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Development of a multiplex PCR assay for the detection of major virulence genes in *Vibrio cholerae* including non-O1 and non-O139 serogroups

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ABSTRACT

Vibrio cholerae strains producing cholera toxin (CT) and toxin co-regulated pilus (TCP) and belonging to O1 and O139 serogroups are responsible for cholera. However, non-CT producing *V. cholerae* from non-O1/non-O139 serogroups have been increasingly isolated from diarrheal stools and extra-intestinal infections. In this study, we have developed a multiplex PCR for the simultaneous detection of heat-stable enterotoxin (*stn*), type three-secretion system (*vopF*), and cholix toxin (*chxA*), along with CT (*ctx*) in *V. cholerae* strains. As other species from genus *Vibrio* carries homologous virulence genes, *V. cholerae* specific *ompW* was also included to differentiate *V. cholerae* from other vibrios. This assay was 100% specific and sensitive, and could detect homologous virulence genes like *ctxA* in *V. mimicus* and *vopF* in *V. parahaemolyticus*. This multiplex PCR assay, which can detect four major virulence genes in *V. cholerae,* is novel and important for epidemiologic and environmental surveillance of pathogenic *V. cholerae.*

1. Introduction

Vibrio cholerae, the causative agent of cholera, is a Gram-negative, motile bacterium with a single polar flagellum, and is transmitted through contaminated water. Every year, millions of people suffer from cholera throughout the world, particularly in developing countries (Ali et al., 2015; Mukandavire and Morris, 2015). Out of more than 200 reported 'O' serogroups, *V. cholerae* strains belonging to serogroups O1 and O139 are predominantly associated with cholera epidemics. *V. cholerae* strains other than O1 or O139 are referred to as 'non-O1/non-O139', which are associated with sporadic cases of diarrhea and extra-intestinal infections (Dalsgaard et al., 1999; Dutta et al., 2013; Chowdhury et al., 2016). The *V. cholerae* virulence is primarily attributed to cholera toxin (CT; encoded by *ctxAB* genes), and toxin co-regulated pilus (encoded by *tcpA* gene). However, *ctx* and *tcpA* negative strains, mostly the *V. cholerae* non-O1/non-O139 have been detected in sporadic cases. Other than CT and TcpA, *V. cholerae* genome encodes numerous other virulence factors, including heat-stable enterotoxin (Nag-ST, encoded by *stn* gene), cholix toxin (ChxA, encoded by *chxA* gene), type three-secretion system (T3SS), and accessory virulence factors (hemolysin and repeat-in-toxin) (Sarkar et al., 2002; Awasthi et al., 2013; Dziejman et al., 2005). These virulence factors are associated with different clinical manifestations such as intestinal and extra-intestinal infections, particularly by some strains of *V. cholerae* non-O1/non-O139 (Sharma et al., 1998; Faruque et al., 2004; Chatterjee et al., 2009).

The role of these virulence factors is not well recognized in *V. cholerae* pathogenesis. Either individually or in combination, these virulence factors have been unequivocally associated with the virulence of these species. Among the known major virulence factors of *V. cholerae* non-O1/non-O139, strains possessing *stn* gene can produce Nag-ST, whose production has been associated with severe diarrhea (Honda et al., 1985; Morris et al., 1990). T3SS was reported in *V. cholerae* non-O1/non-O139 strains as an important virulence factor for diarrhea (Dziejman et al., 2005; Alam et al., 2011). T3SS gene cluster comprises of genes encoding the structural components and effector proteins. One of the major effector proteins in *V. cholerae* T3SS is VopF, encoded by *vopF*, which is translocated into the host cells to induce changes in the

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actin cytoskeleton (Tam et al., 2007). Cholix toxin (ChxA) is an ADP-ribosyltransferase produced by *V. cholerae* strains (Jørgensen et al., 2008). Previous studies suggested that ChxA might be associated with extra-intestinal infections and was fatal to mice due to extensive damage to liver (Awasthi et al., 2013). Homologues of some virulence genes of *V. cholerae* have been also reported in other closely related species in genus *Vibrio*. For example, production of CT and Nag-ST and presence of genes encoding those toxins have been reported in *V. mimicus* (Arita et al., 1991; Yuan et al., 1994; Shi et al., 1998). On the other hand, highly homologous T3SS related genes present in *V. cholerae* were detected in *V. parahaemolyticus* (Dziejman et al., 2005).

The majority of the PCR based methods targeted only *ctx* and *tcpA* for characterization of toxigenic *V. cholerae* O1 and O139 strains. However, other virulence determinants were not included in these assays. To understand the epidemiological and clinical importance of these virulence factors in *V. cholerae* pathogenesis, we have developed a multiplex PCR (Mpx-PCR) assay for the detection of 4 major virulence genes (*ctx, stn, chxA, vopF*) and a *V. cholerae* species-specific gene, *ompW* (Jalajkumari and Manning, 1990). Furthermore, the Mpx-PCR assay was evaluated for specificity, sensitivity, detection limit and applicability to the clinical samples.

2. Material and methods

2.1. Bacterial strains and culture conditions

V. cholerae strains N16961 (O1; *ctxA*$^+$), Vc20 (O131; *vopF*$^+$), Vc54 (O39; *chxA*$^+$), GP156 (O1; *stn*$^+$), and Vc129 (O141; *ctxA*$^+$/*chxA*$^+$/*vopF*$^+$) were used as respective control strains in the optimization of the Mpx-PCR assay.

A total of 43 *V. cholerae* strains, including O1 and non-O1/non-O139 (Table 1), were used to evaluate the specificity of this newly developed PCR assay. The virulence gene profiles of the tested *V. cholerae* strains are shown in Table 1. Among the relevant strains of other *Vibrio* species, *Vibrio mimicus* ($n = 5$) including *ctx* positive strains ($n = 2$) isolated from Bangladesh, and *Vibrio parahaemolyticus* ($n = 1$) harboring *vopF* of the T3SS gene cluster were examined by the Mpx-PCR assay. Strains belonging to 10 other species such as *V. alginolyticus* ATCC17749^T, *V. carchariae* ATCC35084^T, *V. mediterraniae* UM517^T, *V. metschnikovii* IAM1039^T, *V. mimicus* ATCC33653^T, *V. natriegens* ATCC14048^T, *V. nigripulchritudo* ATCC27043^T, *V. proteolyticus* ATCC15338^T, *V. vulnificus* IFO15645^T, and *V. fischeri* NCIMB1281^T were also included. Moreover, strains from other major bacterial species associated with gastroenteritis, were also used to evaluate the PCR assay (Table 1). These bacterial strains included *Campylobacter jejuni* ($n = 3$) including strains 81–176, ATCC33560^T, ATCC43432^T, *C. coli* ($n = 7$), *C. fetus* ($n = 9$) including *C. fetus* subsp. *venerealis* ATCC19438^T, *C. fetus* subsp. *fetus* ATCC27374^T, *C. lari* ATCC43675^T and *C. hyointestinalis* ATCC35217^T, *Escherichia coli* ($n = 7$) including strain C600 used for a negative control in the PCR assay, *Salmonella* spp. ($n = 7$), *Shigella* spp. ($n = 7$), *Aeromonas* spp. ($n = 3$) and *Photobacterium damselae* ($n = 1$).

The *Vibrio* strains stored as glycerol stock at −80 °C were grown on thiosulfate citrate bile salts sucrose (Eiken Chemical CO. Ltd., Tochigi, Japan) at 37 °C. *E. coli* and *Shigella* spp. were cultured in LB agar (Becton, Dickinson and Company, NJ, USA) at 37 °C whereas *Salmonella* spp. and *P. damselae* were grown in trypticase soy agar (Becton, Dickinson and Company) at 37 °C. The *Campylobacter* strains were cultured under microaerobic condition, according to Asakura et al. (2007).

2.2. Selection of target genes and primers

We have attempted to develop a pentaplex PCR to detect genes encoding the major virulence factors (CT, Nag-ST, ChxA, VopF) and species-specific outer membrane protein (OmpW) for *V. cholerae* (Table 2). All the PCR primers for *vopF*, *chxA*, and *stn* were designed in

Table 1
Strains used in this study and evaluation of sensitivity and specificity of the multiplex PCR.

Bacterial strains (*n*)	Serogroup	*n*	Colony hybridization data					Mpx PCR data				
			ctxA	*chxA*	*vopF*	*nag-ST*	*ompW*	*ctxA*	*chxA*	*vopF*	*nag-ST*	*ompW*
V. cholerae (43)	O1	6[a]	+	−	−	−	+	+	−	−	−	+
	non-O1/non-O139	5[b]										
	O1	4[b]	−	−	+	−	+	−	−	+	−	+
	non-O1/non-O139	7										
	O1	1	−	+	−	−	+	−	+	−	−	+
	non-O1/non-O139	7										
	non-O1/non-O139	4	−	+	+	−	+	−	+	+	−	+
	non-O1/non-O139	3	−	+	+	+	+	−	+	+	+	+
	non-O1/non-O139	1	+	+	+	−	+	+	+	+	−	+
	non-O1/non-O139	1	−	+	−	+	+	−	+	−	+	+
	O1	1[c]	−	−	−	+	+	−	−	−	+	+
	O1	2[c]	−	−	−	−	+	−	−	−	−	+
	non-O1/non-O139	1										
Vibrio parahaemolyticus (1)	NA	3	ND	ND	ND	ND	ND	−	−	+	−	−
Vibrio mimicus (2)	NA	2	+	ND	ND	ND	ND	+	−	−	−	−
Vibrio mimicus (3)	NA	3	−	ND	ND	ND	ND	−	−	−	−	−
Other Vibrio spp. (10)#	NA	10	ND	ND	ND	ND	ND	−	−	−	−	−
Aeromonas spp.	NA	3	ND	ND	ND	ND	ND	−	−	−	−	−
Photobacterium damselae(1)	NA	1	ND	ND	ND	ND	ND	−	−	−	−	−
Campylobacter jejuni (3)	NA	3	ND	ND	ND	ND	ND	−	−	−	−	−
Campylobacter coli (7)	NA	7	ND	ND	ND	ND	ND	−	−	−	−	−
Campylobacter fetus (9)	NA	9	ND	ND	ND	ND	ND	−	−	−	−	−
Campylobacter hyointestinalis (1)	NA	1	ND	ND	ND	ND	ND	−	−	−	−	−
Campylobacter lari (1)	NA	1	ND	ND	ND	ND	ND	−	−	−	−	−
Escherichia coli(7)	NA	7	ND	ND	ND	ND	ND	−	−	−	−	−
Salmonella spp. (7)	NA	7	ND	ND	ND	ND	ND	−	−	−	−	−
Shigella spp. (7)	NA	7	ND	ND	ND	ND	ND	−	−	−	−	−

ND: not done; a: 5 isolates are of clinical origin, b: 1 isolate is of clinical origin, c: All isolates are of clinical origin; #Other *Vibrio* spp. includes *V. alginolyticus* ATCC17749, *V. carchariae* ATCC35804, *V. mediterraniae* UM517, *V. metschnikovii* IAM1039, *V. mimicus* ATCC33653, *V. natriegens* ATCC14048, *V. nigripulchritudo* ATCC27043, *V. proteolyticus* ATCC15338, *V. vulnificus* IFO15645, *V. fischeri* NCIMB1281.

Table 2
Primers used in this study.

Target gene	Primer ID	Primer sequence (5′-3′)	Primer binding position	Amplicon size (bp)	Reference
ctxA	VCT1	ACAGAGTGAGTACTTTGACC	132–151	505	Hoshino et al., 1998
	ctxA_505	CGATGATCTTGGAGCATTC	618-636		This study
chxA	*chxF*	CAGTAAGAGCGACTAGAC	311–328	634	This study
	chxR	TCTAATGGCACACCACAG	927–944		
vopF	*vopF-U*	GGAAATTCGCCAAGGTGTA	552–570	839	This study
	vopF-R	CAAAACCGTCCATACAAGG	1372–1390		
stn	*nagst-F*	ATGAAAAACCTATTCATTGC	1–20	220	This study
	nagst-R	AGCAAGCTGGATTGCAAC	203–220		
ompW	*ompW-F*	CACCAAGAAGGTGACTTTATTGTG	64–87	304	Nandi et al., 2000
	ompW-R	GGTTTGTCGAATTAGCTTCACC	346–367		

ctxA: cholera toxin A subunit gene, *chxA*: cholix toxin gene, *vopF*: Type three effector gene, *stn*: heat stable enterotoxin gene, *ompW*: outer membrane protein gene.

this study. For the detection of *ctxA* by PCR, the forward primer was selected from Hoshino et al. (1998), however, the reverse primer was designed in this study. PCR primers targeting species-specific *ompW* for *V. cholerae* were described by Nandi et al. (2000). The expected amplicon sizes of the target genes are 839 bp (*vopF*), 634 bp (*chxA*), 505 bp (*ctxA*), 220 bp (*stn*) and 304 bp (*ompW*) as shown in Table 2.

2.3. PCR reaction for Mpx-PCR

The Mpx-PCR was carried out in 20 µL reaction volume with 10 primers (5 sets), each at a final concentration of 0.5 µM, 0.5 U *Ex-Taq* DNA polymerase with its buffer system (Takara Bio Inc. Shiga, Japan). The optimized PCR condition for the Mpx-PCR are as follows: initial denaturation at 94 °C for 2 min, followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 56 °C for 30 s, extension at 72 °C for 45 s and a final extension step at 72 °C for 3 min. The PCR products were subjected to 2.0% LE agarose (Promega Corporation) gel electrophoresis in TAE buffer (40 mM Tris-acetate, 1.0 mM EDTA [pH 8.0]). The gel was stained with ethidium bromide (2 µg ml^{-1}) and destained with distilled water. Images were captured using Gel-Doc 2000 (Bio-Rad Laboratories Inc., CA, USA).

2.4. Sensitivity and specificity of Mpx-PCR

Sensitivity of the Mpx-PCR was examined by using *V. cholerae* strains listed in Table 1. Presence of the virulence genes in the *V. cholerae* strains were previously determined in our laboratory by colony hybridization using dCTP-^{32}P labelled specific gene probes (Table 1). A total of 43 target strains, with varied genotypes as described in Table 1, were used for sensitivity assay. Apart from *V. cholerae*, T3SS gene-positive *V. parahaemolyticus* ($n = 1$) and *ctxA*-positive *V. mimicus* strains ($n = 2$) were also included for the sensitivity analysis.

Moreover, the specificity of the Mpx-PCR assay was determined using the *V. cholerae* strains and other bacterial species. Type strains belonging to non-target *Vibrio* species ($n = 10$) and other enteropathogenic bacterial species including *Aeromonas* spp. ($n = 3$), *Campylobacter* spp. ($n = 21$), *E. coli* ($n = 7$), *Salmonella* spp. ($n = 7$), *Shigella* spp. ($n = 7$), and *P. damselae*. ($n = 1$) were included in the evaluation of the Mpx-PCR assay (Table 1).

2.5. Detection limit

Two strains of *V. cholerae*, Vc129 and GP156 with genotype profiles $ctxA^+/chxA^+/vopF^+/ompW^+$ and $stn^+/ompW^+$, respectively, were used to evaluate the detection limit of the Mpx-PCR. The DNA templates from pre-determined cell numbers (CFU) of the bacterial strains were prepared by boiling method according to Hoshino et al. (1998). The DNA templates represented a gradient of cell concentrations, i.e., approximately 10^5, 10^4, 10^3, 10^2, 10^1 and 10^0 CFU of the *V. cholerae* strains.

2.6. Evaluation of Mpx-PCR with patient samples

The Mpx-PCR assay was evaluated using patient stool samples, which were culture positive for *V. cholerae* O1 ($n = 41$) and *V. cholerae* non-O1/non-O139 ($n = 24$), collected at the National Institute of Cholera and Enteric Diseases (NICED), Kolkata, India between June 2012 and December 2013. Informed consent was obtained for experiments to use their stool samples in research purpose. The stool samples were suspended in phosphate buffered saline, and DNA templates were prepared in 1× TE buffer (pH 8.0) by boiling method (Hoshino et al., 1998) and used for the Mpx-PCR.

3. Results

3.1. Sensitivity and specificity of the Mpx-PCR

The sensitivity of the Mpx-PCR targeting each of five genes developed in this study was 100%. A representative result is shown in Fig. 1. The Mpx-PCR produced the specific amplicon size expected for each target gene. All the *V. cholerae* strains ($n = 43$) generated species-specific *ompW* amplicon (304 bp). Out of these 43 *V. cholerae* strains, 11, 11 and 8 strains were positive for any one of the virulence genes such as *ctxA, vopF* or *chxA*, respectively, by colony hybridization. Application of the Mpx-PCR assay correctly identified these virulence genes by producing the amplicons of specific sizes, i.e., 505, 839, and 634 bp, respectively. Among the tested *V. cholerae* strains, 9 strains were positive for more than one virulence genes by colony hybridization. Application of Mpx-PCR assay also produced multiple amplicons of specific size for these *V. cholerae* strains carrying multiple virulence factors (Table 1). As expected, 3 *V. cholerae* strains, negative for the tested virulence genes by colony hybridization, produced amplicons only for the species-specific *ompW* gene. In the cases of T3SS related gene-positive *V. parahaemolyticus* strain ($n = 1$) and *ctxA*-positive *V. mimicus* strains (2 out of 5), the Mpx-PCR assay also correctly amplified the *vopF* and *ctxA* genes, respectively, but not for *ompW* gene.

No specific amplicon was observed for any of the non-target bacterial species including *Campylobacter* spp. ($n = 21$), *E. coli*. ($n = 7$), *Salmonella* spp. ($n = 7$), *Shigella* spp. ($n = 7$), *Aeromonas* spp. ($n = 3$), *P. damselae* ($n = 1$) and other *Vibrio* spp. ($n = 10$) (Table 1). Accurate identification of all the target genes of the *V. cholerae* examined, but no production of specific amplicon for any of the non-target bacterial species indicates that the specificity of the Mpx-PCR assay was 100%.

3.2. Detection limit

Detection limit of the Mpx-PCR was determined using a mixed DNA template of two *V. cholerae* strains, Vc129 ($ompW^+/ctxA^+/chxA^+/vopF^+$) and GP156 ($ompW^+/stn^+$). The detection limit for *ctxA*, *chxA* and *vopF* was estimated as 1.18E + 01 CFU PCR $tube^{-1}$ and for *stn* 1.23E + 01 CFU PCR $tube^{-1}$. Since a mixed DNA template was used, the detection limit of PCR amplifying the *ompW* was 2.41E + 01 CFU PCR

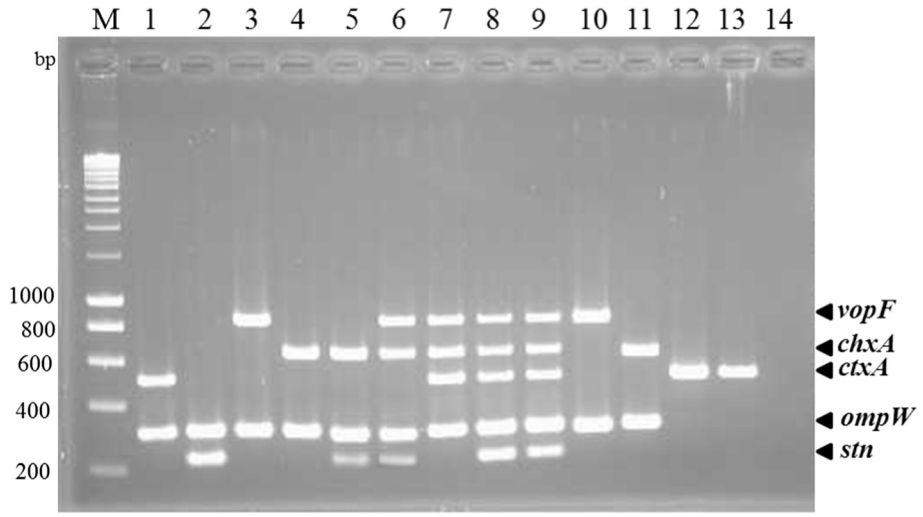

Fig. 1. A representative picture demonstrating detection of virulence genes in *V. cholerae* and *V. mimicus* strains. M, 200 bp ladder (Takara Bio Inc.). Lane 1-11: *V. cholerae* strains, Lane 12 and 13: *ctx* positive *V. mimicus* strains, Lane 14: *E. coli* C600 as a negative control.

tube^{-1}. Thus, a minimum detection limit for all the target virulence genes by this Mpx-PCR was 10 CFU PCR tube^{-1} and there was no difficulty in simultaneous detection of all the major virulence genes such as *ctxA, chxA, vopF* and *stn* and species identification of *V. cholerae* in a single reaction mixture.

3.3. Performance on crude patient sample

To establish if this Mpx-PCR can be relevant to patient stool samples, following examination was performed and the result is shown in Table 3. Out of the 41 *V. cholerae* O1 culture-positive stool samples, 35 (85.4%) produced positive amplicon for *ompW* and 31 (75.6%) were positive for *ctxA*. None of the stool samples containing *V. cholerae* O1 were positive for *chxA, vopF* and *stn*. Similarly, for *V. cholerae* non-O1/non-O139 culture-positive samples, 15 out of 24 (62.5%) were positive for *ompW* by this PCR. Two samples were positive for *chxA* and 3 for *vopF*. None of the stool samples containing *V. cholerae* non-O1/non-O139 produced any specific amplicon for *ctxA* and *stn*.

4. Discussion

Despite a lot of improvements in treatment facilities, the global burden of cholera remains significantly high. In recent years, Sub-Saharan Africa accounts for the majority of this burden (Ali et al., 2015). In 2016, 38 countries reported a total of 132,121 cases including, 2420 deaths, resulting in an overall case fatality rate (CFR) of 1.8% (WHO, 2017). Cholera was reported from countries in all regions. Seventeen countries in Africa, 12 in Asia, 4 in Europe, 4 in the Americas, and 1 in Oceania. Five countries, the Democratic Republic of the Congo, Haiti, Somalia, the United Republic of Tanzania and Yemen, together accounted for 80% of all cases (WHO, 2017). According to the Haiti's Health Ministry report, the Haitian cholera outbreak, which was started in October 2010, over 809,000 people have been infected and 9670 have died (CFR of 1.2%) (Zarocostas, 2017). In an ongoing Yemen cholera outbreak, the cumulative total from 27 April 2017 to 3rd June 2018 was 1,105,371 suspected cholera cases with 2300 deaths (Emro, 2018). Historically, the major virulence factors in *V. cholerae* pathogenesis are CT and TCP. Several PCR assays have been reported to target *ctx* and *tcpA* genes, which are related to CT and TcpA production (Keasler and Hall, 1993; Kumar et al., 2010). However, non-CT producing *V. cholerae* strains, especially belonging to non-O1/non-O139 serogroups has been also isolated from a number of intestinal and extra-intestinal infections (Dalsgaard et al., 1999; Dutta et al., 2013; Hao et al., 2015; Chowdhury et al., 2016). Therefore, there is an increased interest in the simultaneous detection of other virulence factors, i.e., Nag-ST (*stn*), T3SS (*vopF*), and ChxA (*chxA*), along with CT and TCP in *V. cholerae* strains.

Simplex PCR assays for the detection of *stn* encoding heat-stable enterotoxin have been reported (Guglielmetti et al., 1994; Vicente et al., 1997). A PCR-RFLP assay was recently reported for the detection and typing of *chxA* in *V. cholerae* strains (Awasthi et al., 2014). On the other hand, no PCR assay was evaluated for the detection of T3SS in *V. cholerae*. Similarly, no Mpx-PCR assay was available targeting the major virulence genes in *V. cholerae*. Only a hexaplex PCR targeting virulence and its regulatory genes such as *ctxA, zot, ace, tcpA, ompU* and *toxR* in *V. cholerae* has been reported (Singh et al., 2002). Therefore, in this study, we have developed the Mpx-PCR assay targeting *ctxA, stn, chxA, vopF* (T3SS) and *ompW*, which is novel and important for epidemiological and environmental surveillance on *V. cholerae* not only O1 and O139 serogroups but also non-O1/non-O139. Moreover, the *ompW* was considered as one of the target genes in this Mpx-PCR as a marker for the detection and differentiation of *V. cholerae* strains from other vibrios, particularly *V. mimicus* and *V. parahaemolyticus*, which can carry homologous virulence genes like *ctx* and T3SS (Arita et al., 1991; Yuan et al., 1994; Shi et al., 1998; Dziejman et al., 2005).

This newly developed Mpx-PCR showed high efficiency in detecting the target genes in a single reaction, without cross interaction between any of the 5 primer sets (Fig. 1). The primers were designed or selected to produce variable amplicon sizes of the target genes so that they could be distinctly resolved in a 2.0% agarose gel. The performance of the Mpx-PCR was validated using a considerable number of target strains with diverse virulence gene profile, and non-target strains. When applied to *V. cholerae* strains of known genotypes, this detection assay produced expected results with 100% sensitivity. The assay also

Table 3
Evaluation of multiplex PCR on *V. cholerae* positive patient stool samples.

Target gene	*V. cholerae* O1 positive	*V. cholerae* non-O1/O139 positive
	(n = 41)	(n = 24)
ctxA	31	0
chxA	0	2
vopF	0	3
stn	0	0
ompW	35	15

ctxA: cholera toxin A subunit, *chxA:* cholix toxin, *vopF:* T3SS effector, *stn:* heat stable enterotoxin, *ompW:* outer membrane protein of *V. cholerae*.

successfully detected virulence gene-homologues of *ctxA* in *V. mimicus* and *vopF* (T3SS) in *V. parahaemolyticus* and as expected no amplification was observed for *V. cholerae*-specific *ompW* in these strains. The detection assay developed in this study was found to be 100% specific to produce PCR amplicon for all the target genes, but no amplicon in non-target strains. Therefore, this Mpx-PCR is recommended for the characterization of *V. cholerae* strains in pure culture, and can be also applied for the detection of *V. cholerae* and virulence genes in crude patient stool samples. However, the Mpx-PCR assay may have lower sensitivity when applied to crude stool samples, which may be due to the pre-existing PCR inhibitory compounds (Schrader et al., 2012). To make the PCR protocol simple and easy, DNA template was prepared by boiling method and this could be also a possible reason for lower sensitivity of this assay when applied to crude stool samples. If genomic DNA extracted by the commercially available DNA extraction kit was used, the sensitivity and positive rate might be improved. The observed detection limit of 1–10 CFU tube^{-1}, corresponding to 10^3–10^4 CFU mL^{-1}, is comparable to other established PCR detection assays, e.g., Asakura et al., 2007, Neogi et al., 2010. The high efficiency of the new Mpx-PCR assay of this study in detecting virulence genes of *V. cholerae* in low number can be very useful in monitoring the hazards of potentially pathogenic strains of this species in the clinical and environmental samples.

5. Conclusions

We have developed a Mpx-PCR assay, which can detect the major virulence genes in *V. cholerae* not only O1 and O139 serogroups but also non-O1/non-O139 with high efficiency. This PCR assay is simple and rapid, and can be highly beneficial in detecting causal genetic factor of *V. cholerae* strains associated with disease incidence. The Mpx-PCR assay can be also useful in detecting virulence homologues in other species of vibrios, particularly *ctx* and T3SS in *V. mimicus* and *V. parahaemolyticus*, respectively. Altogether, our data clearly demonstrate that the newly developed Mpx-PCR is a simple and reliable method for the detection and characterization of virulence genes in *V. cholerae*.

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Declarations of interest

None.

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