Abundance of pathogenic *Escherichia coli, Salmonella typhimurium* and *Vibrio cholerae* in Nkonkobe drinking water sources

Maggy N. B. Momba, Veronica K. Malakate and Jacques Theron

ABSTRACT

In order to study the prevalence of enteric pathogens capable of causing infection and disease in the rural communities of Nkonkobe, bacterial isolates were collected from several surface water and groundwater sources used by the community for their daily water needs. By making use of selective culture media and the 20E API kit, presumptive *Escherichia coli*, *Salmonella* spp. and *Vibrio cholerae* isolates were obtained and then analysed by polymerase chain reaction assays (PCR). The PCR successfully amplified from water samples a fragment of *E. coli uidA* gene that codes for β-D-glucuronidase which is a highly specific characteristic of enteropathogenic *E. coli*, enterotoxigenic *E. coli* and entero-invasive *E. coli*. The PCR also amplified the *epsM* gene from water samples containing toxigenic *V. cholerae*. Although *E. coli* was mostly detected in groundwater sources, toxigenic *V. cholerae* was detected in both surface and groundwater sources. There was a possibility of *Salmonella typhimurium* in Ngqele and Dyamala borehole water samples. The presence of these pathogenic bacteria in the above drinking water sources may pose a serious health risk to consumers.

Key words | ground water, pathogenic bacteria, PCR, rural community, surface water

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INTRODUCTION

In almost all South African metropolitan areas, the consumer is provided with high-quality drinking water. However, in many rural communities, the situation is very different. In 1994, an estimated 14 million people had no access to clean or safe water. Although initiatives were taken and improvement measures implemented, 7 million of the 14 million people in rural areas still lack safe and clean water (Duse *et al.* 2003). The Nkonkobe district is among those South African rural areas without access to an adequate water supply.

The Nkonkobe district is situated in the Eastern Cape Province of South Africa. The population of the Eastern Cape is largely non-urban and poor, with an inadequate water supply infrastructure. The rural communities of this province comprise both scattered villages and subsistence farmers, and formalised towns serving subsistence farmers.

The poverty rate in 1998 was 78% and only 25% of households had a tap inside their dwelling (Mey 1998). This implies that many people depend on surface and groundwater sources for their daily water needs. Water from these sources is used directly by communities and the water sources are faecally contaminated and devoid of treatment (Momba & Kaleni 2002; Momba & Notshe 2003).

Among the pathogens disseminated in water sources, enteric pathogens are the ones most frequently encountered. Enteric pathogens such as *E. coli*, *V. cholerae* and *S. typhimurium* are usually transmitted to humans by the ingestion of contaminated water and foods. These enteric bacteria are reportedly causative agents of various diseases and their complications (Grabow 1996). The impact of waterborne diseases (such as diarrhoea) is significant in South Africa. Studies have shown that diarrhoea is

responsible for about 20% of all deaths in children under 5 years of age living in settlements with rudimentary access to water supply and sanitation (Borne & Coetzee 1996). Furthermore, waterborne diseases are thought to be responsible for an estimated 43,000 deaths and 3 million incidences of illness annually, with an associated treatment cost of some $420 million (Pegram *et al.* 1998). This scenario will continue to bequeath to rural communities an increasing incidence of water related diseases and their complications if communities are not made aware of the quality of their drinking water sources. A routine programme for monitoring drinking water sources would therefore provide increased public health protection since it would result in the detection of specific pathogenic microorganisms capable of causing infections and diseases in such communities.

This study was aimed at determining the presence of *E. coli*, *S. typhimurium* and *V. cholerae* in drinking water sources used by Nkonkobe rural communities for their daily water needs. To achieve this goal, water samples were collected from several surface water and groundwater sources. By making use of selective culture media, presumptive *Escherichia coli*, *Vibrio cholerae* and *Salmonella* spp. isolates were obtained, identified by 20E API kit and then analysed by polymerase chain reaction assays (PCR).

MATERIALS AND METHODS

Bacterial strains

Pathogenic *Escherichia coli* ATCC 25922 (Seeman & Sumner 2002) and *Salmonella typhimurium* ATCC 14028 were obtained from the American Type Culture Collection, Rockville, MD. *Vibrio cholerae* NCTC 5941 was obtained from the National Collection of Type Cultures, London, UK. These strains were reconfirmed by cultural, morphological and biochemical tests according to standard procedures (*Standard Methods 1998*; South African Bureau of Standards 2001) and were used as reference strains.

Study sites and sampling

Different surface and groundwater sources were used as test waters in this study. Surface water samples were collected

from the Lenge Dam, the Tyume River, the Sityi River and the Mnikina River, whereas groundwater samples were collected from boreholes in the villages of Ngqele, Njwaxa, Dyamala and Ngwenya. All these water sources are situated in the Nkonkobe district in the Eastern Cape Province of South Africa.

Surface water not only serves the communities for drinking but is also used for a variety of other purposes. These include drinking water for domestic animals (cattle, sheep, goats, etc.), washing of clothes by local residents and even the disposal of human and animal excreta. The communities from Dyamala receive underground water from a borehole using a rotary hand pump which is connected to a standpipe, while the Ngqele, Njwaxa and Ngwenya communities receive their drinking water directly from standpipes which are connected to the boreholes. Although all the boreholes are covered, they are surrounded by animal excreta and all of them are located close to pit latrines (with the exception of the Dyamala borehole). Groundwaters are surrounded by sandstones and shale and dolerite intrusions. During the study period, none of the boreholes were measured for depth.

Water samples from the above-mentioned sources were collected over a period of 4 months (November 2001, January–March 2002). The water samples from rivers and dams were collected directly and aseptically in 2 L sterile glass bottles. The standpipes were flushed for approximately 5 min before the collection of samples in 2 L sterile glass bottles. All the bottles were sealed and properly labelled. The samples were then placed in ice bags and transported to the laboratory at the University of Fort Hare for analyses within 2 h of collection.

Culture-based isolation and biochemical tests for the identification of *E. coli*, *V. cholerae* and *Salmonella* spp.

Standard procedures were used to isolate *E. coli*, *Salmonella* spp. and *V. cholerae* from the water samples (*Standard Methods 1998*; South African Bureau of Standards 2001). For *E. coli* counts, the membrane filtration technique was initially considered but the method proved to be too sensitive for analysis of the raw water samples. The spread plate method was then used for the enumeration of the *E. coli* after serial dilutions of the water samples. Fluorocult

E. coli 0157:H7 agar medium (Merck), which is a selective medium for the isolation of *E. coli*, was used during this study. The plates were incubated for 24 h at 35 ± 2°C. To isolate *Salmonella* spp., 100 mL of the sample volume was concentrated onto a 0.45 μm nitrocellulose membrane (Millipore) by vacuum filtration and the filters were inverted directly onto Xylose lysine desoxycholate (XLD) agar (Merck). The plates were then incubated for 24 h at 35 ± 2°C. To isolate *V. cholerae* from surface water sources, sterile Moore gauze swabs made with absorbent cotton were placed in flowing water sources for 48 h followed by enrichment in a double-strength alkaline peptone broth (pH 8.5) at a 1:1 (w/v) ratio for 6–8 h at 35 ± 2°C. For groundwater samples, a l L water sample was transferred into a 1 L sterile glass bottle containing a sterile swab and incubated at room temperature for 48 h. Thereafter the swab was aseptically transferred into a sterile plastic container. Both prior to and after use, the weights of the sterile swab and the plastic container were determined. The difference between the weights was then used for the enrichment step. Next, *Vibrio cholerae* was enumerated by the spread plate procedure on Vibrio diagnostic agar (Biolab) and all of the agar plates were incubated aerobically at 35 ± 2°C for 16–24 h. Typical *V. cholerae* colonies, which were smooth and yellow, were counted (cfu/mL). All these tests for the above microorganisms were performed in triplicate.

The individual bacterial colonies from different water samples were randomly selected from different plates and transferred onto the corresponding medium by the streak plate technique and incubated at 35 ± 2°C. The colonies were further purified by the same methods at least three times using nutrient agar (Biolab) before Gram staining was done. Oxidase tests were then conducted on those colonies that were Gram-negative. The 20E API kit was used for the oxidase-negative colonies and the strips were incubated at 35 ± 2°C for 24 h. The strips were then read and the identification was secured using API LAB PLUS computer software (BioMérieux).

Preparation of lysates for PCR

A direct lysis method was used for the isolation of DNA from the bacteria (Theron *et al.* 2000). Briefly, bacterial colonies were each suspended in 200 μL of sterile Milli-Q water and the bacteria were lysed by heating for 10 min at 100°C. Particulate material present after processing was removed by centrifugation at 10 000 g for 5 min. The lysate supernatant was removed and 10 μL used as the template in the PCR assays.

Oligonucleotide primers

Oligonucleotide primers (Table 1) specific for the *uidA* gene of *E. coli* encoding for β-D-glucuronidase, the *ipaB* gene of *S. typhimurium* encoding the invasion plasmid antigen B and the *epsM* gene of *V. cholerae* encoding the enterotoxin extracellular secretion protein of toxigenic *V. cholerae* were used, as they have been reported to be specific for the respective bacteria (Tsai *et al.* 1993; Kong *et al.* 2002).

PCR amplification and electrophoretic detection of amplicons

The reaction mixtures (50 μL) used in the PCR steps contained 1 U Super Therm GOLD Buffer, 1.5 mM $MgCl_2$, each deoxynucleoside triphosphate (Promega) at a concentration of 0.25 mM, either 50 pmoL of each *V. cholerae*-specific primer or 100 pmoL of each of the *E. coli*- or *S. typhimurium*-specific primers and 1 U of Super Therm *Taq* polymerase (Southern Cross Biotechnology). The sample volume was 10 μL. The reaction tubes were placed in an Eppendorf model AG 22331 thermal cycler. The cycling conditions performed in this study were standardised in our laboratory. The following conditions were used for *V. cholerae* and *E. coli*: heat denaturation at 94°C for 2 min followed by 25 cycles of heat denaturation at 94°C for 1 min, primer annealing at 58°C for 1 min and DNA extension at 72°C for 1 min. After the last cycle, the samples were kept at 72°C for 2 min to complete the synthesis of all strands. The PCR amplification for *S. typhimurium* was performed as described by Kong *et al.* (2002): heat denaturation at 94°C for 2 min followed by 35 cycles of heat denaturation at 94°C for 1 min, primer annealing at 62°C for 1 min and DNA extension at 72°C for 2.5 min. This was followed by incubation at 72°C for 10 min and cooling at 4°C. Control reaction mixtures containing sterile Milli-Q water and all other reagents but no template were amplified

Table 1 | Primer sequences and size of PCR-amplified gene targets of the three types of pathogenic bacteria

Bacterium	Gene target	Amplicon size (bp)	Primer	Sequence (5′ → 3′)	Reference
Escherichia coli	*uidA*	147	UAL-754	AAAACGGCAAGAAAAAGCAG	Tsai *et al.* (1993)
			UAR-900	ACGCGTGGTTAACAGTCTTGCG	
Vibrio cholerae	*epsM*	248	EpsM-F	GAATTATTGGCTCCTGTGCAGG	Kong *et al.* (2002)
			EpsM-R	ATCGCTTGGCGCATCACTGCCC	
Salmonella typhimurium	*epaB*	314	IpaB-F	GGACTTTTTAAAAGCGGCGG	Kong *et al.* (2002)
			IpaB-R	GCCTCTCCCAGAGCCGTCTGG	

along with the test samples throughout the amplification reaction. Positive controls consisting of suspensions of the reference bacterial strains were also included. The amplicons were resolved on a 2% (w/v) agarose gel in 1 U TAE (40 mM Tris-HCl, 20 mM Na.acetate, 1 mM EDTA, pH 8.5) and visualised by UV-induced fluorescence after staining with 0.5 µg of ethidium bromide per mL. A 100 bp DNA ladder (Promega) was included on each gel as a molecular size standard.

RESULTS AND DISCUSSION

The purpose of this study was to determine whether pathogenic *E. coli*, *V. cholerae* and *S. typhimurium* were present in water sources used by Nkonkobe rural communities for their daily water needs. Such information may allow us to determine to what extent the water sources may influence infection and disease in the community.

To determine the presence of the above microorganisms in water sources, culturing methods involving the isolation of presumptive *E. coli*, *V. cholerae* and *Salmonella* spp. and biochemical tests for the identification of those microorganisms preceded the PCR method. This is because, in rural areas of South Africa such as the Nkonkobe region, culturing methods are frequently used to assess the general quality of water. Conventional culture media and techniques used in this study for the isolation of *E. coli*, *V. cholera* and *Salmonella* spp. are identical to those routinely used by a number of laboratories in South Africa. These

culturing methods are often associated with selectivity, sensitivity and specificity problems. Consequently, the detection of the presence of presumptive *E. coli*, *V. cholerae* and *Salmonella* spp. in water sources by culturing methods does not definitely indicate the presence of the strains capable of causing infections and diseases in human. In order to ensure the safety of the various water sources used by the Nkonkobe communities in terms of *E. coli*, toxigenic *V. cholerae* and *S. typhimurium*, a subsequent PCR analysis of the isolates was important, since this method relies on the *in vitro* amplification of a DNA fragment and offers a higher level of specificity of strain detection (Rompré *et al.* 2002).

An analysis of the water samples obtained from the different drinking water sources resulted in the isolation of presumptive *E. coli* and *V. cholerae* by culturing methods (Table 2). The results obtained by culturing methods using selective media also indicated that 100% of both ground and surface water sources were contaminated with presumptive *E. coli* and *V. cholerae* during the study period (Table 3). Biochemical identification results indicated the presence of *E. coli*, from the Ngqele, Dyamala and Lenge sources, whereas *Vibrio spp.* was identified from the Njwaxa, Nqele and Tyume sources. However, *Salmonella* spp. was identified only from the Lenge source. Subsequent PCR analysis of the isolates indicated the presence of *E. coli* in four water sources (Tyume, Ngqele, Dyamala, and Ngwenya) and toxigenic *V. cholerae* strains in two water sources (Tyume and Ngqele) (Table 2). While the PCR analysis of the isolates showed that 75% and 25% of groundwater samples tested positive for *E. coli* and

Table 2 │ Presence/absence of *E. coli*, *V. cholerae*, *Salmonella spp.* and *S. typhimurium* from ground and surface water samples by culture-based and PCR methods (November 2001, January–March 2002)

No.	Source	Culturing *	PCR **		
			E. coli	*V. cholerae*	*S. typhimurium*
1	Lenge	+	−	−	−
2	Tyume	+	+	+	−
3	Sityi	+	−	−	−
4	Mnikina	+	−	−	−
5	Ngqele	+	+	+	−
6	Njwaxa	+	−	−	−
7	Dyamala	+	+	−	−
8	Ngwenya	+	+	−	−

* *E. coli*, *Salmonella spp.*, *V. cholerae*. ** PCR from the isolates.

toxigenic *V. cholerae* respectively, only 25% of the surface water samples tested positive for either bacterial strain.

Several factors may have contributed to the apparently high *E. coli* and *V. cholerae* frequency using culturing methods and their low frequency or absence using the PCR method. This might either have been due to the culture media or to species-specific genes that were targeted. Inthis investigation, the standard procedure was based on the isolation of presumptive *E. coli* and *Fluorocult* ® *E. coli* 0157:H7 was the medium of choice as it contains β-D-glucuronide, a substrate used for the

identification of β-D-glucuronidase, which is characteristic for *E. coli* (Merck, Catalogue Number 1.10426 and Number 1.04036.0500). Although the activity of β-D-glucuronidase is a highly specific characteristic of most *E. coli* strains (enteropathogenic-EPEC, enterotoxin-forming-ETEC, entero-invasive -EIEC), enterohemorrhagic *E. coli* (EHEC) is not capable of forming β-D-glucuronidase (Merck, Catalogue Number 1.04036.0500). However, the PCR detection of water source isolates was limited only to the *uidA* gene as it has been observed by previous investigators that this gene codes for β-D-glucuronidase (Tsai *et al.*, 1993). The polymerase chain reaction analysis using *uidA*-specific primers confirmed that a genetic region homologous in size to the *E. coli uidA* structural gene, including the regulatory region, was present in *E. coli* isolates. It is therefore interesting to note that the PCR assay was successful in amplifying only the 147 bp *uidA* fragment that was present in the genomic DNA of the isolates. No amplified products could then be expected from EHEC isolates. Similar results were obtained in at least four independent experiments. This might explain the low frequency or absence of enterotoxigenic *E. coli* from the PCR assay. In contrast, the high frequency of presumptive *E. coli* in water sources by culturing methods might be linked to the presence of all *E. coli* types in water sources.

The concentration technique used in the present investigation for the isolation of presumptive *V. cholerae* was based on the isolation and selection of all colonies, which appeared yellow on Vibrio diagnostic agar plates. However, these yellow colonies might not only be different strains of *V. cholerae* (*V. cholerae* Inaba NIH 35, *V. cholerae*

Table 3 │ Number of water sources contaminated by *E. coli*, *V. cholerae*, *Salmonella* spp. and *S. typhimurium* during the study period (November 2001, January–March 2002)

Organism	Culturing		PCR *	
	Groundwater	Surface water	Groundwater	Surface water
E. coli	4 (100%)	4 (100%)	3 (75%)	1 (25%)
V. cholerae	4 (100%)	4 (100%)	1 (25%)	1 (25%)
S. typhimurium	4 (100%)	4 (100%)	0	0

* PCR from isolates.

EI Tor Inaba CH 38, *V. cholerae Ogawa* NIH 41, *V. cholerae EI Tor Ogawa* CH 60) but also *V. alginolyticus* (Merck, Catalogue Number. 1.10263.0500). This is therefore one of the possibilities that could explain the high percentage of presumptive *V. cholerae* using the culturing method. Since in the present study the PCR technique targetted only the *epsM* gene present in the genomic DNA of the isolates, no amplification product could be expected from the DNA isolates without this specific gene. Consequently the frequency of *V. cholerae* could automatically decrease.

All the water samples collected from both ground and surface water sources over a period of 4 months (November 2001, January–March 2002) tested positive for *Salmonella* spp (Tables 2 and 3). In contrast, the subsequent PCR analysis of these isolates revealed the absence of *S. typhimurium* in all water samples collected from both water sources, with the exception of two isolates obtained from Ngqele and Dyamala borehole water samples (results not shown in Tables 2 and 3), which showed the presence of the PCR products with a band above 314 bp, which is the expected size for the positive control strain. In this case one of the primers probably fitted onto a part of the gene closer to the other primer or both primers probably fitted onto a totally different gene, as stipulated by Vanfleteren (2004).

Although *E. coli* is part of the normal faecal flora of humans and animals, some strains can cause severe and life-threatening diarrhoea (Gray 1995). The pathogenic strains are important causes of diarrhoeal diseases in the world and remain a major public health problem for children and young infants (Levine *et al.* 1986). In South Africa, diarrhoea is responsible for about 20% of all deaths of one- to five-year-olds (MacKintosh & Colvin 2002) and an annual estimated 43 000 deaths and 3 million incidences of the illness, with an associated treatment cost of some R3.4 billion (Pegram *et al.* 1998). *Escherichia coli* is capable of causing urinary tract infection, neonatal meningitis and intestinal disease (caused by enterotoxin-forming *E. coli* (ETEC), entero-invasive *E. coli* (EIEC), enteropathogenic *E. coli* (EPEC), Enteroaggregative *E. coli* (EAggEC) and enterohemorrhagic *E. coli* (EHEC)). Therefore, the presence of this organism in drinking water sources may pose a serious health risk to the communities. A comparison between surface and ground water sources (Tables 2

and 3) revealed that three of the four groundwater sources were contaminated by pathogenic *E. coli* while only one out of the four surface water sources revealed the presence of pathogenic *E. coli*. Momba & Notshe (2003) reported the deterioration in the microbiological quality of groundwater in the Nkonkobe district. These water sources are characterised by a high number of faecal coliforms and heterotrophic bacteria, exceeding by far the limit allowed by the *South African Water Quality Guideline* for domestic use (Department of Water Affairs and Forestry 1996). It is well known that the inadequate design, construction, operation and maintenance of wells and boreholes can lead to quality problems related to groundwater. Poor sanitary sealing is more generally a common cause of microbiological quality deterioration (Momba & Notshe 2003).

The potential health problem posed by the consumption of water from groundwater sources by rural residents and consumers in the Ngqele, Dyamala and Ngwenya villages must not be underestimated. In semi-arid areas such as parts of this region of South Africa, groundwater remains the main water supply source for many communities. Some communities receive drinking water directly from uncovered or covered boreholes and wells whereas for others the water is drawn from the boreholes (using an engine) to a reservoir, and from the reservoir the water is delivered to the people through standpipes. In both cases groundwater is distributed to the community without any purification. The quality of the water may be improved by cleaning of the groundwater distribution system from the reservoir to the standpipes, the removal of organic and sediment organics from the water, the addition of a disinfectant or the boiling of drinking water before use.

It has long been known that cholera is a waterborne disease that is transmitted via water, with mostly surface water being implicated (Hughes *et al.* 1982). The present study, however, showed that groundwater could also be a vehicle for the transmission of cholera as *V. cholerae* was found at a rate of 25% in groundwater sources. Cholera is endemic in many rural communities in the Eastern Cape. Since October 2000, when a cholera epidemic first broke out in South Africa, 351 people have died, out of 124 613 reported cases. From the beginning of 2003 until 14 February, 666 cholera cases were reported in the Eastern Cape alone. On 23 February 2003, 14 people were reported

to have died and 1000 people from O R Tambo and Chris Hani districts were admitted to local clinics as the cholera epidemic hit the Eastern Cape Province (Jubasi & Padayachee 2003).

This study, and other studies on domestic consumption of water in rural communities of South Africa (Nevondo & Cloete 1999; Lehloesa & Muyima 2001; Obi *et al.* 2002; Momba & Notshe 2003), showed the challenges for health and water resources in South Africa. The provision of potable water for rural communities is important in order to satisfy their basic needs and it is easily seen as crucial for assessing social development in developing countries (Forch & Bremann, 1998). The Nkonkobe district and the Provincial Government as a whole are thus alerted to the need to address water supply problems in these rural communities.

CONCLUSION AND RECOMMENDATIONS

Using the PCR method, this study revealed that the prevalence of pathogenic *E. coli* was more implicated in groundwater sources (three out of four sources) than in surface water sources (one out of four sources) and the presence of *V. cholerae* being implicated in each of both surface and groundwater sources. Although culturing methods can detect pathogenic bacteria, PCR techniques are more reliable, since they are specific in the detection of a target bacterium. The study therefore recommends regular monitoring of drinking water sources in rural communities for the presence of pathogenic bacteria. Educating people in rural communities about pathogenic waterborne bacteria is also recommended.

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