amplification of the *ompW* gene were tested for the presence of the toxin gene in a duplex PCR using the *ompW* and *ctxAB* primers, and none of the 15 *V. cholerae* isolates yielded the 1 069 bp expected for *ctxAB* amplicon. Likewise, none of the confirmed *V. cholerae* also yielded the 638 bp amplicon expected for the *rfbO1* gene.

Almost similar numbers of the *V. cholerae* were obtained from water samples that were collected in sterile

Table 3. Results of biochemical tests used to screen for *V. cholerae* from oxidase positive isolates.

Biochemical test	Isolates tested	Positive isolates	*V. cholerae* reaction[1]
Oxidase test	200	88	+
Esculin hydrolysis	88	73	-
Indole production	88	15	+
Glucose fermentation	88	75	+
String test	88	15	+

[1] Taken from Tamrakar et al. (2006).

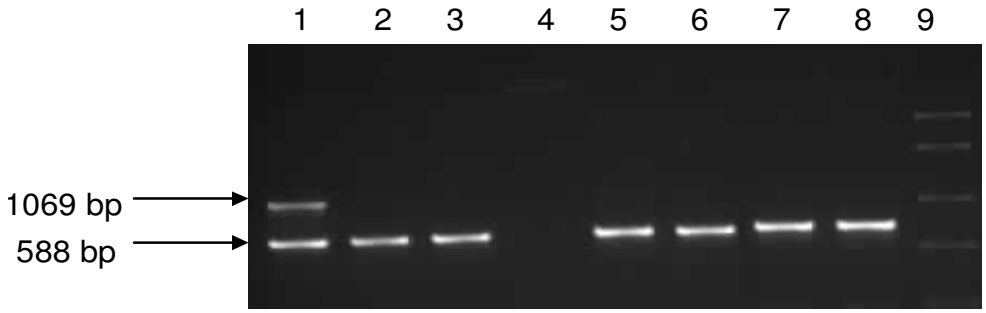

Figure 1. Example of agarose gel picture showing duplex PCR targeting the *ompW* and *ctxAB* genes. Lanes: 1, *V. cholerae* O1 ATCC 5941 positive control strain; 2, 3, 5, 6, 7 and 8, environmental isolates that exhibit typical positive *V. cholerae* with PCR amplicon size of 588 bp, but negative for *ctxAB* gene; 9, Fast Ruler middle range DNA marker.

containers directly (7 isolates) and from gauzes that were submerged in rivers for 5 - 6 days (8 isolates). No *V. cholerae* was isolated from water samples collected using tampons.

DISCUSSION

V. cholerae are natural inhabitants of aquatic environments, hence water plays a central role in the transmission of cholera. In order to circumvent potential cholera threats, it is paramount to determine the prevalence of *V. cholerae* in aquatic environments. Such surveillance systems require methods that are reliable, reproducible and rapid (Tamrakar et al., 2006; Goel et al., 2007). In this study, a polyphasic approach consisting of selected biochemical tests and molecular methods were successfully used to characterize isolates from various rivers in Mpumalanga province. This approach revealed that 15 *V. cholerae* isolates were obtained during September, October and November from Gutshwa, Komati and Crocodile River at Anglican club. This information is important for raising awareness on the extent to which water sources in Mpumalanga are contaminated with *V. cholerae* with a view to take appropriate measures to curb public health threats of diarrhoea.

In this study, 594 samples were collected during a 4-month period from August to November 2009 in order to determine the prevalence of *V. cholerae* in major rivers of Mpumalanga province. Out of these samples, 200 isolates produced yellow colonies due to sucrose fermentation in TCBS agar (Tamrakar et al., 2006). Although the elevated pH of 8.5 selects the growth of vibrios, other non-vibrio bacteria were present in the TCBS agar because some bacteria such as *Aeromonas* may also form yellow colonies that are similar to vibrios on TCBS agar (Pfeffer and Oliver, 2003).

Additional biochemical tests were performed in order to further screen the 88 oxidase positive isolates. Seventy three isolates showed some blackening in the media as a result of hydrolysis of esculin. This reaction is not associated with *V. cholerae*, hence only 15 esculin hydrolysis negative isolates showed the expected result. Indole was produced in 15 of the 88 tested isolates and this positive reaction is strongly associated with *V. cholerae* (Tamrakar et al., 2006). As expected, many of the isolates (75) fermented glucose as shown by acidity in the media. The possibility of *Aeromonas* spp. was excluded by using the string test, which was positive in 15 isolates. Taken together, these biochemical tests revealed the presence of 15 presumptive *V. cholerae* isolates.

Unequivocal diagnosis of the presence of *V. cholerae* was made by amplifying the 588 bp *ompW* gene. The *ompW* gene is unique to *V. cholerae* and is absent in other members of this genus (Nandi et al., 2000). All the 15 presumptive *V. cholerae* isolates were confirmed to be *V. cholerae* using PCR targeting the *ompW* gene. Although there was harmony between the results of

biochemical tests and molecular techniques, it is important to note the limitations associated with using bioche-mical tests alone, especially regarding the differentiation of closely related *V. cholerae* and *Vibrio mimicus*. Hence a polyphasic approach is recommended for unequivocal identification of *V. cholerae*.

Duplex PCR using *ompW* and *ctxAB* primers showed that none of the 15 *ompW* positive isolates harboured the *ctxAB* genes, indicating that the environmental strains of *V. cholerae* in this study did not produce cholera toxin, which is generally linked to clinical isolates (Nair et al., 1988; Sharma et al., 1998; Tamrakar et al., 2006). Similar results were obtained by Tamrakar et al. (2006) who found that out of 151 *ompW* positive isolates, only 2 possessed the *ctx* gene. Likewise, the PCR targeting *rfbO1* gene showed that none of the isolates possessed the gene that encodes the O1 serogroup, indicating that *V. cholerae* isolates in this study are non-toxigenic and belong to non-O1 serogroup. However, water that consists of non-toxigenic *V. cholerae* is not necessarily safe as these strains are supposedly converted to toxigenic strains in the intestine (Faruque et al., 1998). For this reason, the presence of non-O1 and non-toxigenic *V. cholerae* in this study implies that rivers in South Africa, especially in Mpumalanga, must be routinely monitored and managed in order to protect public health (Choopun et al., 2002; Goel et al., 2007). Eight of the fifteen *V. cholerae* were isolated from Moore swabs that were left in running water for 5 - 6 days, while 7 of the isolates were obtained from water taken directly from the various rivers, implying that both methods are suitable for isolating these bacteria from environmental samples. Tampons were found to be unsuitable for obtaining *V. cholerae* from water sources as no isolates were recovered with this method. This may be due to some inhibiting substances present in tampons.

No *V. cholerae* isolates was obtained during the month of August for all the sites sampled. This may be due to the relatively low temperatures experienced during this month, which may have made this bacteria species to enter into a viable but non-culturable state.

Some of the *V. cholerae* isolates were obtained from Gutshwa river downstream of Kabobweni sewage treatment plant. The treated sewage from Kabokweni treatment plant is discharged into Gutshwa River. Malfunctioning of the oxidation ponds at Gutshwakop has been reported and the effluent that discharges into Gutshwa river was shown not to conform to general standards because of the presence of *E. coli*, high chemical oxygen demand (COD) and ammonia (Makhubele, 2009). This highlights the need for thorough wastewater treatment before discharging the effluent into the river. In addition, numerous used baby pampers with human excreta were also present in close proximity to the river edge during the study period. It is therefore tempting to mention that this may have contributed to the high probability of isolating *V. cholerae* in this river. It is important to note that Gutshwa river is a tributary of the Crocodile River, hence it is possible to relate some of the *V. cholerae* isolated from this river at Anglican club to those detected from Gutshwa river.

Some of the *V. cholerae* isolates were obtained from Komati river, which is situated in the Phiva trust rural settlement. In general, the water quality in Komati river has been reported to be in a good state (Komati Water Basin Authority (KOBWA) annual report, 2004/2005). However, due to the poor sanitation conditions in the surrounding rural settlement and some periods of no water flow, the river is subject to contamination by micro-organisms including *V. cholerae* (KOBWA annual report, 2004/2005). This poses a public health threat as the water from this river is used for irrigation, domestic and industrial purposes (Dhlamini, 2008).

Conclusion

The polyphasic approach that encompassed conventional microbiological techniques and DNA-based PCR was effective at revealing the presence of strains of *V. cholerae* in Gutshwa, Komati and Crocodile rivers in Mpumalanga Province, South Africa. Such information is important for raising awareness to local authorities about the presence of these autochthonous aquatic bacteria and the need for environmental management and efficient water treatment. As Komati and Crocodile rivers are shared by South Africa's neighbouring countries such as Mozambique and Swaziland, a molecular epidemio-logical survey regarding the prevalence of *V. cholerae* in rivers that are shared by South Africa and its neighbouring countries may be paramount in future. It is also important to educate inhabitants of rural communities about the public health threats of *V. cholerae*.

ACKNOWLEDGEMENTS

We are grateful to the National Research Foundation, South Africa for providing a grant that supported this work. Mr Vilakazi from Mbombela Municipality in Mpumalangais thanked for assistance with identifying the water sampling sites.

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