Utilization of Small RNA Genes to Distinguish *Vibrio cholerae* Biotypes via Multiplex Polymerase Chain Reaction

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Abstract. The diarrheal disease “cholera” is caused by *Vibrio cholerae*, and is primarily confined to endemic regions, mostly in Africa and Asia. It is punctuated by outbreaks and creates severe challenges to public health. The disease-causing strains are most-often members of serogroups O1 and O139. PCR-based methods allow rapid diagnosis of these pathogens, including the identification of their biotypes. However, this necessitates the selection of specific target sequences to differentiate even the closely related biotypes of *V. cholerae*. Oligonucleotides for selective amplification of small RNA (sRNA) genes that are specific to these *V. cholerae* subtypes were designed. The resulting multiplex PCR assay was validated using *V. cholerae* cultures (i.e., 19 *V. cholerae* and 22 non-*V. cholerae* isolates) and spiked stool samples. The validation using *V. cholerae* cultures and spiked stool suspensions revealed detection limits of 10–100 pg DNA per reaction and 1.5 cells/mL suspension, respectively. The multiplex PCR assay that targets sRNA genes for amplification enables the sensitive and specific detection, as well as the differentiation of *V. cholerae*—O1 classical, O1 El Tor, and O139 biotypes. Most importantly, the assay enables fast and cheaper diagnosis compared with classic culture-based methods.

INTRODUCTION

Vibrio cholerae is a rod-shaped Gram-negative bacterium that belongs to the family Vibrionaceae. *V. cholerae* strains cause 3–5 million cases of diarrheal cholera with more than 100,000 deaths each year.^{1,2} Cholera is characterized by watery stools and vomiting that lead to massive, and often even lethal, dehydration.³

Vibrio cholerae can be serologically differentiated according to the presence of the O antigen within outer membrane lipopolysaccharides of the bacterium.^{4,5} To date, more than 200 serogroups of *V. cholerae* have been identified, but only some of them harbor the cholera toxin, which is chiefly responsible for the symptoms of the disease.⁶

Cholera epidemics and pandemics are mainly caused by the *V. cholerae* serogroups O1 and O139.⁶ Acquisition of the “O” surface antigen within *V. cholerae* O1 and O139 genetic traits marks their common evolutionary origins.^{5,7} The serogroup O1 can be further divided into two different biotypes: “classical” and “El Tor.” The former serogroup is responsible for the first six pandemics, whereas the latter caused the last pandemic.^{8–12} The serogroup O139 was first described in a devastating outbreak in 1992.^{8–12} However, the O1 El Tor strain remains globally dominant and its variants, which refer to a collection of El Tor strains that also exhibit classical phenotypes, caused an even more rapid and severe cholera progression.^{8–10,13,14} Previous studies suggest that the emergence of such variants during the seventh pandemic were attributable to horizontal gene transfers.^{14–16}

Despite being most prominent in South Asia, cholera spread rapidly to Latin America, and has now become endemic in South and Central America.^{6,9} Devastating outbreaks in Kenya,

Zimbabwe, Nigeria, Cameroon, the Democratic Republic of Congo, Guinea, and Sierra Leone represented a high burden for affected communities during 2000–2008.^{17–19} Immediate access to intense medical care is therefore in high demand, and poses the biggest challenge to public health systems, especially in the developing African societies. Methods that permit the investigation of the source of infection and the differentiation of cholera biotypes are urgently needed.^{17,18,20,21}

To date, there are various diagnostic strategies to detect and differentiate pathogenic *V. cholerae* serogroups.^{17,22–30} Conventional cultivation-based methods are widely applied but unfortunately are time-consuming and require sterile cultivation settings, which are often impractical in places where outbreaks occurred.^{21–23} Antibody-based immunoassays for detection of *V. cholerae* strains have also been established, but often fail to differentiate between individual biotypes.^{24,25,31,32} Therefore, the generation of accurate and rapid diagnostic regimes for cholera is essential to monitor the outbreak and for epidemiological analysis.²¹

During the last decades, PCR screens have been developed, and are to date, the best established alternative to the conventional cultivation approaches for pathogen diagnosis.^{33–35} These techniques have been successfully applied for the characterization of serogroups, biotypes, and the toxigenic potential of *V. cholerae* strains.^{26,27,36}

Small RNAs (sRNAs) are approximately 40–500 nucleotides in length and exert regulatory functions, often in complex with proteins as ribonucleoparticle.³⁷ A plethora of sRNAs have been reported to play pivotal roles in the regulation of cell differentiation, growth, metabolism, and defense within prokaryotes, eukaryotes, and archaea.^{37–48} Recently, sRNAs and their regulation in *V. cholerae* have been described.^{49–52} Here, we report the design and validation of a multiplex PCR assay that targets sRNA genes and the O139-specific *rfb* protein-coding gene to identify and differentiate between *V. cholerae* O1 El Tor, O1 classical, and O139 strains.

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TABLE 1
Specificity testing on *Vibrio cholerae* isolates

Bacterial strains	No. of isolates	Target genes		
		3853_VCD	3991_VCD	O139_rfb
<i>V. cholerae</i> O1 classical	3	+(3/3)	–	–
<i>V. cholerae</i> O1 El Tor	8	+(8/8)	+(8/8)	–
<i>V. cholerae</i> O139	8	+(8/8)	+(8/8)	+(8/8)

Note: + Presence of target; – Absence of target.

EXPERIMENTAL PROCEDURES

Bacterial strains and culture conditions. All bacterial strains used in this study were retrieved from the Hospital Universiti Sains Malaysia, Kelantan, Malaysia, and are listed in Table 1. Strains were maintained on Luria-Bertani (LB) agar and grown overnight in LB broth medium at 37°C and 200 revolutions per minute (rpm) before DNA isolation. For long-term storage, strains were stored at –80°C in LB broth medium containing 20% (v/v) glycerol. Alkaline peptone water (APW) was used for enrichment culture of the spiked stool suspensions.

DNA extraction. Bacterial cell pellets were re-suspended in 200 µL of suspension buffer (2 g/L sucrose; 50 mM tris-hydrochloride [Tris-HCl], pH 8.0; 0.1 g/L sodium dodecyl sulfate; 0.2 M sodium hydroxide [NaOH]; 25 mM ethylenediaminetetraacetic acid, pH 8.0; and 0.1 M sodium chloride), and incubated at room temperature (24°C) for 3–10 minutes. Equal volumes of pre-chilled (4°C) 3 M sodium acetate (pH 6.4) were added and briefly mixed. The resulting suspensions were centrifuged at 18,000 × *g* and 4°C for 5 minutes to collect the supernatants. Genomic DNA were precipitated with about 0.5 volumes of pre-chilled (4°C) 3 M sodium acetate (pH 5.2) and 1.5 volumes of ice-cold absolute ethanol, followed by incubation at –80°C for 30 minutes. Extracted DNA was collected by centrifugation at 18,000 × *g* and 4°C for 10 minutes and washed with 1 mL of ice-cold 70% ethanol. DNA pellets were briefly air-dried and re-suspended in 100 µL deionized-distilled water before being stored at –20°C.

Crude DNA was prepared from APW enrichment cultures as previously described.²⁹ In brief, 1 mL of APW culture was centrifuged at 9,000 × *g* for 2 minutes to harvest the cells (pellet), which was then re-suspended in 200 µL of 25 mM NaOH and boiled for 5 minutes. For neutralization, 8 µL of 1 M Tris-HCl (pH 7.5) was added and the resulting suspension was centrifuged at 18,000 × *g* and 4°C for 10 minutes to remove the cellular debris. The resulting supernatants were aliquoted and used as a crude DNA template in the multiplex PCR assay.

Selection of targets and primer design. Small RNAs *Vc_npcR_3853* and *Vc_npcR_3991* were previously identified in our experimental RNomic study.⁵² These sRNA genes were analyzed using Basic Local Alignment Search Tool (BLAST) on National Center for Biotechnology Information (NCBI) ([https://](https://blast.ncbi.nlm.nih.gov/Blast.cgi)

blast.ncbi.nlm.nih.gov/Blast.cgi) to identify strain-specific amplification targets with (default) Expect values (“E-values”) of 10 and a minimum word size (W) of 28.⁵³ Finally, these three genes were selected for analysis: 1) O1-specific sRNAs *Vc_npcR_3853* (NCBI accession number: HQ442047), 2) El Tor-specific *Vc_npcR_3991* (NCBI accession number: HQ442117), and 3) O139-specific *rfb* gene (NCBI accession number: Q56678). The sRNA genes *Vc_npcR_3853* and *Vc_npcR_3991* were detected within the genomes of O1 classical and O1 El Tor, with E-values of 3e-97 and 7e-46, respectively.

Annotations of the *Vc_npcR_3853* and *Vc_npcR_3991* genes are summarized in Table 2. *Vc_npcR_3991* is specific to the seventh pandemic El Tor and O139 strains and contained within the Seventh Pandemic Island 1 (*Vibrio* seventh pandemic 1 [VSP-1]).

Primer design. The Primer-BLAST online software (Primer-BLAST is developed and maintained by the NCBI at the National Library of Medicine, NLM, www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi) was used for the primer design.⁵⁴ Amplicon sequences and the corresponding sizes are summarized in Supplemental Table 1. In addition, each reaction was supplemented with 10 ng of plasmid p100 as internal amplification control (with respective primer pair L₅₀_F and L₅₀_R) to test for false-negative results as the PCR may be affected by the presence of potential PCR inhibitors (Supplemental Table 1). All primers were purchased from Bio Basic Canada, Inc. (Markham, Canada).

Primer combinations and serotype differentiation. Primer pair 3853_VCD (Forward/Reverse [F/R]) generates an 85-bp amplicon for both *V. cholerae* O1 and O139 serogroups. The primer pair 3991_VCD (F/R) amplifies a 96-bp region, which is common to O1 El Tor and O139 strains. The combination of both primer pairs, that is, *Vc_npcR_3853* and *Vc_npcR_3991*, within a single multiplex PCR assay allows the detection and differentiation between *V. cholerae* O1 classical and O1 El Tor as described in Table 1. Finally, we used the primer pair O139 (F/R), which yields a 120-bp amplicon, to differentiate between O139 serogroup and O1 El Tor (Table 1).

The combination of these three molecular targets allows the design of a multiplex PCR assay for the simultaneous detection and differentiation of *V. cholerae* O1 classical, O1 El Tor, and O139 strains. These primer pairs were evaluated with 19 *V. cholerae* and 22 non-*V. cholerae* isolates (Supplemental Table 2).

Multiplex PCR amplification. *Preparation of PCR mastermixes.* All PCR amplifications were performed with the MyCycler™ Personal Thermal Cycler (Bio-Rad, Hercules, CA). Each 20-µL reaction mix contained 1× PCR buffer (10 mM Tris-HCl [pH 8.3], 50 mM potassium chloride, 1.5 mM magnesium chloride [Fermentas, Vilnius, Lithuania]), 0.35 mM deoxynucleotides (Fermentas), 0.5 µM of primers,

TABLE 2
Genomic location of target genes

Target gene	Chr.	Coordinates		Strand	Flanking genes	
		Start	Stop		Upstream	Downstream
<i>Vc_npcR_3853</i>	II	897527	897723	+	Maltose ABC periplasmic transporter (VCA0945)	Maltodextrin transporter ATP-binding protein gene (VCA0946)
<i>Vc_npcR_3991</i>	I	177024	177127	–	Transcriptional regulator (VC0176)	Deoxycytidylate deaminase related protein (VC0175)

1 U *Taq* DNA polymerase (Biotoools, Madrid, Spain), DNA templates, and p100. In addition, a DNA-free reaction mix was also included in each assay as negative control.

PCR optimization and amplification. A gradient PCR (with annealing temperature ranging from 55°C to 68°C) was carried out to determine the optimal annealing temperatures for each primer pair within the multiplex PCR reaction. Similarly, the minimal concentration of p100 was also determined to yield optimal results. Subsequently, the following PCR amplification parameters were derived: denaturation at 95°C for 2 minutes; 35 cycles of denaturation at 95°C for 30 seconds, primer annealing at 57°C for 30 seconds, and extension at 72°C for 30 seconds; followed by a final extension at 72°C for 2 minutes. PCR products were separated on 4% agarose gels (1× Tris-acetate-EDTA [TAE] buffer) containing ethidium bromide (0.5 µg/mL final concentration) at 60 V for 1 hour.

Detection limit and specificity of the multiplex PCR assay. To establish detection thresholds in pure culture, DNA samples of *V. cholerae* O1 classical, El Tor biotypes, and O139 serogroup were tested in 10-fold serial dilution, representing DNA concentrations ranging from 1 fg to 100 ng per reaction. The actual detection limit was defined as the lowest dilution of genomic DNA that still produces visible amplicons in gel after the PCR amplification. The equivalent DNA copy number was calculated via an online tool provided by the Rhode Island Genomics and Sequencing Center (RIGSC) (<http://cels.uri.edu/gsc/cndna.html>). The specificity of PCR amplification was analyzed with the genomic DNA of various bacterial isolates (Supplemental Table 2).

Detection limits in spiked human stool suspension. The applicability of these multiplex PCR assays in clinical samples was evaluated using spiked human stool suspensions as previously described.²⁹ Stool obtained from a healthy donor was spiked with selected isolates each of O1 classical, El Tor biotypes, and the O139 serogroup to determine its detection level as described below. This study was approved by the Human Research Ethics Committee of Universiti Sains Malaysia (USM) (USM/JEPeM/14120496). *Vibrio cholerae* cell suspensions equivalent to 1.5×10^8 cells/mL were used in 10-fold serial dilutions yielding densities ranging from 0.15 to 1.5×10^9 cells/mL, which were spiked into healthy donor stool suspensions. Each stool suspension (10%, w/v) was prepared by mixing 10 g of feces with 90 mL of 1× phosphate-buffered saline (PBS), followed by centrifugation at $900 \times g$ for 1 minute to remove larger debris, to simulate the watery stool from diarrhea patients. Each suspension was aliquoted to 900 µL and spiked with 100 µL *V. cholerae* cell suspension (final cell densities in stool suspensions: 0.015– 1.5×10^7 cells/mL). A total of 100 µL of spiked stool suspensions was transferred to 5 mL of APW and incubated at 200 rpm and 37°C for 6 hours. One milliliter of the APW enrichment culture was harvested for crude DNA template preparation as previously described.²⁹

RESULTS AND DISCUSSION

To date, there are only two serogroups of *V. cholerae*, that are O1 and O139, known to cause epidemic and pandemic cholera.⁶ The former serogroup is responsible for the first six pandemics, whereas the latter was first described in the last pandemic in 1992.^{8–12} Other serogroups, that are, non-O1 and non-O139, have only been reported to cause sporadic outbreaks with cholera-like symptoms.^{55–57} It is crucial to design

a rapid and specific diagnostic assay for these two serogroups so as to prevent or control them from developing into epidemics or pandemics in a timely manner. In this study, we have designed a sensitive and specific PCR assay for the detection and differentiation between O1 (classical and El Tor) and O139 as described in the following text. To date, the O1 El Tor strain remains globally dominant and its variants, which refer to a collection of El Tor strains that also exhibit classical phenotypes, caused even a more rapid and severe progression of cholera.^{8–10,13,14} However, our study did not include those variants as they represent a loosely defined collection of El Tor strains that emerged during the seventh pandemic. Since their genomic makeups have not been fully understood and their genome sequences are not fully available, it is difficult to identify molecular markers for designing diagnostic tests that can differentiate the variants from the wild-type El Tor strain.

Optimization of the multiplex PCR assay. The optimization of the multiplex PCR assay focused on two major aspects: the identification of the optimal annealing temperature and minimal concentrations of plasmid p100 supplement. The plasmid was spiked in each PCR reaction to check for false-negative results, which might be due to contamination with PCR inhibitors.

Optimal annealing temperatures were analyzed via gradient PCR amplifications ranging from 55°C to 70°C. The outcome of each reaction was analyzed via agarose gel electrophoresis. The annealing temperature of 57.5°C (Supplemental Figure 1) yielded the most effective PCR amplification. Moreover, we also tested 10-fold serial dilutions of p100 ranging from 10^{-15} to 10^{-9} g to determine the minimum required concentration of plasmid p100 and observed that 10^{-12} g of spiked control plasmid per PCR reaction was the lowest concentration that still yielded a detectable amplicon under the conditions of this assay. Consequently, all reactions were incorporated with 1 pg of p100 (Supplemental Figure 2).

Detection limit and specificity. To determine the detection limit of our assay, genomic DNA templates for each strain were analyzed in 10-fold serial dilutions (1 fg to 100 ng per reaction) and subjected to the multiplex PCR assay. This analysis, established DNA threshold concentrations of 10 pg template per reaction in case of O1 classical (Figure 1A). However, the corresponding detection limits for O1 El Tor and O139 were approximately 10 times higher (Figure 1B and C).

In summary, the detection limit of the multiplex PCR assay ranges between 10 and 100 pg per reaction, equivalent to 2.32×10^3 to 2.32×10^4 cells, respectively. This assay was further evaluated with 19 isolates of *V. cholerae* strains and 22 isolates of other bacterial strains (Supplemental Table 2). All O1 classical, O1 El Tor and O139 were correctly identified (Table 1). Other bacterial isolates, including *Vibrio vulnificus* and *Vibrio parahaemolyticus* revealed no false-positive or any nonspecific amplification. Gel images for PCR products on selected bacterial isolates are shown in Figure 2.

Determination of the multiplex PCR detection limit for spiked fecal samples. The multiplex PCR assay was further evaluated with DNA templates prepared from APW enrichment cultures from human stool suspensions spiked with *V. cholerae* cells. Briefly, stool suspension from healthy human that have been spiked with 10-fold serially diluted cell suspensions of *V. cholerae* were used for the inoculation of APW enrichment cultures. The results of this analysis are displayed in Figure 3, and indicated that DNA templates prepared from spiked

FIGURE 1. Determination of PCR detection thresholds: Thresholds of PCR detection with genomic DNA from (A) O1 classical isolate; (B) O1 El Tor; and (C) O139: Lane L: 25-bp molecular ladder; Lane N: no-template control; PCR products were generated with serially diluted genomic DNA templates: Lane 1 (10 ng), Lane 2 (1 ng), Lane 3 (100 pg), Lane 4 (10 pg), Lane 5 (1 pg), Lane 6 (100 fg), Lane 7 (10 fg), and Lane 8 (1 fg). The lowest DNA concentration that (still) produced visible amplicon bands was 10 pg per reaction for *Vibrio cholerae* O1 classical (A, Lane 4), and 100 pg for O1 El Tor (B, Lane 3) and O139 (C, Lane 3). This figure appears in color at www.ajtmh.org.

human stool suspensions yielded correct amplifications for all three strains without generating nonspecific amplicons.

No amplicon was observed for DNA samples prepared from enrichment cultures inoculated with “unspiked” healthy human stool suspension (negative control). For *V. cholerae* O1 classical and El Tor, we established via gel electrophoresis that the detection limit for all targets was approximately 1.5×10^5 cells/mL stool suspension. However, the detection limit was even lower for *V. cholerae* O139, which still yielded positive signals at 1.5 cells/mL stool suspension (Figure 3C).

The presence of PCR inhibitors in stool samples such as bilirubin, bile salts, heme, and complex polysaccharides constrain PCR amplifications and limit their applicability for diagnosis.^{58,59} Therefore, we included an enrichment procedure before DNA template preparation to dilute potential PCR inhibitors and increase the total number of cells from the stool samples (see Experimental Procedures). In addition, an

FIGURE 2. Determination of PCR specificity: Evaluation with genomic DNA templates from selected *Vibrio cholerae* O1 classical isolates, O1 El Tor, and O139: Lane L: 25-bp molecular ladder; Lane N: no-template control; Lane 1–3: O1 classical identified by the 85-bp PCR signal; Lane 4–7: O1 El Tor identified by the combination of PCR signals at 85 and 96 bp; Lane 8–11: O139 identified by the combination of PCR signals at 85, 96, and 120 bp; and Lane 12–13: *Vibrio vulnificus* and *Vibrio parahaemolyticus*, respectively. This figure appears in color at www.ajtmh.org.

internal amplification control was incorporated to check for the presence of potential PCR inhibitors (see Experimental Procedures). The sensitivity test for the multiplex PCR assay revealed that positive signals were still detected for cell densities as low as 1.5 cells/mL. The average detection limit of our PCR assay represented significant improvements compared with the previously reported threshold of 10^4 *V. cholerae* cells/mL for PCR assays with fecal-spiked material.^{60,61} Also, Chua et al.²⁹ detected 2×10^4 colony-forming units (CFU) and 100 pg DNA at the genomic level, whereas Mehrabadi et al.³⁰ detected 10–100 CFU *V. cholerae* and 8.5–85 pg genomic DNA in multiplex PCR assays. Both PCR assays used protein-coding genes as targets for amplification. Our analysis revealed that sRNA genes are at least equally suitable for the detection of *V. cholerae*.

Furthermore, the assay differentiates between biotypes of *V. cholerae*, and hence represents a very useful tool, especially in the context of epidemiological surveillance of cholera outbreaks or food safety. For instance, a mixed outbreak of *V. parahaemolyticus* and norovirus caused acute gastroenteritis for 99 people in Guangdong, China, in 2013, after consuming roasted duck. Subsequent investigations revealed that one of the food handlers was the actual carrier/source for both pathogens.⁶² A larger *Vibrio* outbreak in Malaysia in November 2009, caused approximately 400 infections and one casualty; several restaurants and food factories were ordered to close as they were suspected to be responsible for the outbreak.⁶³ In such cases, multiplex PCR assays would allow the fast identification of potential sources and the differentiation of disease-causing biotypes. As a consequence, the time window for medical intervention would be significantly widened compared to culture-based analysis. We envision that this assay can be used routinely or on-demand to deal even with bigger endemic or locally restricted outbreaks as described previously. In the context of food safety, the detection of *V. cholerae* is especially important to seafood products, which are widely consumed and exported worldwide.⁶⁴

FIGURE 3. Evaluation of the multiplex PCR assay with spiked human stool suspensions: Test for the applicability of the assay on DNA templates prepared from enrichment cultures inoculated with human stool suspension spiked with: (A) O1 classical; (B) O1 El Tor; and (C) O139 strains. Lane L: 25-bp molecular ladder; Lane N: no-template control; Lane C: enrichment culture of human stool suspension without spiking (control); enrichment culture of human stool suspensions spiked with *Vibrio cholerae* cell suspensions representing cell densities of: 1.5×10^7 (Lane 1), 1.5×10^6 (Lane 2), 1.5×10^5 (Lane 3), 1.5×10^4 (Lane 4), 1.5×10^3 (Lane 5), 150 (Lane 6), 15 (Lane 7), 1.5 (Lane 8), 0.15 (Lane 9) and 0.015 (Lane 10) cells/mL stool suspension. Detection limits for the multiplex PCR with stool samples were 1.5×10^5 cells/mL stool suspension for *V. cholerae* O1 classical (A, Lane 3) and O1 El Tor (B, Lane 3), and 1.5 cells/mL stool suspension for O139 (C, Lane 8). This figure appears in color at www.ajtmh.org.

Early detection and differentiation of *Vibrio* biotypes is crucial and should rely on rapid and cost-effective methods. Because of the simple application of our assay, it requires only simple gel electrophoresis to generate analytical read outs and the connected low(er) costs, it might become a versatile tool to monitor cholera outbreaks, and prove beneficial for the poorer societies of Asia or Africa.

CONCLUSION

Here, we demonstrated the efficiency of combining sRNA genes (*Vc_npcR_3853* and *Vc_npcR_3997*) with the O139-specific *rfb* protein-coding gene as targets to detect and differentiate *V. cholerae* biotypes via multiplex PCR assays. Under optimal conditions, the detection limit for pure culture ranges between 10 and 100 pg per reaction. Enrichment cultures of spiked stool suspensions revealed a detection limit of 1.5 cells/mL suspension. The success rate of sample detection was close to 90%. Small RNAs represent superior candidates for molecular diagnostic assays, especially in PCR

and reverse-transcription quantitative real-time PCR diagnostics. Moreover, given the importance to identify the source of infection, we will further assess the capacity of this assay by analyzing seafood products, such as seashell, crabs, shrimp, and mussels. Furthermore, we will include more non-protein coding RNAs as target genes to allow the detection of other *Vibrio* species that are prevalent in seafood using multiplex PCR assay.

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