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Full Length Research Paper

The use of a high resolution melt real-time polymerase chain reaction (PCR) assay for the environmental monitoring of *Vibrio cholerae*

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A real-time polymerase chain reaction (PCR) assay utilizing high resolution melt (HRM) curve analysis was developed and tested for the monitoring of *Vibrio cholerae* in water samples. The assay utilized previously published primers that are specific to regions of the *V. cholerae ompW* and *ctxAB* genes, allowing it to differentiate between toxigenic and non-toxigenic strains. The *ompW* and *ctxAB* primers amplify target regions of 588 and 564 bp in length (respectively) and the amplicons could be accurately identified using HRM curve analysis. High resolution melt curve analysis provided additional accuracy for the determination of amplicon melting temperatures, and allowed amplification of the two targets in a multiplex reaction. Two laboratories employed the assay to analyse 178 water samples obtained from diverse environmental water sources, for the presence of *V. cholerae*. The assay was found to be a rapid, highly accurate, sensitive and cost effective method for the detection and distinction between toxigenic and non-toxigenic *V. cholerae* strains in water.

Key words: *Vibrio cholerae,* high resolution melt, real-time polymerase chain reaction (PCR).

INTRODUCTION

The diarrhoeal disease cholera remains a constant threat in developing countries. Africa in particular seems to be the hardest hit with the continent accounting for the vast majority of officially notified cases in recent history (World Health Organization, 2006). Cholera outbreaks continued with many African countries reporting outbreaks during 2010. In the same year, more than 1800 cholera attributed mortalities were reported in Africa alone, this in a 4 month period (World Health Organization, 2010). The causative agent of cholera, *Vibrio cholerae* is a natural inhabitant of the aquatic environment. It is associated but not limited to estuarine systems. *V. cholerae* is known to persist in inland freshwater systems (le Roux et al., 2004). Efforts to manage and mitigate cholera outbreaks invariably start with bacteriological monitoring of aquatic ecosystems, a procedure that is considered essential for the tracking of *V. cholerae* O1 (Binsztein et al., 2004). Monitoring is of vital importance to aid in preventing the dissemination of toxigenic *V. cholerae* before the onset of epidemics (Faruque et al., 2006).

Culture based diagnostic techniques have long been regarded as the gold standard for the detection and confirmation of *V. cholerae*. In this approach, samples are enriched in alkaline peptone water (APW) followed by sub-culturing onto selective thiosulfate-citrate-bile salts-sucrose (TCBS) agar. Sucrose positive colonies exhibiting the typical yellow morphology are further characterised by oxidase testing and serotype identification by means of biochemical and serological tests (Kay et al., 1994). Confirmation of cholera toxin production can be confirmed by ELISA (Bhadra et al., 1991; Ramamurthy et al., 1993). Culture based techniques have several disadvantages; most notably they are time-consuming and laborious and often require to be performed by skilled experienced and laboratory technicians. Typically, completion of the method can require up to 72 h. Culture based methods may also fail to detect viable but non-culturable (VBNC) cells and difficulties arise where *V. cholerae* bacteria are out-

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Table 1. Bacterial strains used in the study.

Bacterial species	Source of strain
Non-*Vibrio* genera	
Escherichia coli	ATCC 25922
Shigella flexneri	ATCC 12022
Shigella boydii	ATCC 9207
Shigella sonnei	ATCC 9290
Enterococcus faecium	ATCC 6569
Enterobacter aerogenes	ATCC 13048
Klebsiella pneumoniae	Vermaak and Partners Pathologists, S.A.
Pseudomonas aeruginosa	ATCC 27853
Staphylococcus aureus	ATCC 25923
Proteus mirabilis	Vermaak and Partners Pathologists, S.A.
Shigella species (environmental strain 1)	Human stool sample, Vermaak and Partners Pathologists
Shigella species (environmental strain 2)	Human stool sample, Vermaak and Partners Pathologists
Shigella species (environmental strain 3)	Human stool sample, Vermaak and Partners Pathologists
***Vibrio* species**	
Vibrio cholerae O1	National Health Laboratory Service, S.A.
Vibrio cholerae O1	NCTC 5941
Vibrio cholerae O139	National Health Laboratory Service, S.A.
*Vibrio cholerae O1 (*environmental strain*)*	Zimbabwe/South Africa cholera outbreak (2007/8)
Vibrio cholerae Non-toxigenic (Environmental strain)	RW1009 Rand Water Analytical Services, South Africa

competed by other bacteria during enrichment and sub-culturing steps (Colwell and Huq, 1994; Mendes et al., 2008).

In contrast to culturing, molecular methods have several advantages; they are rapid, sensitive, highly selective and do not require extensive hands-on time. Traditional PCR has emerged as a useful molecular based detection technique and there have been a number of reports describing the application of PCR for the detection of *V. cholerae*. These assays were mostly directed against single gene targets involved in *V. cholerae* virulence, regulation of virulence, outer membrane proteins or genes involved with O-antigen synthesis (Shirai et al., 1991; Fields et al., 1992; Chowdhury et al., 1994; Albert et al., 1997; Nandi et al., 2000; Chow et al., 2001; Lipp et al., 2003).

Several multiplex PCR assays for the detection of *V. cholerae* have also been described (Keasler and Hall, 1993; Shangkuan et al., 1995; Hoshino et al., 1998; Kapley and Purohit, 2001; Rivera et al., 2001; Singh et al., 2002; Rivera et al., 2003; Goel et al., 2005). However, the majority of these methods made use of a conventional PCR approach, as opposed to the faster, less laborious and more sensitive real-time PCR method. Only a few multiplex real-time PCR methods for the detection of *V. cholerae* have been published (Lyon, 2001; Fukushima et al., 2003; Gubala and Proll, 2006; Gubala, 2006; Mendes et al., 2008). These methods were all developed and validated by single centres and reports of these assays being used in the routine application environment are lacking. To our knowledge, PCR assays that employ HRM curve analysis for the detection of *V. cholerae* have not been reported.

Here, we report a multi-centre implementation and performance evaluation of an EvaGreen real-time PCR assay with HRM curve analysis for the detection of and distinction between non-toxigenic and toxigenic *V. cholerae* in water samples. This assay which was implemented both as a single-tube and separate tube multiplex PCR is based on primers directed against the outer membrane protein (*ompW)* and cholera toxin (*ctxAB)* genes (Nandi et al., 2000; Goel et al., 2005).

MATERIALS AND METHODS

Bacterial strains

V. cholerae O1 Ogawa biotype (VIBCH07, National Health Laboratory Services, South Africa) and *Vibrio cholerae* O1 (NCTC 5941), were used as positive controls for developing the real-time PCR detection assay and in all subsequent experimental procedures. Although the selectivity of the primer sets used in this study has been demonstrated (Nandi et al., 2000; Goel et al., 2005), several environmental strains of *V. cholerae* as well as other bacterial genera were also used to further evaluate the specificity of the detection assay (Table 1). All bacterial cultures were maintained aerobically on nutrient agar plates (Oxoid, United Kingdom) at

Table 2. Oligo-nucleotide primers used in this study

Target	Primer sequence (5' – 3')	Amplicon size (bp)	Tm (°C)	References
ompW gene	CACCAAGAAGGTGACTTTATTGTG GAACTTATAACCACCCGCG	588	81.27 ± 0.27	Nandi et al. (2000)
ctxAB gene	CCTGTCCTTTTACCAGACAACCA GGTCTCTCTTTTCGTTGGGATCT	564	77.68 ± 0.16	Goel et al. (2004)

37°C.

Sample preparation

Prior to real-time PCR analysis, water samples were enriched in alkaline peptone water (APW, Oxoid, UK). A volume of 100 mℓ of water sample was filtered using a stainless steel vacuum manifold system and a 0.45 µm nitrocellulose membrane filter. The membrane filter was transferred aseptically to 100 mℓ sterile APW for aerobic enrichment at 30 ± 1.0°C for 16 – 18 h. Turbid water samples were added directly to double strength APW (50:50 v/v) followed by incubation at the stated conditions.

Genomic DNA extraction

Genomic DNA was extracted using the InstaGene Matrix commercial kit (Bio Rad, U.S.) according to the manufacturer's instructions. Briefly, 1 ml from the top phase (3 mm) of the pre-enriched APW was transferred to a sterile 1.5 ml microcentrifuge tube. Alternatively, genomic DNA was extracted from pure bacterial cultures following the manufacturer's protocol (Bio Rad, U.S.A.). The supernatant contained PCR amplifiable DNA of which 5 µℓ was used in subsequent real-time PCR reactions.

Primers, real-time PCR and HRM curve analysis

For the detection of *V. cholerae* and specifically toxigenic (cholera toxin producing) *V. cholerae*, two genes were targeted. Firstly, an outer membrane protein gene (*ompW*) unique to *V. cholerae* as a species and secondly the *ctx* gene (complex A and B) involved in cholera toxin production and associated with toxigenic *V. cholerae*. Two primer sets obtained from previously published studies were used to target and amplify the *ompW* and *ctxAB* genes (Nandi et al., 2000; Goel et al., 2005). All primers used were synthesized by integrated DNA Technologies (IDT), USA. Primer sequences are listed in Table 2.

Real-time PCR amplification of the targets was performed in either 0.2 or 0.1 mℓ thin walled PCR tubes (Corbett Research, Australia / Qiagen, Germany). Each reaction contained 1x SensiMix HRM reaction buffer containing dNTP's, $MgCl_2$ (6 mM), heat activated DNA polymerase and EvaGreen dye (Bioline, UK). For both single and separate tube multiplex reactions each reaction contained 0.2 µM of all primers and 5 µl of DNA extract as template. Nuclease free water (Applied Biosystems, USA) was used to make up the reaction to a final volume of 25 µl.

Amplification was performed in RotorGene 6000 rotary thermal cyclers (2-plex and 5-plex) with HRM capability (Corbett Research, Australia / Qiagen, Germany). A heat activation step of the DNA polymerase was performed at 95°C for 10 min, followed by 45 cycles of a DNA denaturation at 95°C for 30 s, annealing at 64°C for 30 s and extension at 72°C for 30 s. A final extension step was performed at 72°C for five minutes after cycling. To differentiate and identify amplification products formed, high resolution melt (HRM) curve analysis was performed by lowering the temperature to 60°C for five minutes, followed by an increase in temperature to 90°C at increments of 0.1°C per second. Fluorescence was measured continuously and melting temperature (Tm) peaks were calculated based on the initial fluorescence curve (F/T) by plotting the negative derivative of fluorescence over temperature versus temperature (-dF/dT versus T). The Tm values for both the *ompW* and *ctxAB* products were calculated using values from 25 independent analyses of both the *V. cholerae* O1 reference strain and environmental strains detected from natural water samples over a two year period (Table 2).

Confirmation of amplicon identity

Agarose gel electrophoreses was used to confirm that amplification products were of the expected length for the *ompW* and *ctxAB* gene targets. 5 µℓ of the real-time PCR products were loaded onto a 2% agarose gel stained with ethidium bromide and subjected to electrophoresis at 80 volts for 45 min. Following electrophoresis, the gel was visualized using an ultra-violet transluminator 2000 (Bio Rad, U.S.A.) and the images were captured using a Digi Doc System (Bio Rad, U.S.A.).

The identity of selected amplification products were also confirmed by Sanger nucleotide sequencing (Inqaba Biotec, South Africa) and alignment of the obtained sequences to sequences in an existing database (GenBank at NCBI, BLASTn option).

PCR sensitivity and proficiency evaluation

For the evaluation of PCR sensitivity in sterile media an overnight culture of *V. cholerae* O1 (Ogawa, VIBCH07) was serially diluted 10-fold using sterile Bacteriological Peptone (BP, Oxoid, UK) after which 100 µl of each dilution was added to 900 µl of both sterile APW and sterile double distilled and deionised water (ddH_2O). Genomic DNA was extracted from each dilution and used in subsequent real-time PCR detection assays. The concentration of *V. cholerae* O1 (colony forming units (cfu)/ml) for each serial dilution was determined by plating either 10 or 100 µl, depending on the dilution onto nutrient agar plates in triplicate. After incubation at 37 ± 1.0°C for 16 - 18 h, the average count for each dilution was determined.

To determine the sensitivity of the PCR assay in environmental water samples, *V. cholerae* O1 (Ogawa, VIBCH07) was cultivated overnight at 30 ± 1.0°C in alkaline peptone water (Oxoid, United Kingdom) after which a 10-fold dilution series was prepared and used to artificially contaminate surface water samples (500 ml). The final concentration of *V. cholerae* O1 in each contaminated water sample was determined using plating techniques. This was achieved by plating either 10 or 100 µl of each serial dilution of *V. cholerae* O1 on a non-selective media (Nutrient Agar, Oxoid, United Kingdom) in triplicate. After overnight incubation at 37 ± 1.0°C, the average count was determined for each dilution and stated as the

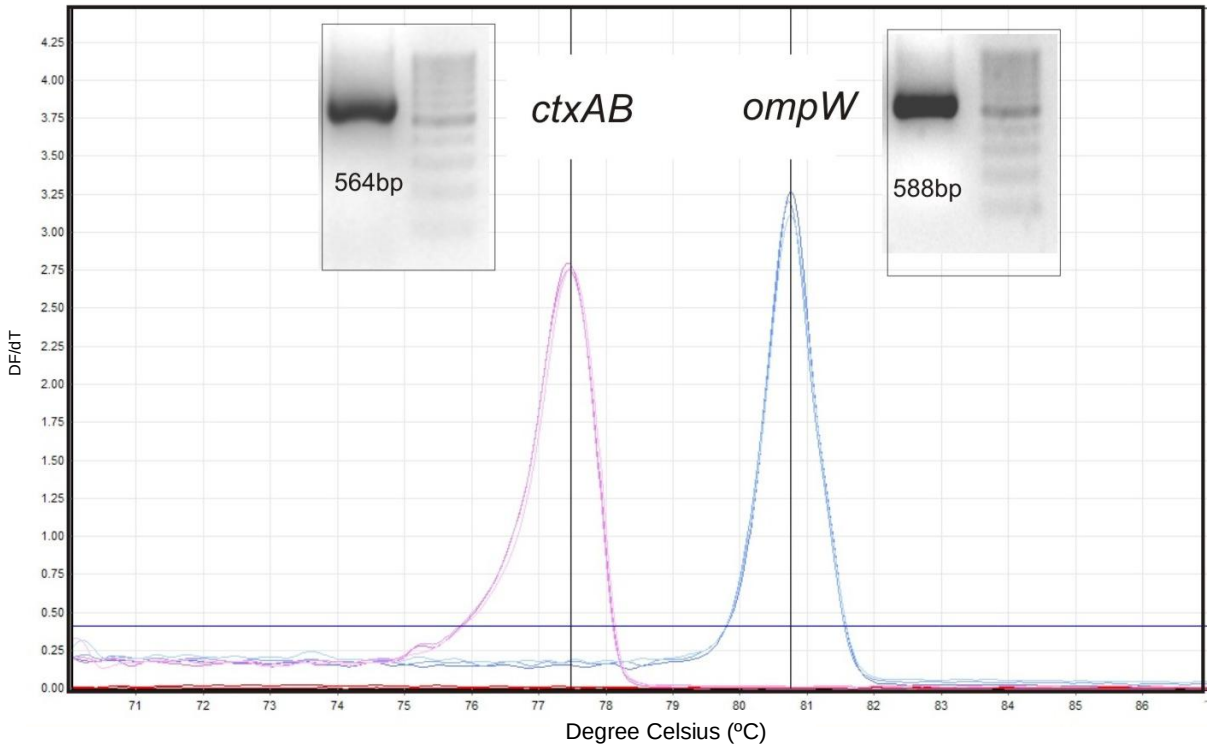

Figure 1. The HRM results for *ctxAB* and *ompW* amplicons depicted as a dF/dT (change in fluorescence over change in time) versus temperature graph.

concentration of *V. cholerae* O1 in cfu/mℓ. A blank sample was also prepared by contaminating an environmental water sample with sterile diluents (Bacteriological Peptone) used to prepare the serial dilutions of *V. cholerae* O1. This was to ensure that all reagents aswell as the surface water samples used were free of any *V. cholerae* O1.

After artificial contamination, each surface water sample was subjected to the real-time PCR detection assay. This entailed the direct enrichment of the spiked environmental water samples (50 mℓ sample in 50 mℓ double strength APW, pH 8.6 ± 0.2, 30 ± 1.0°C, 16 - 18 h) after which the genomic DNA was extracted from 1 mℓ enriched broth using the method as described. Extracted genomic DNA was used as template for subsequent real-time PCR reactions.

For quality control purposes, an inter-laboratory proficiency exercise was undertaken. Briefly, 10 water samples (consisting of five river water and five tap (potable) water samples) that did not contain *V. cholerae* (as tested by PCR and culture) were inoculated with nutrient broth containing either *V. cholerae* O1 toxigenic (NCTC 5941), *V. cholerae* non-toxigenic (RW1009), or *Escherichia coli* (ATCC 25922). The water samples contained the inoculated bacteria at a final concentration of ~1.0E + 04 cfu/mℓ. The 10 water samples were prepared in duplicate and a set of samples were sent for PCR analysis (as described elsewhere in this article) to each of the two laboratories (CSIR and ERWAT Laboratory Services). All aspects of the blinded proficiency test with the exception of the PCR analysis and subsequent result reporting were handled by a third party (Aquadoc Analytics cc., South Africa).

RESULTS

The specificity of the primer pairs used in this HRM real-time PCR assay was validated in the studies published by Nandi et al. (2000) and Goel et al. (2005), as such the focus was not to re-validate these assays, but rather to test their applicability in a HRM real-time PCR context. The *ompW* primers produced an amplicon of 588 bp in length and this product exhibited a mean melting peak temperature of 80.367°C in our reactions (Figure 1). The *ctxAB* primers amplified a 564 bp amplicon with a mean peak melting temperature of 77.339°C under our reaction conditions.

It can be seen from Figure 1 that even though the *ctxAB* and *ompW* products are closely matched in terms of size they are well separated by melt analysis. High resolution melt analysis (utilizing Evagreen) compared to melt analysis (using SYBR green) provides better peak separation and resolution and increases the detection sensitivity, thereby allowing multiplexing of the *ctxAB* and *ompW* reactions (Khan et al., 2011). As such, the *ompW* and *ctxAB* PCR assays were carried out either in separate tubes or in a single-tube multiplex assay as the two approaches gave comparable results (data not shown). To verify that the amplicons were correctly designated either as *ompW* or *ctxAB* by HRM analysis, 20 amplicons from selected PCR assays were sized using gel electrophoreses and five amplicons were sequenced in order to compare them to sequences in Genbank's Nucleotide Database. All the amplicons that were investigated were of the expected length and were

Table 3. Summary of a two-year *Vibrio cholerae* monitoring exercise using the HRM PCR assay (December 2008 to January 2011).

Sampling region (province)	Number of samples	Samples positive for the *ompW* gene (% in brackets)	Samples positive for the *ctxAB* genes (% in brackets)
Gauteng	11	11 (100)	2 (18.2)
Limpopo	76	48 (63.2)	7 (9.2)
Mpumalanga	25	12 (48)	0 (0)
North-West	66	40 (60.6)	2 (3)
Totals	178	111 (62.4)	11 (6.2)

The percentage of positive detection results in relation to the samples tested (either per region or total number of samples) is given in brackets.

identified by sequence comparison to be the target sequences of the *ompW* or *ctxAB* genes. Sequence comparison between three *ompW* amplicons and the sequence of *V. cholerae* 16961 (Heidelberg et al., 2000) showed the presence of various small nucleotide polymorphisms (SNP's) within the amplified target sequences.

Additionally, the specificity of the HRM assay was tested as a single and separate tube multiplex reaction against various non-*V. cholerae* bacterial strains (Table 1). No HRM peaks were observed in the melting temperature ranges of the *ctxAB* and *ompW* reactions indicating that the primer pairs are specific to their targets.

Two separate laboratories (CSIR and ERWAT Laboratory Services) utilized this HRM assay on a routine basis; this allowed for a blinded inter-laboratory validation. Ten environmental water samples which were inoculated by either *E. coli* (negative control), non-toxigenic *V. cholerae* or toxigenic *V. cholerae* were correctly identified by both laboratories.

The assay was found to be very sensitive, with the ability to repeatedly detect six *V. cholerae* colony forming units per reaction using PCR without a prior enrichment step. However, direct PCR was not regularly performed as environmental samples often contain PCR inhibitors and provide no information on the viability of the detected *V. cholerae*. The PCR assay was routinely performed after an overnight enrichment in Alkaline Peptone Water (APW), which allowed for the repeatable detection of as little as 2 *V. cholerae* CFU's per enrichment.

Inter-run variance in peak melting temperature (Tm) was found to be low, with the Tm values of both *ctxAB* and *ompW* amplicons having a run to run standard deviation of less than 0.53°C. Comparing PCR runs between laboratories resulted in a run to run standard deviation which was only slightly higher with Tm's between the two laboratories having a standard deviation equal to or less than 0.55°C. The standard deviation values were calculated from the results of 178 *V. cholerae* containing samples tested in 35 separate PCR reactions. These samples were taken from diverse geographical settings and likely contained diverse strains of *V. cholerae* (Jiang et al., 2000).

Single nucleotide polymorphisms (SNP's) may have been present in target sequences and may explain the slight variation in Tm values as observed by HRM analysis. Upon investigation sequence variations in the *V. cholerae ompW* gene of different isolates were found (data not shown) and have also been reported elsewhere (Nandi et al., 2000). Lower Tm standard deviation values were calculated when only using data from the HRM curves of a single reference strain. The data from 25 separate HRM PCR reactions (using only the reference strain) resulted in a standard deviation of 0.230°C for the *ctxAB* amplicon and 0.297°C for the *ompW* product. No significant intra-run variation in Tm's could be observed when using replicates of the same *V. cholerae* strain (as can be seen in Figure 1).

Over a two-year period (December 2008 to January, 2011) the assay was employed by the two laboratories for the routine monitoring of drinking, environmental and wastewater for the presence of *V. cholerae*. During this time, 178 water samples were analysed using the HRM detection assay for the presence of *V. cholerae* and *ctx* mediated toxigenicity, targeting the *ompW* and *ctxAB* genes (respectively). Of the 178 water samples analysed during the study period, 111 samples (62.4%) were found to contain non-toxigenic *V. cholerae* (as determined by the detection of the *ompW* gene). Eleven (9.9%) of the 111 *V. cholerae* detections were found to be toxigenic strains, as indicated by the detection of the *ctxAB* target. The majority of samples were taken from environmental sources such as rivers and dams. Table 3 provides a relative break-down of the water samples analysed with respect to their origin (per province) and also shows which portion of the samples tested positive for the presence of the *ompW* and *ctxAB* genes. It can be seen that most of the samples were taken in the Limpopo and North-West Province of South Africa due to cholera cases being reported in the Northern parts of Limpopo province during 2008/2009 (Department of Health, South Africa, 2009).

A seasonal pattern was observed with 73.7% of samples taken during the wet months of October to March containing *V. cholerae* as opposed to the 46.8% testing positive for this organism during the dry months of April to September.

DISCUSSION

Although HRM curve analysis is mostly used in genotypic studies, this study demonstrated HRM to be a useful tool for the identification of waterborne bacterial pathogens such as toxigenic *V. cholerae*. Combined with the specificity of the primer pairs used, HRM curve analysis of resulting amplification products yielded unique melting temperature profiles. The profiles obtained could easily be compared to high resolution melting temperature peaks generated by reference culture strains included in each run. This allowed for rapid and accurate detection of non-toxigenic and/or toxigenic *V. cholerae.*

Variation in HRM Tm's (for *ompW* and *ctxAB* products) between *V. cholerae* strains were observed, but the standard deviation was relatively small (≤ 0.55°C). Slight sequence variations present in target sequences may explain the variation in Tm values as observed by HRM curve analysis, as it is sensitive enough to detect small differences in sequence length and composition. The samples evaluated were obtained from diverse geographical settings and likely contained diverse strains of *V. cholerae* as observed elsewhere (Jiang et al., 2000). The amplicons produced in this assay are generally longer in length (~550 bp) than is considered ideal for the detection of SNP's with HRM, however, successful SNP detection has been reported in fragments up to 544 bp long (Wittwer et al., 2003; Liew et al., 2004). The standard deviation in amplicon melting temperature was found to be relatively small (≤ 0.55°C) allowing for the reliable detection of the *ctxAB* and *ompW* targets. Ultimately, HRM curve analysis could distinguish between *V. cholerae* variants harbouring target regions with slight sequence differences.

The two laboratories that participated in this study performed a standardized HRM based PCR assay, utilizing the same DNA preparation and PCR protocols and reagents from the same manufacturers. The standardization allowed trouble free comparison of data generated by both laboratories, a platform for inter-laboratory quality control exercises and provided each laboratory with a reference laboratory for the confirmation of positive results.

In our opinion, HRM based PCR detection has several advantages compared to probe based assays; it is cost effective, does not require additional conserved sequences for probe binding, is not subject to unspecific probe binding, is simple to design and it can detect and distinguish between diverse strains within the same PCR reaction (based on differences in target sequences). However, the interpretation of melting curve data requires experience and a good understanding of melt based PCR technologies.

The HRM real-time PCR assay reported here is highly specific, cost effective, sensitive and reliable tool for the rapid detection of (and distinction between) *V. cholerae* non-toxigenic and toxigenic types. The successful implementation of this assay in two routine laboratories also demonstrated its usefulness as an environmental monitoring tool for toxigenic *V. cholerae*. In countries such as South Africa where cholera is endemic, the routine monitoring of toxigenic *V. cholerae* may greatly contribute to a proactive approach to cholera outbreaks, which is difficult and time-consuming to achieve using culture based methods. Using the real-time PCR assay described here, problem areas and possible cholera outbreaks can be quickly identified reducing socio-economic impacts and the loss of lives.

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