Detection of *Vibrio cholerae* O1 in animal stools collected in rural areas of the Limpopo Province

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Abstract

Vibrio cholerae (*V. cholerae*), the causative agent of cholera, has been responsible for various outbreaks worldwide and may be associated with animal faeces. In an attempt to understand the occurrence of this organism in the environment, 230 faecal samples were collected from pigs, chickens, goats, donkeys, cows and pigeons in rural areas of the Limpopo Province. Bacterial DNA was extracted from the faecal samples using a guanidium thiocyanate-based method. The DNA was screened for the presence of the *sodB*, *rfb*, *FlaE,* 16S rRNA and *ctxA* genes associated with *V. cholerae*, *V. cholerae* O1, *V. cholerae* O139 using 2 multiplex polymerase chain reactions (m-PCR). The *V. cholerae sodB* gene was detected in 74 of the 230 samples tested. Detection rates for the faecal samples obtained from individual species were as follows: cows (55/74), chickens (8/74), goats (2/74), donkeys (4/74), pigs (3/74) and pigeons (2/74). *V. cholerae* O1 was detected in (17/74) cow and (3/74) chicken samples, of which (9/17) cow samples and (3/3) chicken samples tested positive for toxigenic *V. cholerae* O1. The presence of this organism in faecal samples, taken close to water sources used by the villagers, raises the possibility that the causative *V. cholerae* O1 strain of the most recent outbreak in South Africa was present in the area 6 months prior to the outbreak.

Keywords: *Vibrio cholerae*, PCR, animal faeces, cholera toxin

Introduction

Cholera, caused by *Vibrio cholerae*, is a severe epidemic diarrhoeal disease which continues to devastate many developing countries where socioeconomic conditions are poor, sanitary systems and public hygiene are rudimentary, and safe drinking water is not available (Chen et al., 2007; Theron et al., 2000; Hunter, 1997). In rural areas of most developing countries people still use water from communal sources for domestic purposes. The sources may be unimproved (hand-dug wells, unprotected springs, rivers), with low and seasonal flow rates, or improved sources (public taps, borehole or pumps, protected wells, protected springs or harvested rainwater) (Gundry et al., 2006). Nevondo and Cloete (1999) reported that rivers in most rural areas of South Africa become polluted due to upstream activities such as washing clothes, bathing and animal activities and due to lack of sanitation.

V. cholerae has been isolated from surface water (Fraga et al., 2007; Percival et al., 2004) and the occurrence of *V. cholerae* in water sources can be linked to faecal pollution (Cox et al., 2005). Domestic and farm animals have been shown to be carriers of *V. cholerae* strains, contributing to their sustained presence in the area (Visser et al., 1999; Sanyal et al., 1974). Among the 193 currently recognised O serogroups of *V. cholerae*, only O1 and O139 have caused epidemics of cholera (Fraga et al., 2007). More than 95% of the strains belonging to serogroups O1 and O139 produce the cholera toxin (CT), which is central to the disease process (Chakraborty et al., 2000). Lipp et al. (2003) reported that 2 genes, *rfb* and *wbe*, are associated with the synthesis of the O-antigen in *V. cholerae* O1 and *V. cholerae* O139 and can be used to distinguish the 2 serogroups from each other. The genes coding for the cholera toxin can also be used to distinguish between toxigenic and non-toxigenic *V. cholerae*.

The polymerase chain reaction (PCR) is a rapid, oligonucleotide primer-directed *in vitro* method for replicating defined DNA sequences from target organisms (Brasher et al., 1998). PCR primers have been reported that allow for specific detection of a range of targets that include species-, serogroup- and virulence-specific genes. As a consequence of the speed, specificity and sensitivity of PCR, the procedure has become one of the most widely-used assays for direct detection of low levels of pathogenic microbes in environmental samples (Theron and Cloete, 2004). PCR is not dependent on the culturability of bacteria, and requires no additional conformational steps (Mumy and Findlay, 2004), leading to a significant decrease in sample analysis time.

The present study investigated the occurrence of *Vibrio cholerae* in animal faecal samples collected in rural areas in the Vhembe region of the Limpopo Province in South Africa using 2 multiplex PCRs.

Material and methods

Bacterial strains

The bacterial strains used during this study were obtained from the National Health Laboratory Services (NHLS) of South Africa, American Type Culture Collection (ATCC) and National Collection of Type Cultures (NCTC). All the bacterial strains were stored at -70°C in Microbank™ cryovials (Pro-Lab

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Diagnostics). The strains were grown on nutrient agar (Oxoid®) at 37°C for 18 to 24 h.

Sample collection

A total of 230 faecal samples were randomly collected from the villages of the Vhembe region, during March and October 2008. The faecal samples were obtained from cows (147), chickens (38), donkeys (23), goats (10), pigs (6), dogs (4) and pigeons (2). The samples were collected aseptically as soon as possible after defecation and stored in sterile urine containers (Bioster) at 4°C until the samples were transported to the Water and Health Research Unit, Johannesburg Laboratory for analysis.

DNA extraction

An adaptation of the protocol reported by Boom et al. (1990) was used for this study. The changes included the use of the stool suspension as starting material. Approximately 0.15 g of each of the faecal samples was suspended in 1 mℓ phosphate buffered saline (pH 7.4; Sigma Aldrich, USA) containing 0.1% (vol/vol) Tween buffer (Merck, Germany) and homogenised by vortexing. Any debris that was not dissolved was removed by centrifugation for 15 s at 13 000 r/min, after which 200 uℓ of supernatant was transferred to a sterile 2 mℓ eppendorf tube to be used for the DNA extraction. To this, 700 µℓ L7A lysis buffer (120g guanidium thiocyanate, 0.1 M tris hydrochloride, 0.2 M EDTA, 2.6 g Triton X-100, 1 mg/ml ά-casein) was added and incubated at 70°C for 10 min. A volume of 250 µℓ 100% (vol/vol) ethanol was added to this mixture and further incubated at 56°C for 10 min. A celite solution (50 µℓ) was added and incubated at room temperature for 10 min (with occasional mixing). A sterile spin column prepared according to the method published by Borodina et al. (2003) was placed into a sterile 2 mℓ micro-fuge tube and the mixture loaded into the column. The mixture was loaded by adding approximately 500 µℓ into the column followed by centrifugation at 13 000 r/min for 30 s to separate the buffer from the celite. This step was repeated twice until all of the lysis mixture was loaded into the column. The column was washed twice with 400 µℓ wash buffer (120 g guanidium thiocyanate and 0.1 M tris hydrochloride) and twice with 400 µℓ of a 70% (vol/vol (ethanol solution separating the liquid and solid phase each time by centrifugation at 13 000 r/min for 30 s. The last wash step was followed by a 2 min centrifugation step at 13 000 r/min to ensure that all the ethanol was removed from the column. The columns were transferred into clean sterile 1.5 mℓ micro-fuge tubes and 100 µℓ elution buffer (AE buffer, Qiagen) was added to the columns and incubated for 2 min at 56°C. The DNA was eluted from the columns by centrifugation for 2 min at 13 000 r/min, after which the columns were discarded. The DNA containing AE buffer was collected into the 1.5 mℓ microfuge tube. A negative control was included by performing the DNA extraction methods with only the DNA extraction reagents. A positive control was prepared by extracting DNA from 1.5 mℓ culture grown overnight suspended in sterile water.

PCR assays

Two multiplex-PCR (m-PCR) assays were used for the detection and identification of *V. cholerae* species in the samples (Ntema, 2009). The 1st m-PCR targeted the *sodB* (*V. cholerae* species), *FlaE* (*V. parahaemolyticus* species) and 16S rRNA (*Vibrio* and *Enterobacteriaceae* species) genes (Tarr et al., 2007). The 2nd m-PCR targeted *V. cholerae* O1 and *V. cholerae* O139 *rfb* genes, *ctxA* (cholera toxin) and the 16S rRNA gene**.** For both the multiplex PCR's, the 16S rRNA primers were included as the positive internal control. The primers used for the amplification of these genes and the predicted sizes of the amplicons are shown in Table 1.

PCR reactions were performed in a Biorad Mycycler™ Thermal cycler in a total volume of 20 µℓ. Each reaction consisted of 10 µℓ 2x Qiagen m-PCR master mix (master mix contains HotStartTaq DNA Polymerase, m-PCR buffer with 2 mM $MgCl_2$, and dNTP Mix); 1 µℓ 5x Qiagen Q-solution; 1-5 µℓ genomic DNA and PCR grade water. Two microlitres (2 µℓ) of the primer mix was used for the PCR reaction which consisted of 0.5 µℓ of a 10 mM stock of each *V.c SodB* primer; 1 µℓ of a 10 mM stock of each *V.p FlaE* primer and 0.16 µℓ of a 10 mM stock of each 16S rRNA primer (Table 1) for the 1st multiplex. The thermal cycling profile consisted of a 15 min enzyme activation step at 95°C followed by 35 cycles of denaturing at 92°C for 40 s, annealing at 57°C for 60 s, extension at 72°C for 90 s and a final elongation at 72°C for 7 min.

For the 2nd m-PCR, 0.25 µℓ of a 10 mM primer stock for each primer (*V. cholerae* O1, *V. cholerae* O139, *ctxA*,

Table 1
Primers used for the multiplex PCR reactions

Primers	Sequence (5'-3')	Amplicon size (bp)	Reference
V.c SodB (F)	AAGACCTCAACTGGCGGTA	248	Tarr et al. (2007)
V.c SodB (R)	GAAGTGTTAGTGATCGCCAGAGT		
V.p FlaE (F)	GCAGCTGATCAAAACGTTGAGT	897	Tarr et al. (2007)
V.p FlaE (R)	ATTATCGATCGTGCCACTCA		
16S rRNA (F)	CGGTGAAATGCGTAGAGAT	663	Tarr et al. (2007)
16S rRNA (R)	TTACTAGCGATTCCGAGTTC		
V. cholerae O1(F)	GTTTCACTGAACAGATGGG	192	Lipp et al. (2003)
V. cholerae O1 (R)	GGTCATCTGTAAGTACAA		
V. cholerae O139 (F)	AGCCTCTTTATTACGGGTGG	449	Lipp et al. (2003)
V. cholerae O139 (R)	GTCAAACCCGATCGTAAAGG		
ctxA (F)	ACA GAG TGA GTA CTT TGA CC	308	Lipp et al. (2003)
ctxA (R)	ATA CCA TCC ATA TAT TTG GGA		

(F)-Forward Primer
(R)-Reverse Primer

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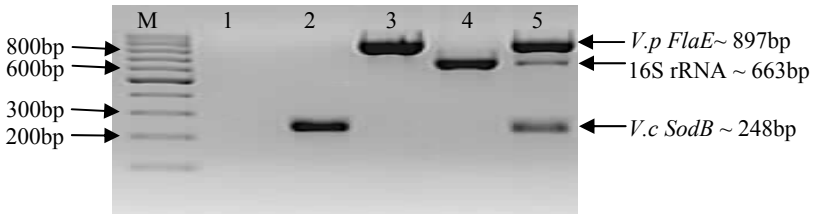

Figure 1
Agarose gel for the PCR product obtained for V. cholerae *species-specific gene (Lane 2),* V. parahaemolyticus *species-specific gene (Lane 3), the 16S rRNA gene (Lane 4) and the multiplex PCR (Lane 5) for the detection of the 2* Vibrio *species. The 100 bp DNA ladder is shown in Lane M and the no-DNA control in Lane 1.*

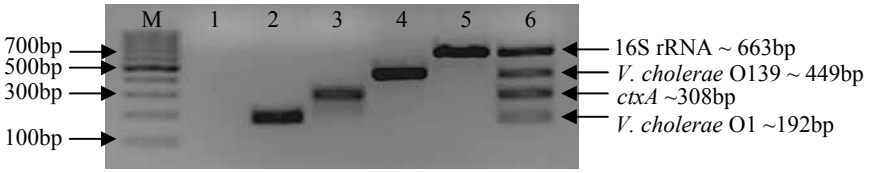

Figure 2
Agarose gel for the PCR products obtained for the V. cholerae*-O1 specific gene (Lane 2), cholera toxin (Lane 3),* V. cholerae*-O139 specific gene (Lane 4), the 16S rRNA gene (Lane 5) and the multiplex PCR (Lane 7). The 100 bp DNA ladder is shown in Lane M and the no-DNA control in Lane 1.*

16S rRNA) was added to the reaction mixture. Each reaction consisted of 10 µℓ 2x Qiagen m-PCR master mix 1 µℓ 5x Q-solution; 1-5 µℓ genomic DNA and PCR grade water. The m-PCR reactions were subjected to an enzyme activation step at 95°C for 15 min, followed by 35 cycles of denaturing at 94°C for 45 s, annealing at 55°C for 45 s, extension at 72°C for 60 s and a final elongation step at 72°C for 5 min.

Electrophoresis and visualisation of PCR products

DNA was analysed in a horizontal agarose slab gel (2.5% (weight/vol)) with ethidium bromide (0.5 µg/mℓ) in TAE buffer (40 mM tris acetate; 2 mM EDTA, pH8.3). The agarose gel was electrophoresed for 1 to 2 h at 80 to 100 V. The DNA was visualised with UV light (Gene Genius Bio Imaging System, Vacutec®). The relative sizes of the DNA fragments were estimated by comparing their electrophoretic mobility with 100 bp markers (Fermentas O' GeneRuler DNA ladder; Canada) that were run with the samples.

Results and discussion

The aim of the study was to investigate the occurrence of *V. cholerae* species in animal faecal matter using PCR. To achieve this DNA was extracted from cow (147), chicken (38), donkey (23), goat (10), pig (6), dog (4) and pigeon (2) faecal samples and used as template for the multiplex PCR reactions. The m-PCR's were performed sequentially with all samples first tested for the presence of *V. cholerae* species specific sequences with the 1st m-PCR (Fig. 1). Samples that tested positive for the presence of *V. cholerae* species were subsequently tested for the presence of *V. cholerae* O1 or *V. cholerae* O139 specific O-antigen biosynthesis genes as well as the cholera toxin gene with the 2nd m-PCR (Fig. 2).

A total of 230 faecal samples were screened with the 1st m-PCR for the presence of *V. cholerae* (Table 2). Amplification of the 16S rRNA target from all samples indicated that bacterial DNA had been extracted and inhibitors had not suppressed amplification. The 16S rRNA primers included in the PCR

Table 2
PCR results obtained for the detection of *V. cholerae* O1, *V. cholerae* O139 and cholera toxin genes in the faecal samples with the multiplex PCRs

Faecal source (No of samples)	Stool description	n	*V. cholerae*	*V. cholerae* O1	Cholera toxin	*V. cholerae* O139
Cow (n=147)	Dry	8	5	0	0	0
	Fresh	121	42	17	9	0
	Moist	18	8	0	0	0
Chicken (n=38)	Dry	18	3	3	3	0
	Fresh	7	2	0	0	0
	Moist	13	3	0	0	0
Goat (n=10)	Dry	2	0	0	0	0
	Fresh	5	2	0	0	0
	Moist	3	0	0	0	0
Donkey (n=23)	Dry	4	2	0	0	0
	Fresh	1	0	0	0	0
	Moist	18	2	0	0	0
Pig (n=6)	Dry	1	0	0	0	0
	Fresh	0	0	0	0	0
	Moist	5	3	0	0	0
Pigeon (n=2)	Dry	2	2	0	0	0
Dog (n=4)	Dry	1	0	0	0	0
	Fresh	2	0	0	0	0
	Moist	1	0	0	0	0

targets the 16S rRNA gene of *Enterobacteriaceae* (Tarr et al., 2007) and were used as the internal control in the PCR.

The *V. cholerae sodB* gene was detected in 32% (74/230) of the samples tested of which 74% (55/74) were cow faecal samples. The rest of the *sodB* positive samples were from chickens (8/74; 11%), goats (2/74; 3%), donkeys (4/74; 5%), pigs (3/74; 4%) and pigeons (2/74; 3%). Although the *sodB* gene was not detected in all of the samples, it was present in samples from all of the types of animals targeted, with the single exception of dogs. The *V. cholerae* O1 *rfb* gene was detected in only 27% (20/74) of the *sodB* positive samples and was restricted to samples obtained from cows (17/20; 85%) and chickens (3/20; 15%). Only (9/17; 53%) of the *V. cholerae* O1 positive cow samples showed the presence of the gene encoding the cholera toxin whereas all of the *V. cholerae* O1 positive chicken samples tested positive for the cholera toxin. No *Vibrio cholerae* O139 was detected in any of the samples.

Contrary to what would be expected, the occurrence of the *V. cholerae* in the samples could not be linked to the freshness of the faecal sample. *V. cholerae* was detected more often in fresh cow faecal samples compared to chickens where the *V. cholerae* was detected more in the dry faecal samples, especially *V. cholerae* O1. No conclusion about the survival and occurrence of *V. cholerae* can, however, be drawn at this stage, as factors such as sample collection during different seasonal conditions, animal age and herd immunity can all have an effect on the occurrence and detection of the organism (Cox et al., 2005).

The detection of *V. cholerae* in the animal faeces collected confirms reports that *V. cholerae* do occur in cow, chicken, dog and pig faecal samples (Visser et al., 1999; Sanyal et al., 1974). The one drawback experienced with this study was that although *V. cholerae* was detected nothing can be said about the viability of the organisms. PCR amplifies fragments of DNA that are present in dead and viable cells making it impossible to comment on the viability of the organism detected (Mieta, 2009).

Conclusion

V. cholerae, as well as *V. cholerae* O1, was detected in faecal samples obtained from animals in rural areas in the Vhembe region of the Limpopo Province, using a PCR-based technique. In most cases the animals were free to roam the area, including the areas around rivers and springs that are used as drinking water sources for some of the villages. Since *V. cholerae* could be detected in the animal faeces it can be concluded that the organism could find its way into the water sources as reported by Cox et al. (2005). Interestingly this area was part of the 2008/2009 cholera outbreak that started in Zimbabwe and spread to this province. This raises the possibility that the causative agent for the outbreak was introduced into the area from Zimbabwe or that it could have been present in the area 6 months before the outbreak started, during the time that the samples were collected.

It is important that attention is paid to the occurrence of *V. cholerae* O1 in animal faeces and to its possible health impact in an area where animal faeces are collected and used for agriculture, as well as construction and decorative purposes, in homes. Mpazi and Mntika (2005) reported that the spread and persistence of *V. cholerae* in rural areas can be attributed to the belief held by villagers that *V. cholerae* cannot be transmitted through cow and chicken faeces. It is therefore important that these uses of animal faeces should be kept in mind and included in analysis during diarrhoeal outbreaks.

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