CHAPTER 3

POLYMERASE CHAIN REACTION BASED IDENTIFICATION AND TOXIN GENE SCREENING OF *VIBRIO CHOLERAE*

3.1 INTRODUCTION

Cholera, caused by enterotoxigenic *Vibrio cholerae*, is still a major health concern in many developing countries where water supply infrastructure is insufficient. During 2001 more than 180 000 cholera cases from 58 countries where reported to the World Health Organization but it is believed that this figure does not reflect the overall burden of the disease due to widespread underreporting and surveillance limitations. Fifty eight percent of the cases reported during 2001 were from South Africa, representing one of the worst outbreaks in the recent history of the country (WHO 2003, Department of Health, South Africa 2004).

Non-enterotoxigenic *Vibrio cholerae* strains have not been implicated in epidemics, but pose a health threat to individuals due to alternative methods of pathogenesis (Ogawa *et al.*, 1990). Further more it has been shown that some non-enterotoxigenic *V. cholerae* strains can be converted to enterotoxin producing strains through invasion of bacteriophage CTXϕ and the associated addition of genes (Faruque *et al.*, 1998).

Vibrio cholerae is known to be an autochthonous inhabitant of brackish as well as fresh water aquatic systems (Colwell and Huq, 1994; Halpern *et al*., 2003). Even aquatic environments not suited for proliferation may harbour viable but non-culturable *V. cholerae* that may at a later stage pose a health threat (Colwell and Huq, 1994). These aquatic systems may act as a natural reservoir for current and future epidemic strains, especially due to the ability of non-toxigenic *V. cholerae* strains to be converted to enterotoxigenic strains (Faruque *et al*., 1998). Being able to effectively monitor inland aquatic systems can help protect vulnerable populations from potential cholera outbreaks. A reliable surveillance system needs to satisfy two criteria: firstly it must be able to rapidly identify all *Vibrio cholerae* strains, and secondly it must give insight into the virulence potential of the detected strains.

Identification of *Vibrio cholerae* is usually achieved through a series of biochemical tests after isolation of the bacterium on selective media. The major disadvantage of this approach is that it is laborious and time consuming. Close-relatedness with other members of the genus *Vibrio* and *Aeromonas* has also made the identification of *Vibrio cholerae* difficult (Farmer III and Hickman-Brenner, 1992). Many of the problems associated with biochemical identification of bacteria can be overcome by targeting species-specific genes with techniques such as the polymerase chain reaction (PCR). The PCR approach is not only labour effective, but is also a rapid, reliable and extremely sensitive technique (Saiki *et al*., 1988). The need was therefore recognized for a PCR based detection method that would offer these advantages and could form part of an environmental surveillance system.

The screening of *V. cholerae* virulence genes using PCR could also prove to be of value, as was showed by Singh *et al*. (2002), here a hexaplex PCR approach was used to rapidly screen isolates for the presence of virulence and regulatory genes. In *Vibrio cholerae* the *ctxAB* and *tcpA* genes are known to play a cardinal role in maintaining virulence. The *ctxAB* genes code for cholera toxin which is the main *Vibrio cholerae* virulence factor, while *tcpA* codes for the toxin-coregulated pili (TCP) - a colonization factor that also acts as a receptor for bacteriophage CTXϕ. Strains carrying the genetic potential for cholera toxin and TCP production are potential epidemic strains, while strains carrying only the *tcp* genes could be converted to enterotoxigenic strains by addition of bacteriophage CTXϕ genetic elements. A multiplex PCR targeting these genes could give an indication of the current enterotoxigenic capability of screened environmental strains, as well as give an indication of the possibility of new epidemic strains arising from autochthonous environmental strains in the near future

The aim of this research was to develop a sensitive and reliable PCR based detection technique for the detection for all *Vibrio cholerae* strains regardless of toxigenic potential. This detection method was evaluated by screening strains previously identified as *Vibrio cholerae* using biochemical tests. The screening of *V. cholerae* isolates for other virulence associated genes was also performed.

3.2 MATERIALS AND METHODS

3.2.1 Acquisition and Maintenance of Cultures

Environmental *Vibrio cholerae* isolates were obtained from Rand Water, Vereeniging, South Africa (Table 6). The strains were isolated on TCBS agar over a two-year period from 15 different sampling sites in the Vaal Barrage Catchment, South Africa (Table 5, Fig 1). The sampling sites were located in three linked river systems – the Vaal river containing sampling sites VRB47, VRB30 and RV2, the Suikerbosrant and Blesbokspruit rivers (B10, S1 and S2), and lastly the Rietspruit, Natalspruit and Klip River system (R5, R6, N8, R25, K18 and K19) The Suikerbosrant and Klip River systems flow into the greater Vaal River between sampling sites VRB47 (upstream) and VRB30 (downstream). An Enterotoxigenic *Vibrio cholerae* O1 was obtained from the National Collection of Type Cultures, Public Health Laboratory Services, London (Strain: NCTC5941). Three clinical *Vibrio cholerae* O1 (Enterotoxigenic) strains were obtained from National Health Laboratory Services, Johannesburg, South Africa. These isolates originated from cholera cases in the Kwazulu-Natal and Mapumalanga provinces. *Vibrio cholerae* cultures were maintained on Nutrient Agar (Biolab, Merck C1) or alternatively in Nutrient Broth No. 2 (Oxoid CM67). Agar plates were incubated at 37 °C for 24 hours and were stored at room temperature. Isolates were sub-cultured on a fortnightly basis. Long-term storage of strains (up to three years) was achieved using Microbank™ Cryobeads (Davies Diagnostics). The manufacturers protocol was followed and the beads stored at –70 °C.

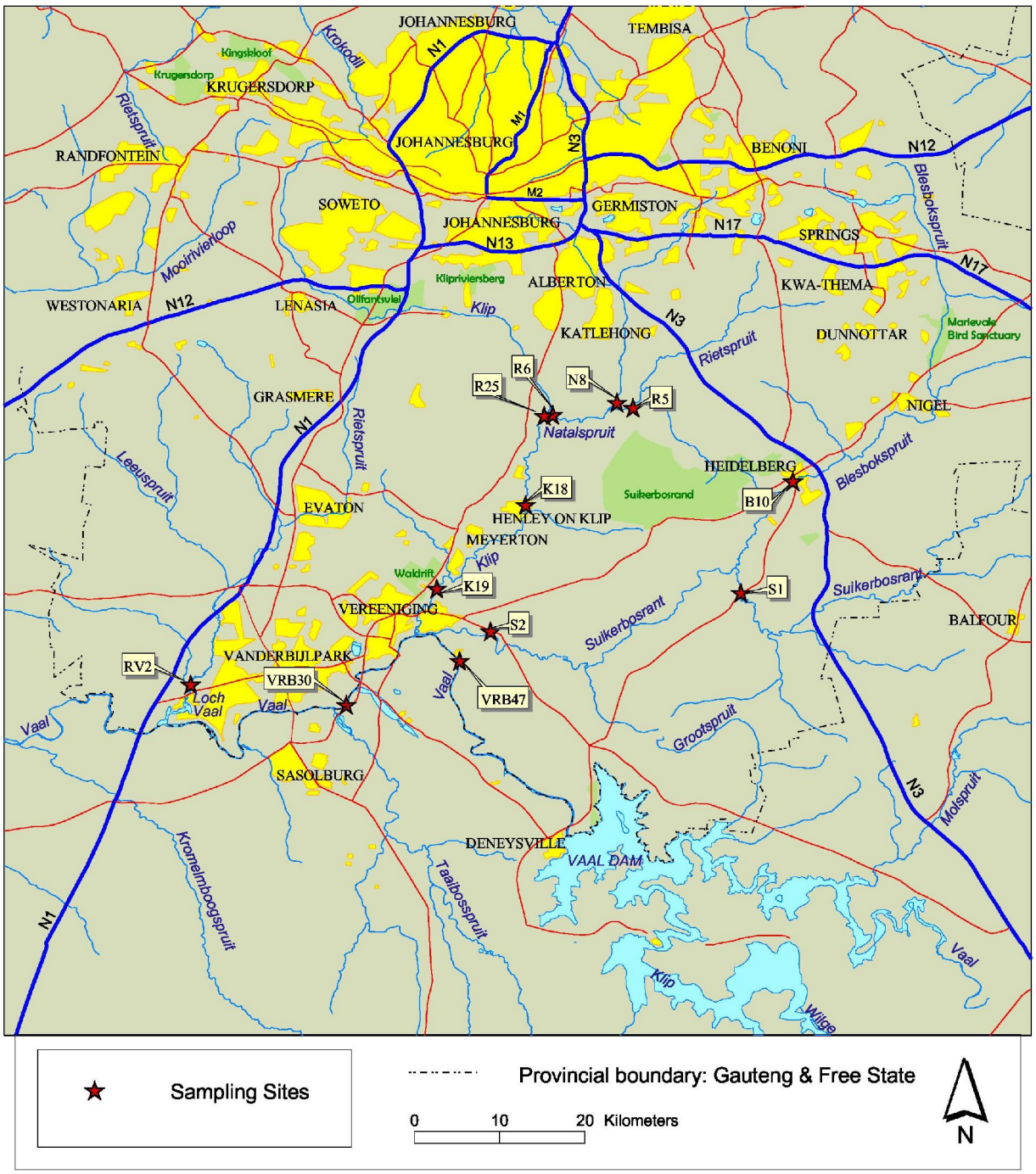

Fig 1. **Sampling sites in the Vaal Barage Catchment where water was sampled for the presence of *Vibrio cholerae*.**

Table 5. River sampling sites in the Vaal Barrage Catchment

Sampling Site	Description
A18 *	Raw water pipeline at Vereeniging
B6 *	Distribution main at Zuikerbosch
B10	Blesbokspruit near Heidelberg
K18	Klip River at Henley-on-klip
K19	Klip River weir at Redan Train Bridge
K25	Klip River
M-Canal *	Canal at Zuikerbosch from Vaal Dam
N8	Natalspruit at Heidelberg road
R5	Rietspruit at Hardman's Farm
R6	Rietspruit weir at Luttig's Farm
RV2	Rietspruit weir at Loch Vaal
S1	Suikerbosrand River near Heidelberg
S2	Suikerbosrand River weir at Three Rivers
VRB30	Vaal River Barrage (Ascott Bridge)
VRB47	Vaal River Barrage (Zb pump station)

* Sites marked with an asterisk are not shown on the accompanying map (Fig 1.)

Table 6. *Vibrio cholerae* isolates obtained from sampling sites in the Vaal Barrage catchment

Isolate Number	Isolation Site	Isolation Date
1	B10	20-Dec-2000
2	R6	20-Dec-2000
3	N8	20-Dec-2000
11	S2	8-Jan-2001
12	A18	8-Jan-2001
13	VRB47	15-Jan-2001
14	VRB30	15-Jan-2001
35	BUV SECUNDA	22-Jan-2001
45	M-CANAL	29-Jan-2001
46	K18	29-Jan-2001
50	VRB30	29-Jan-2001

Isolate Number	Isolation Site	Isolation Date
53	A18	5-Feb-2001
68	SSE	5-Feb-2001
67	Vass	5-Feb-2001
156	R5	19-Mar-2001
160	S2	19-Mar-2001
171	R6	26-Mar-2001
174	S2	26-Mar-2001
178	B10	2-Apr-2001
194	K19	9-Apr-2001
195	K25	9-Apr-2001
209	N8	23-Apr-2001
217	S2	23-Apr-2001
226	K25	30-Apr-2001
235	A18	7-May-2001
240	K25	7-May-2001
241	R6	7-May-2001
244	S2	7-May-2001
256	RV2	14-May-2001
269	R6	21-May-2001
289	B10	4-Jun-2001
303	K18	11-Jun-2001
304	K19	11-Jun-2001
306	R5	11-Jun-2001
373	K18	27-Aug-2001
384	A18	9-Sep-2001
391	R6	9-Sep-2001
395	M-CANAL	9-Sep-2001
400	N8	1-Oct-2001
401	K18	1-Oct-2001
402	K19	1-Oct-2001
433	R6	29-Oct-2001
435	S1	29-Oct-2001
446	R5	12-Nov-2001
448	RV2	12-Nov-2001
449	S1	12-Nov-2001
454	A18	26-Nov-2001
455	B10	26-Nov-2001
455A	B10	26-Nov-2001
456	N8	26-Nov-2001
456A	N8	26-Nov-2001

Isolate Number	Isolation Site	Isolation Date
464A	S2	26-Nov-2001
465A	M-CANAL	7-Jan-2002
496	A18	7-Jan-2002
500	N8	7-Jan-2002
501	N8	7-Jan-2002
502	K18	7-Jan-2002
504	K25	7-Jan-2002
505	K25	7-Jan-2002
506	R5	7-Jan-2002
510	S2	7-Jan-2002
515	B10	14-Jan-2002
517	N8	14-Jan-2002
520	K19	14-Jan-2002
521	K19	14-Jan-2002
522	K25	14-Jan-2002
523	K25	14-Jan-2002
528	RV2	14-Jan-2002
529	RV2	14-Jan-2002
530	S1	14-Jan-2002
531	S1	14-Jan-2002
532	S2	14-Jan-2002
534	M-CANAL	14-Jan-2002
535	M-CANAL	14-Jan-2002
569	A18	11-Feb-2002
576	K19	11-Feb-2002
578	K25	11-Feb-2002
590	M-CANAL	11-Feb-2002
591	M-CANAL	11-Feb-2002
598	B10	25-Feb-2002
599	B10	25-Feb-2002
600	N8	25-Feb-2002
601	N8	25-Feb-2002
602	K18	25-Feb-2002
605	K19	25-Feb-2002
606	K25	25-Feb-2002
607	K25	25-Feb-2002
610	R6	25-Feb-2002
612	RV2	25-Feb-2002
613	RV2	25-Feb-2002
615	S1	25-Feb-2002

Isolate Number	Isolation Site	Isolation Date
616	S2	25-Feb-2002
617	S2	25-Feb-2002
618	M-CANAL	25-Feb-2002
619	M-CANAL	25-Feb-2002
622	VRB47	27-Feb-2002
625	A18	11-Mar-2002
628	N8	11-Mar-2002
629	N8	11-Mar-2002
632	K19	11-Mar-2002
637	R5	11-Mar-2002
640	RV2	11-Mar-2002
641	RV2	11-Mar-2002
643	S1	11-Mar-2002
644	S2	11-Mar-2002
645	S2	11-Mar-2002
650	VRB47	13-Mar-2002
655	B10	25-Mar-2002
656	B10	25-Mar-2002
661	N8	25-Mar-2002
671	S1	25-Mar-2002
672	S1	25-Mar-2002
681	A18	15-Apr-2002
746	R5	13-May-2002
750	RV2	13-May-2002
882	R6	27-Jul-2002
983	K25	23-Sep-2002
984	K25	23-Sep-2002
991	RV2	23-Sep-2002
995	S2	23-Sep-2002
1010	N8	30-Sep-2002
1011	R5	30-Sep-2002
1012	R5	30-Sep-2002
1020	S2	30-Sep-2002
1027	A18	14-Oct-2002
1028	A18	14-Oct-2002
1029	B10	14-Oct-2002
1030	B10	14-Oct-2002
1040	VRB30	14-Oct-2002
1053	VRB47	14-Oct-2002
1054	VRB47	14-Oct-2002

Isolate Number	Isolation Site	Isolation Date
1057	B10	20-Oct-2002
1059	K18	20-Oct-2002
1072	RV2	20-Oct-2002
1073	S1	20-Oct-2002
1074	S1	20-Oct-2002
1075	S2	20-Oct-2002
1085	B10	11-Nov-2002
1094	N8	11-Nov-2002
1097	R6	11-Nov-2002
1103	S2	11-Nov-2002
1104	S2	11-Nov-2002
1143	B10	9-Dec-2002
1147	K19	9-Dec-2002
1148	K25	9-Dec-2002
1149	K25	9-Dec-2002
1152	R5	9-Dec-2002
1153	R5	9-Dec-2002
1154	R6	9-Dec-2002
1155	R6	9-Dec-2002
1156	RV2	9-Dec-2002
1157	RV2	9-Dec-2002
1158	S2	9-Dec-2002
1159	S2	9-Dec-2002
1168	B10	6-Jan-2003
1169	B10	6-Jan-2003
1171	K18	6-Jan-2003
1174	K25	6-Jan-2003
1175	K25	6-Jan-2003
1176	N8	6-Jan-2003
1177	N8	6-Jan-2003
1178	R5	6-Jan-2003
1180	R6	6-Jan-2003
1181	R6	6-Jan-2003
1182	RV2	6-Jan-2003
1184	S2	6-Jan-2003
1190	B10	13-Jan-2003
1193	K18	13-Jan-2003
1199	N8	13-Jan-2003
1203	R6	13-Jan-2003
1204	RV2	13-Jan-2003

Isolate Number	Isolation Site	Isolation Date
1205	RV2	13-Jan-2003
1212	VRB47	15-Jan-2003
1213	VRB47	15-Jan-2003
1216	B10	27-Jan-2003
1218	K18	27-Jan-2003
1228	R6	27-Jan-2003
1232	S1	27-Jan-2003
1235	S2	27-Jan-2003
1242	A18	10-Feb-2003
1243	A18	10-Feb-2003
1244	B10	10-Feb-2003
1245	B10	10-Feb-2003
1246	K18	10-Feb-2003
1247	K18	10-Feb-2003
1250	K25	10-Feb-2003
1251	K25	10-Feb-2003
1252	N8	10-Feb-2003
1253	N8	10-Feb-2003
1254	R5	10-Feb-2003
1255	R5	10-Feb-2003
1256	R6	10-Feb-2003
1257	R6	10-Feb-2003
1258	RV2	10-Feb-2003
1259	RV2	10-Feb-2003
1264	M-CANAL	10-Feb-2003
1265	M-CANAL	10-Feb-2003
1267	VRB30	12-Feb-2003
1268	VRB47	12-Feb-2003
1269	VRB47	12-Feb-2003
1272	B10	24-Feb-2003
1274	K18	24-Feb-2003
1290	S2	24-Feb-2003
1294	VRB30	26-Feb-2003
1295	VRB30	26-Feb-2003
1296	VRB47	26-Feb-2003
1297	VRB47	26-Feb-2003
1300	B10	10-Mar-2003
1301	B10	10-Mar-2003
1302	K18	10-Mar-2003
1303	K18	10-Mar-2003

Isolate Number	Isolation Site	Isolation Date
1304	K19	10-Mar-2003
1305	K19	10-Mar-2003
1306	K25	10-Mar-2003
1307	K25	10-Mar-2003
1310	R6	10-Mar-2003
1311	R6	10-Mar-2003
1312	RV2	10-Mar-2003
1317	S2	10-Mar-2003
1320	VRB30	12-Mar-2003
1321	VRB30	12-Mar-2003
1322	VRB47	12-Mar-2003
1323	VRB47	12-Mar-2003
1331	K19	24-Mar-2003
1341	RV2	24-Mar-2003
1343	S1	24-Mar-2003
1344	S2	24-Mar-2003
1355	K19	24-Mar-2003
1358	N8	31-Mar-2003
1361	R5	31-Mar-2003
1362	R6	31-Mar-2003
1398	VRB30	29-Apr-2003
1405	B10	29-Apr-2003
1406	K18	29-Apr-2003
1408	K19	29-Apr-2003
1410	K25	29-Apr-2003
1411	K25	29-Apr-2003
1412	N8	29-Apr-2003
1413	N8	29-Apr-2003
1420	S2	29-Apr-2003
1424	VRB30	30-Apr-2003
1425	VRB30	30-Apr-2003
1438	N8	12-May-2003
1447	S2	12-May-2003
1489	K25	9-Jun-2003
1570	N8	28-Jul-2003
1571	N8	28-Jul-2003
1577	RV2	28-Jul-2003
1585	VRB30	30-Jul-2003
1586	VRB47	30-Jul-2003
1587	VRB47	30-Jul-2003

Isolate Number	Isolation Site	Isolation Date
1644	A18	8-Sep-2003
1651	K19	8-Sep-2003
1654	N8	8-Sep-2003
1657	R5	8-Sep-2003
1660	RV2	8-Sep-2003
1721	VRB30	14-Oct-2003

3.2.2 PCR Detection of Target Genes

3.2.2.1 DNA extraction

DNA was extracted from the bacterial strains by a rapid boil-lysate technique for PCR tests. Bacterial colonies, after overnight cultivation on Nutrient Agar plates, were suspended in 100 µL sterile water (Whitehead Scientific P1193). The bacteria were lysed by boiling the bacterial suspension for 10 minutes in a water bath. Centrifugation (10,000g for 1 minute) was used to remove cell debris, and the supernatant containing the DNA was used as template in the PCR reaction. The DNA was either used immediately or stored at –20 °C until required. The DNA used for 16S rDNA sequencing was extracted and prepared using the Dneasy Tissue Kit (Qiagen 69504). The manufacturer's protocol was followed, and a final elution of 100µL buffer was used to obtain a high concentration of DNA.

3.2.2.2 Selection of Primers

The three primers VIB1, VIB2, and VIB3 (Table 7) used in the PCR detection of *Vibrio cholerae* isolates were designed by Nandi *et al.* (2000) on the basis of *ompW* nucleotide sequence data. For amplification of part of the *ctxA* gene primer CTX2 and CTX3 was used, primer CTX2 and CTX3 was designed by Fields et al. (1992). Primers targeting the Classical and El Tor variants of the *tcpA* gene (Table 7) were designed by

Mukhopadhyay *et al*. (2001). Universal 16S primers fD1 and rP2 (Table 7) were used for 16S rDNA amplification (Weisburg *et al*., 1991). All primers were synthesized by Inqaba Biotech, South Africa.

Table 7. Sequences of PCR primers

Primer	Sequence	**Amplicon Size**
VIB1	5' – CACCAAGAAGGTGACTTTAATTGTG – 3'	-
VIB2	5' – GAACTTATAACCACCGCG – 3'	**VIB1 + VIB2 588bp**
VIB3	5' – GGTTTGTCGAATTAGCTTCACC – 3'	**VIB1 + VIB3 304bp**
CTX2	5' – CGGGCAGATTCTAGACCTCCTG – 3'	-
CTX15	5' – GAGTATGGAATCCCACCTAAAGC – 3'	**CTX2 + CTX15 564bp**
tcpA-F(Cla)	5' – CACGATAAGAAAACCGGTCAAGAG – 3'	-
tcpA-R(Cla)	5' – ACCAAATGCAACGCCGAATGGAGC – 3'	**tcpA-F(Cla) + tcpA-R(Cla) 617bp**
tcpA-F(Elt)	5' – GAAGAAGTTTGTAAAAGAAGAACAC – 3'	-
tcpA-R(Elt)	5' – GAAAGGACCTTCTTTCACGTTG – 3'	**tcpA-F(Elt) + tcpA-R(Elt) 471bp**
fD1	5' – AGAGTTTGATCCTGGCTCAG – 3'	-
rP2	5' – ACGGCTACCTTGTTACGACTT – 3'	**Aprox. 1500bp**

3.2.2.3 DNA Amplification

3.2.2.3.1 Targeting the *ompW* gene

Amplification of the *ompW* target sequence was carried out in a PCR reaction containing either primer set VIB1 and VIB2 or primer set VIB1 and VIB3. PCR amplification of the targeted DNA was carried out in a thermal cycler (Perkin-Elmer 2400 Gene-amp and Perkin-Elmer 2700 Gene-amp, PE Applied Biosystems, Weiterstadt, Germany) in 200 µL PCR tubes with a reaction mixture volume of 50 µL. Each of the reaction mixtures

contained 10 µL of bacterial lysate, 0.2 µM of each of the dNTP's (Abgene Ab-11.24), 2 mM MgCl2 (JMR 801), 1X Reaction buffer (JMR 801), 1 µM of each of the primers and 1 unit Taq DNA polymerase (JMR 801). PCR parameters for *OmpW* amplification: Initial denaturation 94°C for 3 minutes followed by 30 cycles of amplification consisting of denaturation at 94°C for one minute, primer annealing at 62°C for one minute and DNA extension at 72°C for one minute. Synthesis of the strands was completed at 72°C for 7 min. Negative controls were included, which were made of reaction mixtures containing all the reagents but without the bacterial lysate added. The amplification products were separated by electrophoresis (Amersham Pharmacia Biotech EPS 601) in 1% or 1.5% agarose gels (Hispanagar D-1 LE), visualisation was achieved by ethidium bromide staining (10mg/ml) and UV transillumination. A molecular size marker (Molecular weight marker XIV, Roche) was used to verify the size of the amplicons.

3.2.2.3.2 Targeting the *ctxA* and *tcpA* genes

Amplification of the targeted virulence genes were carried out essentially the same as described for the *OmpW* gene, in this case being a multiplex PCR containing the primers directed at the *ctxA*, *tcpA* Classical and *tcpA* El Tor genes (Table 7). The PCR parameters for amplification of virulence genes (*ctxA* and *tcpA*) were as follows: Initial denaturation at 94°C for 3 minutes followed by 30 amplification cycles consisting of denaturation at 94°C for one minute, primer annealing at 55°C for one minute and DNA extension at 72°C for one minute. Synthesis was completed at 72°C for 7 min.

3.2.3 16S rDNA Sequencing

For sequence-based identification of isolates a thermal cycler (Perkin-Elmer Gene-Amp 2700, Applied Biosystems) was used to amplify a portion of the 16S rDNA. Each of the 25µL reactions contained 0.2µM of each dNTP (Abgene Ab-11.24), 2 mM MgCl2 (JMR 801), 1X reaction buffer (JMR 801), 1 µM fD1 universal 16S primer, 1 µM rP2 universal 16S primer (Table 7, synthesized by Inqaba Biotech), 1 unit Taq polymerase (JMR 801) and 5µL bacterial DNA extraction (Dneasy Tissue Kit 69504, Qiagen). During the PCR reaction, heat denaturation at 94°C for three minutes was followed by 30 cycles consisting of heat denaturation at 94°C for one minute, primer annealing at 55°C for one minute and DNA extension at 72°C for one minute. After the last cycle was completed, the reaction sample was kept at 72°C for seven minutes to complete synthesis of all strands. The synthesised PCR product was purified using QIAquick PCR purification kit (Qiagen 28104). Each of the 5µL sequencing reactions contained 2µL BigDye Terminator v3.1 reaction mixture (Applied Biosystems), 10 µM fD1 universal 16S primer and 1 µL of the purified PCR product. For the reaction, an initial denaturation step at 96°C for five seconds was followed by 25 cycles that consisted of denaturation at 96°C for five seconds, annealing at 50°C for five seconds and extension at 60°C for 4 minutes. The product of the reaction was purified and loaded onto an automated sequencer (ABI prism 3100) for final analysis. The DNA sequences were subjected to BLAST searches at the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov), the *Megablast* and *Blastn* functions were used to search within the *NR* Database with the sequences of highest similarity taken to be the closest relative.

3.3 RESULTS

3.3.1 Identification of *Vibrio cholerae*

Two primer-pair combinations, VIB1/VIB2 and VIB1/VIB3, were initially evaluated. The combination of primer VIB1 and primer VIB2 gave unspecific amplification, for five of the 40 isolates screened, resulting in multiple amplicons instead of the expected 588bp amplicon (Fig 2). All *Vibrio cholerae* screened with the primer combination of VIB1 and VIB3 gave only the expected amplicon of 304 bp. Based upon this evaluation, the VIB1 and VIB3 primer combination was selected for further use in this study.

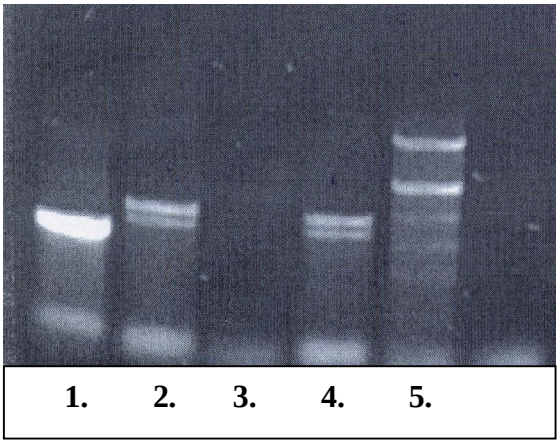

Figure 2 **Amplification products of PCR with primers VIB1 and VIB2 directed at the *ompW* gene. PCR was performed using template DNA from phenotypically identified *V. cholerae* isolates. Note the multiple unspecific bands in lanes 2,4 and 5.**

A total of 251 isolates, which were initially identified as *Vibrio cholerae* using the API 20e and VITEK biochemical identification systems, were screened using the primer set VIB1/VIB3 (Fig 3). Twenty-five of these isolates did not produce an amplicon with the

selected primer pair; these were designated as PCR-negative. Out of these PCR-negative isolates 14 were randomly selected and subjected to partial 16S rDNA sequencing (Table 8), 10 were found to be non-*Vibrio cholerae* isolates (mostly Aeromonas) while 4 were identified as *Vibrio cholerae*. To act as positive and negative controls the identity of four *V. cholerae* and seven *Aeromonas* isolates identified as such by all three identification methods were verified by means of sequencing. All were confirmed to be either *V. cholerae* or *Aeromonas* sp. respectively.

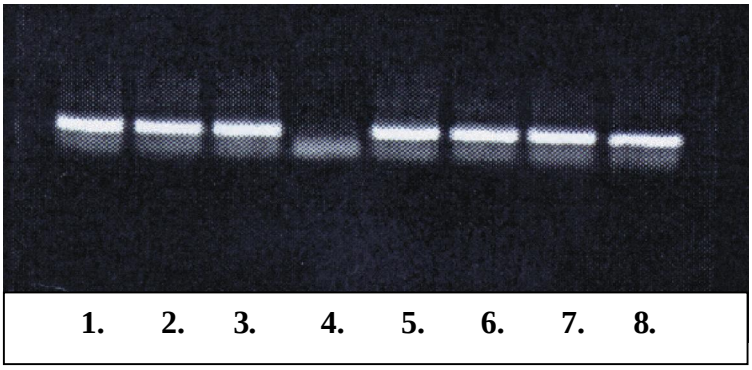

Figure 3 **Amplification products of PCR performed using primers VIB1 and VIB3 to amplify a section of the *ompW* gene (304bp fragment). Template DNA from phenotypically identified *Vibrio cholerae* isolates. The isolate in lane 4 regarded as PCR negative due to the lack of an amplicon.**

Table 8 **BLAST results for selected *ompW*-PCR negative isolates**

Isolate Number	Api/Vitek identification	Identification via 16S sequencing
14	Vibrio cholerae	*Aeromonas sp.*
35	Vibrio cholerae	Unidentified Marine Bacterium
45	Vibrio cholerae	*Aeromonas sp.*
68	Vibrio cholerae	*Aeromonas sp.*
194	Vibrio cholerae	*Aeromonas sp.*
303	Vibrio cholerae	*Aeromonas sp.*
384	Vibrio cholerae	*Aeromonas sp.*
395	Vibrio cholerae	*Aeromonas sp.*
500	Vibrio cholerae	*Vibrio cholerae*

Isolate Number	Api/Vitek identification	Identification via 16S sequencing
504	Vibrio cholerae	*Vibrio cholerae*
569	Vibrio cholerae	*Vibrio cholerae*
984	Vibrio cholerae	*Vibrio cholerae*
1020	Vibrio cholerae	*Aeromonas sp.*
1213	Vibrio cholerae	*Salmonella sp.*

The reasons for the erroneous identification of *Aeromonas* spp. as *V. cholerae* were further investigated. All of the 25 PCR-negative isolates were identified as *V. cholerae* using the API 20e system. The API database utilizes five test results to distinguish specifically between *V. cholerae* and most of the *Aeromonas* spp. These are arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase and the utilisation of amygdalin and arabinose. Most of the 25 isolates gave patterns typically associated with *V. cholerae* and were, therefore, classified as such. No single test could be found to be responsible for the inaccurate classification of the isolates using the API 20e system. The VITEK system has at least four tests that can assist in distinguishing between these two closely related genera, but identified at least 10 strains incorrectly as *V. cholerae*. These test were arginine dihydrolase, D-glucosidase, utilisation of aesculin and the inhibition by p-coumaric.

3.3.2 Screening for virulence genes

The multiplex PCR technique developed was optimized and tested using clinical *V. cholerae* O1 isolates of both the Classical as well as the El Tor biotypes. With Classical and El Tor strains two bands, the *ctxA* and *tcpA* amplicons, were observed on a 1.5% agarose gel (Fig 3). The tcpA Classical amplicon produced being 617bp in length, with the tcpA El Tor amplicon 471bp, and the ctx A amplicon 564bp long.

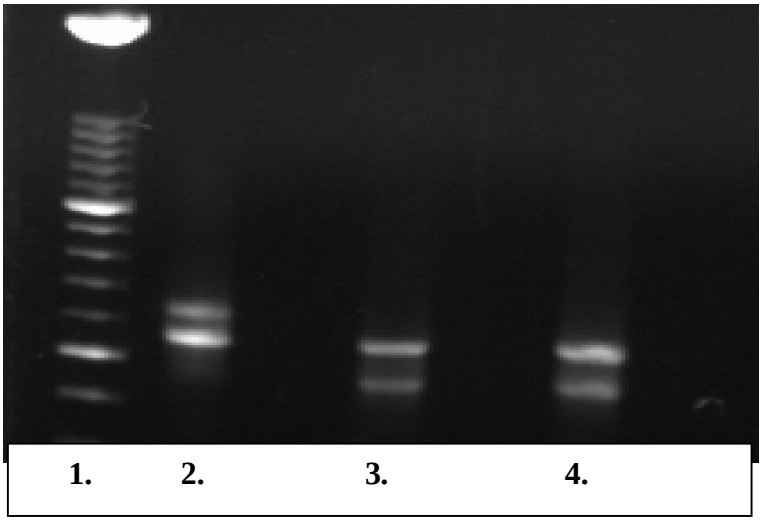

Figure 4 **Amplicons produced by multiplex PCR targeting the *ctxA* and *tcpA* genes. Lane 1, molecular size marker (Roche XIV). Lane 2, Enterotoxigenic *Vibrio cholerae* O1 Classical biotype. Lanes 3 – 4, Enterotoxigenic *V. cholerae* O1 El Tor biotype.**

A hundred environmental *V. cholerae* strains were screened for the presence of *tcpA* and *ctxA*, which code for toxin co-regulated pili and cholera enterotoxin respectively. None of the environmental isolates screened with the multiplex PCR produced an amplicon of the correct size (or any other size amplicon).

3.4 DISCUSSION

With the PCR identification procedure being evaluated, it was found that 25 out of 251 isolates phenotypically identified (with either the API 20e or VITEK approach) as *V. cholerae* did not give positive results with the PCR method. At least 10 of the 25 PCR negative isolates were shown to be non-*Vibrio cholerae* through 16S rDNA sequencing. The automated biochemical identification process (VITEK) and the API approach,

therefore, had a reliability of 96% at best. Partial sequencing of the 16S rDNA region indicated that most of the misidentified isolates were *Aeromonas* spp. This was not surprising since *Aeromonas* and *Vibrio* spp. are phenotypically closely related (Farmer and Hickman-Brenner, 1992). No specific test could be singled out to be responsible for the inaccurate identification of the isolates by the API or VITEK systems. The inaccuracy in the VITEK and API systems was rather the result of a lack of resolution when trying to identify closely related species with these systems. The *OmpW* based primer pair used in this study was found to be a highly reliable (at least 98%) and rapid technique for the identification of *V. cholerae* isolates. Four isolates that were PCR negative were found to be *Vibrio cholerae* by 16S sequencing, these strains could have minor mutations at the primer binding sites. No internal control was used in this PCR therefore PCR failure or inhibition cannot be excluded as to producing these negative results. When working with closely related species, techniques with higher resolution are required for accurate identification. PCR certainly has the edge over conventional phenotypic identification methods in this respect, and should find wider application in the water industry.

With the multiplex PCR used to screen for virulence genes no copies of the *ctxA* nor the *tcpA* gene were found in environmental *V. cholerae* strains. It is believed that screening with this multiplex approach is reliable since the PCR was developed using primers sets from two previous studies in which the primers were shown to be specific, reliable and sensitive (Theron *et al.*, 2000; Mukhopadhyay *et al.*, 2001). The PCR was also evaluated using enterotoxigenic *V. cholerae* of the Classical as well as the El Tor biotype, and found to be able to detect the target genes. The results show that no *ctxA* genes were

present in the sampled environmental *V. cholerae*, these strains thus lack the genetic potential to produce cholera toxin and therefore the potential to cause enterotoxigenic epidemics. Furthermore no strains were found to harbor the *tcpA* gene, which forms part of the larger TCP pathogenicity island coding for toxin co-regulated pili. The toxin co-regulated pili has been shown to be the receptor for bateriophage (CTXϕ) It has been proposed that non-cholera toxin producing strains can be converted to CT producing strains through the addition of the *ctx* genetic element during CTXϕ invasion (Waldor and Mekalanos, 1996). From these results in can be seen that environmental *V. cholerae* in the Vaal Barage catchment do not pose a serious health risk in terms of enterotoxigenic epidemics. They do not possess the genetic potential for cholera toxin production, and they are unlikely to undergo toxigenic conversion due to the fact that they lack the receptor for CTXϕ.

The two PCR approaches discussed, one for the detection of all *Vibrio cholerae* strains, and one for the detection of *V. cholerae* virulence factors, could prove to be valuable tools for the monitoring of environmental water systems. When used in combination they not only detect *V. cholerae* but also provide an indication of cholera associated risk factors in a rapid, reliable and sensitive manner.