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Original Research

Development of Real-Time PCR Assay for Detection of *Vibrio cholerae*

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Abstract

Vibrio cholerae is the etiological agent of epidemic cholera. Symptoms include stomach ache, bloody diarrhea, and vomiting, which lead to severe dehydration and even death. Environmental monitoring as well as rapid and accurate identification of this pathogen are important for public health protection. In this study, a real-time PCR method for the detection of toxigenic *V. cholerae* was developed. In total, 63 environmental and clinical strains were tested for the presence of seven targets, namely *ompW*, *ompU*, *tcpA*, *ctxA*, *zot*, *rfbO1*, and *rfbO139*. The proposed method is specific, simple, and fast, and can be used for detection of toxigenic and non-toxigenic *V. cholerae* strains. The minimum detection limit of this assay for *V. cholerae* in environmental water samples was 1.4 CFU/ml.

Keywords: real-time PCR, *Vibrio cholerae*, *ompW*, *ompU*, *zot*, *tcpA*, *rfbO1*, *rfbO139*, *ctxA*

Introduction

Cholera is an acute, severe infectious disease that leads to death through dehydration and electrolyte disturbances. The etiological agent of cholera is *Vibrio cholerae*, a member of the domain *Bacteria*, phylum *Proteobacteria*, class *Gamma-proteobacteria*, order *Vibrionales*, family *Vibrionaceae*, genus *Vibrio* [1]. *V. cholerae* species are grouped on the basis of the structure of surface antigens into serogroups. These include *V. cholerae* O1 strains that are known for their toxigenic properties caused by the ability to secrete a strong enterotoxin, i.e. cholera toxin (CT), and are therefore considered to be of major epidemiological importance [2]. There are two biotypes of toxigenic strains, namely Classical and El Tor. The first is responsible for the pandemics of cholera that spread in Asia since 1961 and subsequently reached the Western Hemisphere. In 1992, during a cholera epidemic in Bangladesh, a new toxigenic serotype was isolated, designated *V. cholerae* O139 "Bengal" [3]. The strains of another 137 serogroups, collectively referred to as non-O1 serotypes, are frequently isolated from cases of diarrhea but are of lesser epidemiological importance [4-8]. Rapid detection of the etiological agent of cholera is essential for effective treatment and to prevent spread of the disease.

Thiosulfate-citrate-bile-salts-sucrose agar (TCBS), a selective medium, and alkaline (pH 8.4-8.6) peptone water with 1-3% NaCl are used for isolation of *V. cholerae* from fecal samples. *V. cholerae* grows on TCBS medium in the form of smooth, yellow colonies with an opaque center and transparent edges that are caused by the decomposition of sucrose. The presence of bile and bile salts in the basic medium inhibit the growth of most Gram-positive and Gram-negative intestinal flora [1]. *V. cholerae* may be distinguished from other bacterial species on the basis of the following biochemical features: the ability to produce indole, fermentation of sucrose and D-mannitol, lysine decarboxylation, and growth without NaCl and in the pres-

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ence of 1% NaCl [1]. *V. cholerae* gives negative test results for lactose fermentation, decomposition of salicin, determination of arginine dehydrolase and growth in the presence of 7 and 10% NaCl [1]. Serological tests are used primarily to detect *V. cholerae* antigens O1 and O139, and these include the agglutination test with specific anti-sera, either anti-O1 nor anti-O139 [9-11].

Currently, molecular biology methods, including the PCR technique [9, 11-14] and one of its derivatives, real-time PCR, are used in the identification of *V. cholerae* and are also useful for epidemiological purposes. The high sensitivity and quick time for obtaining results are the main advantages of this technique [15, 16].

The aims of our study were the development of a real-time PCR assay, and the design of primers and molecular probes for identification of *V. cholerae* species and also for differentiation of toxigenic *V. cholerae* strains from the non-toxigenic strains. Moreover, the sensitivity of the real-time PCR for the detection of *V. cholerae* in spiked water, obtained from the Vistula River, was also determined.

Materials and Methods

Bacterial Cultures

A total of 63 *V. cholerae* strains were analyzed in this study. Fifty-five strains were isolated from the Bug and Vistula rivers, and eight reference strains were obtained from the Institute of Maritime and Tropical Medicine in Gdynia. These comprised of *V. cholerae* 8/6332, El Tor (Institute of Pasteur), *V. cholerae* 10/C219, El Tor (Institute of Pasteur), *V. cholerae* 11/AA73, El Tor Inaba (Institute of Pasteur), *V. cholerae* 13/154, El Tor Ogawa (Institute of Pasteur), *V. cholerae* 14/Jord 1, El Tor, *V. cholerae* 15/Gwinee 1014, El Tor Ogawa (Institute of Pasteur), and *V. cholerae* O1 Classical, No. 15/2002/S (WHO Kopenhaga), obtained from PZH (National Institute of Public Health – National Institute of Hygiene), *V. cholerae* O139 CIP 104152 "Bengal" (Institute of Pasteur). All environmental and reference strains were examined using the immunochromatographic test (SMART – New Horizons Diagnostic Corporation, Maryland, USA). The O1 and O139 serogroups were confirmed. Additionally, the following bacterial strains were also included as controls in the analyses: *Klebsiella pneumoniae* ATCC 10031, *Enterobacter cloaceae* ATCC 35030, *E. faecium* ATCC 27270, *E. faecalis* ATCC 19433, *Salmonella enterica* subsp. *enterica* Serovar Choleraesuis var Kunzendorf ATCC 12011, *Shigella flexneri* ATCC 12022, *Escherichia coli* NCTC 10538, *Vibrio parahaemolyticus* ATCC 17802, and *V. alginolyticus* ATCC 17749.

Real-Time PCR Primers and Probes

To identify *V. cholerae*, we designed and used the following primers and molecular probes: one hybridization probe (Hyb) for the *ctx* sequence and four hydrolysis probes specific for the *ompW*, *ompU*, *ctxA*, and *zot* genes (Table 1). The primers and probes were designed using the programs LightCycler Probe Design Software 2.0 Version 1.0 and OligoAnalyzer 1.2, and were synthesized in Genomed in the Service of Sequencing and Synthesis IBB PAS.

In addition, we identified a gene sequence in *tcpA* to differentiate *V. cholerae* biotype El Tor and Classical O1 and O139, as well as *rfbO139* and *rfbO1* gene sequences in order to distinguish serotype O1 from O139. All of the reference strains (O1 and O139 *V. cholerae* strains), each containing the *ompW*, *ompU*, *ctxA*, and *zot* genes, and the environmental samples, which were tested for the presence of the *ompU* gene (random selection), were used for analysis. Tests for the *tcpA*, *rfbO139*, and *rfbO1* genes were performed using SYBRGreen dye.

Real-Time PCR Assays

Genomic DNA was extracted from the bacterial strains using the Genomic Mini kit (Gdynia). The real-time PCR reactions were performed in the Light Cycler 2.0 (Roche) capillary system, in a final reaction volume of 20 µl. The reaction mix for two sets of probes and primers contained the same proportions of components. The reaction proceeded with the same thermal conditions. The reaction mix of Hyb probe, contained 1x FastStart DNA Master Hyb Probe (Roche); 25 mM of $MgCl_2$; 0.5 µM of primers; 0.15 µM of Hyb probe and 10-100 pg/µl of genomic DNA. The reaction mix of hydrolysis probes contained 1x FastStart DNA Taq Man Master (Roche), 0.5 µM of primers; 0.1 µM of hydrolysis probe and 10-100 pg/µl of genomic DNA. The reaction mix of SYBRGreen dye contained 1x FastStart DNA Master SYBRGreen (Roche), 0.5 µM of primers, 1 mM of $MgCl_2$, and 10-100 pg/µl of genomic DNA.

Preparation of Environmental Water Samples

Water samples were collected from the Vistula River between Sandomierz and Warsaw. A total of ten river water samples were collected and used in artificial contamination experiments. Ten-fold serial dilutions of *V. cholerae* O139 were made from overnight APW (alkaline peptone water pH 8.6) cultures. To enumerate the bacteria (CFU/ml), 100 µl of each dilution was plated onto TCBS agar plates and incubated at 37ºC.

Water samples from the Vistula River, in a volume of 495 ml, were spiked with 5 ml of ten-fold serial dilutions of the *V. cholerae* O139 culture in peptone water, to yield titers ranging between $1.4 \cdot 10^6$ CFU/ml and $1.4 \cdot 10^{-1}$ CFU/ml. The spiked water samples were then incubated for 1 h at room temperature. Subsquently, the water samples were filtered through 0.45-µm pore size filters using a Millipore vacuum-pressure filter system (20 kPa). The membranes were recovered, intensively vortexed, and incubated in 10 ml of alkaline peptone water (pH 8.6) for 10 min. at room temperature. One ml of each type of sample was centrifuged at 10,000 x g for 10 min. to collect the bacterial cells. The cell pellets were suspended in 100 µl of Tris buffer and used for genomic DNA isolation and real-time PCR, as described above.

Table 1. Primers and probes for *V. cholerae* real-time PCR assays.

Target	Primers and probes	Amplicon size	Thermal profiles
ompWF	5'-AACAAACCATTTGCGGC -3'	104 bp	95ºC for 10min, 95ºC for 15s, 58ºC for 30s (35 cycles)
ompWR	5'-TACCGAGGCAATACCCG -3'		
ompWP ***(hyd)***	5'-FITC-CACCAAGAAGGTGACTTTATTGTGCG-Pho-3'		
ompUF	5'-GTAAGGCACAAGACAACTCTC-3'	232 bp	95ºC for 10 min, 95ºC for 15 s, 56ºC for 30 s (35 cycles)
ompUR	5'-AAGTCAGTGATTACGCCC-3'		
ompUP ***(hyd)***	5'-FITC-ATACCTACGCTGGTATCGGTGG-Pho-3'		
ctxAF	5'-CAGGTGGTCTTATGCC-3'	161 bp	95ºC for 10 min, 95ºC for 10 s, 60ºC for 15 s, 72ºC for 10 s (35 cycles). Melting curve: 95ºC for 0 s 38ºC for 30 s, 92ºC for 0 s
ctxAR	5'-CACTAAGTGGGCACTTCT-3'		
ctxAP ***(hyb1)***	5'-AGAGGAACTCAGACGGATTTG-Fluo-3'		
ctxAP ***(hyb2)***	5'-LCRed-640-AGGCACGATGAGGATATGTTTCC-Pho-3'		
zotF	5'-GATAGGGTATCGCCACTTTAACCG-3'	241 bp	95ºC for 10 min, 95ºC for 15 s, 58ºC for 30 s (35 cycles)
zotR	5'-AACCATGCCGAACAAGAA-3'		
zotP ***(hyd)***	5'-FITC-TTTAAGATGTACGCAAGCACCACC-Pho-3'		
rfbO1F	5'-GTTTCACTGAACAGATGGG-3'	192 bp	95ºC for 10 min 95ºC for 15 s, 55ºC for 30 s 72ºC for 15 s (35 cycles). Melting curve: 95ºC for 0 s 45ºC for 15 s, 95ºC for 0 s
rfbO1R	5'-GGTCATCTGTAAGTACAAC-3'		
rfbO139F (wbf)	5'-AGCCTCTTTATTACGGGTGG-3'	449 bp	95ºC for 10 min 95ºC for 15 s, 55ºC for 30 s 72ºC for 15 s (35 cycles). Melting curve: 95ºC for 0 s 45ºC for 15 s, 95ºC for 0 s
rfbO139R (wbf)	5'-GTCAAACCCGATCGTAAAGG-3'		
tcpAF	5'-GAAGAAGTTTGTAAAAGAAGAACAC-3'	473 bp	95ºC for 10 min, 95ºC for 10 s, 62ºC for 10 s 72ºC for 10 s (35 cycles). Melting curve: 95ºC for 0 s 65ºC for 15 s, 95ºC for 0 s
tcpAR	5'-GAAAGGACCTTCTTTCACGTTG-3'		

hyd – hydrolysis probes, **hyb1**, **hyb2** – Hyb probe

Results

A total of 63 *V. cholerae* strains were used for detection of the following genes, *ompW*, *ompU*, *zot*, *ctxA*, *wbf*, and *tcpA*, using appropriate primers and probes according to indicated thermal profiles. The *ompW* gene was identified in all reference strains, as well as in environmental isolates.

The presence of the *ompU* gene was detected in all of the *V. cholerae* reference strains of and seven environmental isolates, i.e. 13/06/4, 2/110, 16/110, 31/110, 49/110, 59/110, 65/110, using a specific hydrolysis probe for real-time PCR.

The Hyb probe was used for the detection of the *ctxA* gene, a positive result was obtained in seven reference strains, i.e. *V. cholerae* 8/6332 El Tor, *V. cholerae* 10/C219 El Tor, *V. cholerae* 11/AA73 El Tor Inaba, *V. cholerae* 13/154 El Tor Ogawa, *V. cholerae* 14/Jord. 1 El Tor, *V. cholerae* 15/Gwinee 1014, El Tor Ogawa, and *V. cholerae* O1 Classical 15/2002/S, but all of the environmental isolates were negative for this gene (Fig. 1). The melting temperature of the amplification product determined for the strains mentioned above was 64.4ºC.

Analysis for the presence of the *zot* gene, performed using the specific hydrolysis probe, gave positive results in all of the *V. cholerae* reference strains and in three environmental isolates, i.e. R 15, 1/110, 62/110.

In the case of the *tcpA* gene, identification was performed using the SYBRGreen nonspecific dye. The presence of the *tcpA* gene was detected in seven reference strains, i.e. *V. cholerae* 8/6332 El Tor, *V. cholerae* 10/C219 El Tor, *V. cholerae* 11/AA73 El Tor Inaba, *V. cholerae* 13/154 El Tor Ogawa, *V. cholerae* 14/Jord 1 El Tor, V. *cholerae* 15/Gwinee 1014 El Tor Ogawa, and *V. cholerae* O139 CIP 104152. The strain O1 Classical No. 15/2002/S of *V. cholerae* was identified as negative for the *tcpA* gene. The melting temperature of the amplification product of the reference strains was 84ºC (Fig. 2). None of the environmental strains were positive in terms of the *tcpA* gene.

In addition, molecular analysis using primers *rfbO139* and *rfbO1* was useful to distinguish the serogroup O139 from O1 within *V. cholerae* species. The real-time PCR with non-specific SYBRGreen dye revealed the presence of the *wbfO1* gene in seven reference strains, i.e. *V. cholerae* 8/6332 El Tor, *V. cholerae* 10/C219 El Tor, *V. cholerae* 11/AA73 El Tor Inaba, *V. cholerae* 13/154 El Tor Ogawa, *V. cholerae* 14/Jord. 1 El Tor, *V. cholerae* 15/Gwinee 1014 El Tor Ogawa, *V. cholerae* O1 Classical No. 15/2002/S, and in one environmental isolate, i.e. R15. The melting temper-

ature of the amplification products was 82.3ºC. The presence of the *wbfO139* gene was found in *V. cholerae* strain O139 CIP 104152. The melting temperature of the amplification product was 83.1ºC (Fig. 3). All other reference and environmental strains were negative in regard to the presence of this gene. The results of genetic analysis of *V. cholerae* O1, O139 and non-O1 (environmental) strains by real-time PCR are listed in Table 2.

The cross-reactivity for examined specific genes against control panel strains was not observed.

To determine the sensitivity of the real-time PCR, water samples were spiked with 10-fold serial dilutions of *V. cholerae* O139 and filtrated, as described in the Materials and Methods section (Figs. 4 and 5). The minimum cell detection limit by real-time PCR was determined to be $1.4 \cdot 10^0$ CFU/ml water sample (Fig. 4).

Discussion

We used the real-time PCR method with the designed primers and molecular probes for the *ompW*, *ompU*, *zot*, and *ctxA* gene sequences, which enabled us to identify *V. cholerae* species and to distinguish *V. cholerae* toxigenic strains from the strains lacking these properties. In our study we used the *ompW* gene as a marker for identification of *V. cholerae* species, which was shown to be present in all of the investigated samples, both in the clinical and environmental non-O1 and non-O139 isolates. The *ompW* gene is characterized by a low polymorphism within *V. cholerae* [17], and therefore it has been selected to identify this species. This gene encodes the OmpW protein, which plays an important role in the physiology of these bacteria, having an impact on the rapid adaptation of bacteria to the envi-

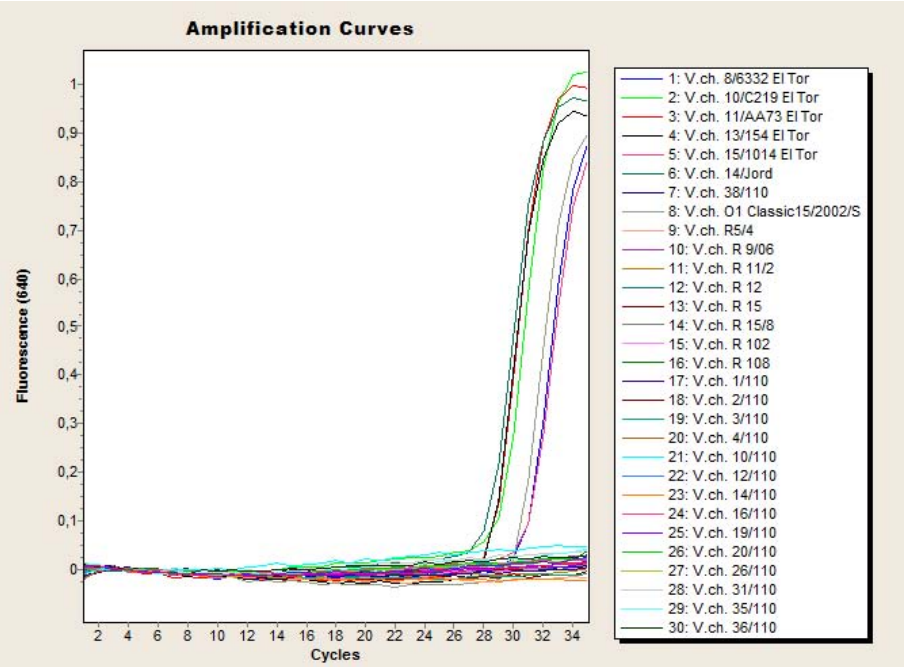

Fig. 1. Presence of *ctxA* gene sequence in examined strains of *V. cholerae* by the real-time PCR method using Hyb probes.

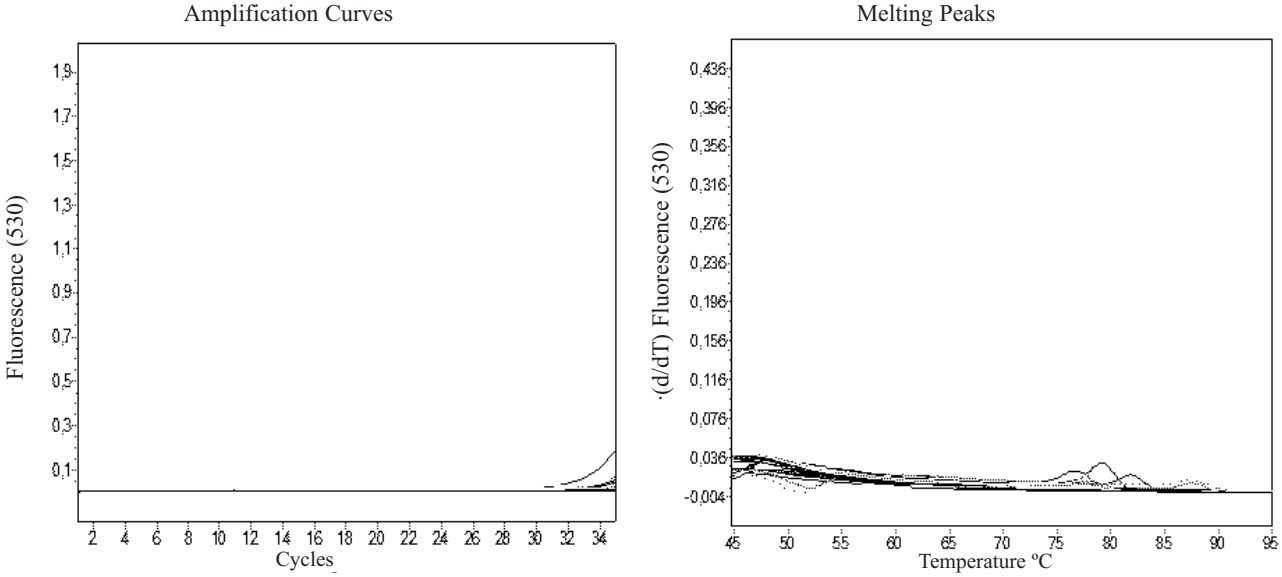

Fig. 2. Presence of *rfbO139* gene sequence in examined strains of *V. cholerae* by real-time PCR method using SYBR Green.

Table 2. The results of genetic analysis of *V. cholerae* O1, O139, and non-O1 (environmental) strains by real-time PCR.

			Presence of the following genes as determined by real-time PCR						
	Strain	Serogroup	*ompW*	*ompU*	*ctxA*	*zot*	*tcpA*	*wbfO1*	*wbfO139*
1.	*V.ch.* 8/6332, El Tor	O1	+	+	+	+	+	+	-
2.	*V.ch.* 10/C219, El Tor	O1	+	+	+	+	+	+	-
3.	*V.ch.* 11/AA73, El Tor Inaba	O1	+	+	+	+	+	+	-
4.	*V. cholerae* 13/154, El Tor Ogawa	O1	+	+	+	+	+	+	-
5.	*V.ch.* 14/Jord 1 El Tor	O1	+	+	+	+	+	+	-
6.	*V.ch.* 15/Gwinee 1014, El Tor Ogawa	O1	+	+	+	+	+	+	-
7.	*V.ch.* O1Classical, no. 15/2002/S	O1	+	+	+	+	-	+	-
8.	*V.ch.* O139 CIP 104152 “Bengal”	O139	+	+	+	+	+	-	+
9.	*V.ch. R 4/2*	non-O1	+	-	-	-	-	-	-
10.	*V.ch. R 5/4*	non-O1	+	-	-	-	-	-	-
11.	*V.ch. R 8/5*	non-O1	+	-	-	-	-	-	-
12.	*V.ch. R 9/06*	non-O1	+	-	-	-	-	-	-
13.	*V.ch. R 11 /2*	non-O1	+	-	-	-	-	-	-
14.	*V.ch. R 12*	non-O1	+	-	-	-	-	-	-
15.	*V.ch. R 15*	non-O1	+	-	-	+	-	+	-
16.	*V.ch. R 15 /8*	non-O1	+	-	-	-	-	-	-
17.	*V.ch. R 102*	non-O1	+	-	-	-	-	-	-
18.	*V.ch. R 108*	non-O1	+	-	-	-	-	-	-
19.	*V.ch. R 114*	non-O1	+	-	-	-	-	-	-
20.	*V.ch. R 118*	non-O1	+	-	-	-	-	-	-
21.	*V.ch. 5/06*	non-O1	+	-	-	-	-	-	-
22.	*V.ch. 13/06/4*	non-O1	+	+	-	-	-	-	-
23.	*V.ch. 15/06/4*	non-O1	+	-	-	-	-	-	-
24.	*V.ch. 22/06*	non-O1	+	-	-	-	-	-	-
25.	*V.ch. 23/06*	non-O1	+	-	-	-	-	-	-
26.	*V.ch. 27/06*	non-O1	+	-	-	-	-	-	-
27.	*V.ch. 28/06*	non-O1	+	-	-	-	-	-	-
28.	*V.ch. 29/06*	non-O1	+	-	-	-	-	-	-
29.	*V.ch. 2/17 /07*	non-O1	+	-	-	-	-	-	-
30.	*V.ch. 3/17/07*	non-O1	+	-	-	-	-	-	-
31.	*V.ch. 1/110*	non-O1	+	-	-	+	-	-	-
32.	*V.ch. 2/110*	non-O1	+	+	-	-	-	-	-
33.	*V.ch. 3/110*	non-O1	+	-	-	-	-	-	-
34.	*V.ch. 4/110*	non-O1	+	-	-	-	-	-	-
35.	*V.ch. 10/110*	non-O1	+	-	-	-	-	-	-
36.	*V.ch. 12/110*	non-O1	+	-	-	-	-	-	-
37.	*V.ch. 14/110*	non-O1	+	-	-	-	-	-	-
38.	*V.ch. 16/110*	non-O1	+	+	-	-	-	-	-
39.	*V.ch. 19/110*	non-O1	+	-	-	-	-	-	-
40.	*V.ch. 20/110*	non-O1	+	-	-	-	-	-	-
41.	*V.ch. 26/110*	non-O1	+	-	-	-	-	-	-
42.	*V.ch. 31/110*	non-O1	+	+	-	-	-	-	-
43.	*V.ch. 35/110*	non-O1	+	-	-	-	-	-	-
44.	*V.ch. 36/110*	non-O1	+	-	-	-	-	-	-

Table 2. Continued.

			Presence of the following genes as determined by real-time PCR						
	Strain	Serogroup	*ompW*	*ompU*	*ctxA*	*zot*	*tcpA*	*wbfO1*	*wbfO139*
45.	*V.ch. 38/110*	non-O1	+	-	-	-	-	-	-
46.	*V.ch. 40/110*	non-O1	+	-	-	-	-	-	-
47.	*V.ch. 41/110*	non-O1	+	-	-	-	-	-	-
48.	*V.ch. 46/110*	non-O1	+	-	-	-	-	-	-
49.	*V.ch. 48/110*	non-O1	+	-	-	-	-	-	-
50.	*V.ch. 49/110*	non-O1	+	+	-	-	-	-	-
51.	*V.ch. 50/110*	non-O1	+	-	-	-	-	-	-
52.	*V.ch. 51/110*	non-O1	+	-	-	-	-	-	-
53.	*V.ch. 53/110*	non-O1	+	-	-	-	-	-	-
54.	*V.ch. 54/110*	non-O1	+	-	-	-	-	-	-
55.	*V.ch. 55/110*	non-O1	+	-	-	-	-	-	-
56.	*V.ch. 56/110*	non-O1	+	-	-	-	-	-	-
57.	*V.ch. 57/110*	non-O1	+	-	-	-	-	-	-
58.	*V.ch. 58/110*	non-O1	+	-	-	-	-	-	-
59.	*V.ch. 59/110*	non-O1	+	+	-	-	-	-	-
60.	*V.ch. 60/110*	non-O1	+	-	-	-	-	-	-
61.	*V.ch. 61/110*	non-O1	+	-	-	-	-	-	-
62.	*V.ch. 62/110*	non-O1	+	-	-	+	-	-	-
63.	*V.ch. 65/110*	non-O1	+	+	-	-	-	-	-
64.	*Klebsiella pneumoniae* ATCC 10031		-	-	-	-	-	-	-
65.	*Enterobacter cloaceae* ATCC 35030		-	-	-	-	-	-	-
66.	*Enterococcus faecium* ATCC 27270		-	-	-	-	-	-	-
67.	*Enterococcus faecalis* ATCC 19433		-	-	-	-	-	-	-
68.	*Salmonella enterica* subsp. *enterica* Serovar Choleraesuis var Kunzendorf ATCC 12011		-	-	-	-	-	-	-
69.	*Shigella flexneri* ATCC 12022		-	-	-	-	-	-	-
70.	*Escherichia coli* NCTC 10538		-	-	-	-	-	-	-
71.	*Vibrio parahaemolyticus* ATCC 17802		-	-	-	-	-	-	-
72.	*Vibrio alginolyticus* ATCC 17749		-	-	-	-	-	-	-

ronment [17]. Similar results were obtained in another study, which examined 115 environmental samples of *V. cholerae*, and demonstrated the presence of *ompW* genes in all of them. The *ompW* gene occurs only in *V. cholerae* and can serve to distinguish *V. cholerae* from *V. mimicus*, a closely related species with very similar biochemical characteristics [18, 19].

In our study, we found the presence of the *ompU* gene in eight clinical isolates and in five (10%) environmental samples (non-O1 and non-O139). The outer membrane protein (OmpU) is encoded by the *ompU* gene and determines the resistance of *V. cholerae* to certain antimicrobial peptides and acts as adhesin. The presence of OmpU in membrane is associated with resistance to bile salts and organic acids. Similarly, Singh et al. [14] in their study on 91 *V. cholerae* strains belonging to serogroups O1, O139, non-O1, and non-O139, detected the *ompU* gene in five isolates of the non-O1 and non-139 serogroups. Other studies [8] demonstrated that among 26 samples of serogroups O1, O139, non-O1, and non-O139, the presence of the *ompU* gene was found in all of them. These results prove the existence of differences between strains and support the idea of heterogeneous distribution of this segment among environmental strains.

In the next stage of the present study, we aimed to determine the usefulness of the designed real-time PCR probes designed to detect the toxigenic *V. cholerae* strains. Using hybridization probes (Hyb), we were able to detect the

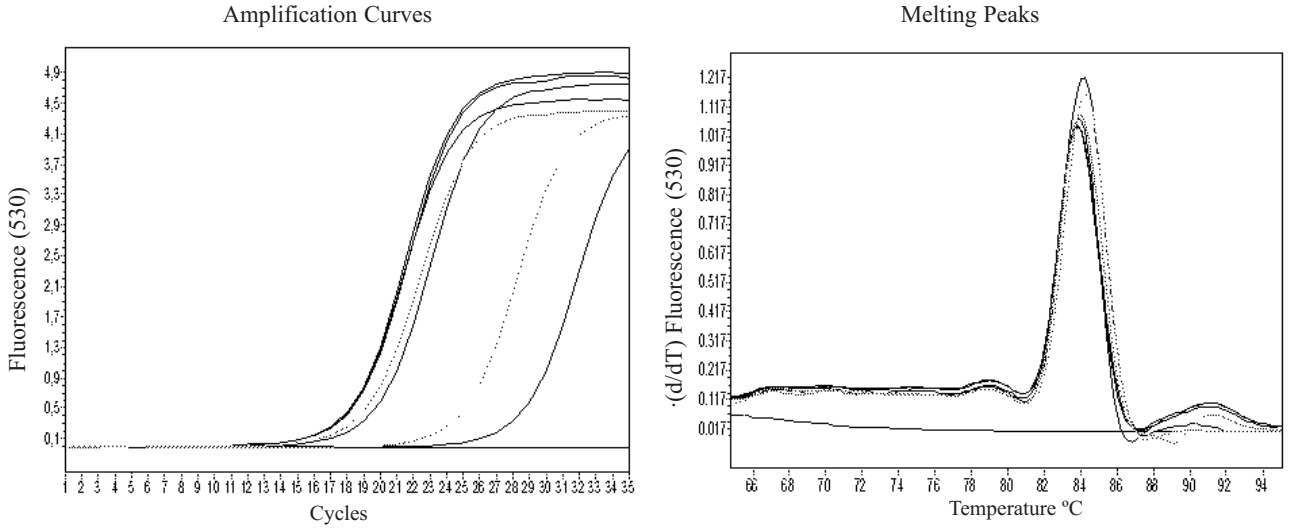

Fig. 3. Presence of *tcpA* gene sequence in examined strains of *V. cholerae* by real-time PCR method using SYBR Green.

occurrence of the *ctxA* gene in all of the tested reference strains belonging to the Classical and El Tor biotypes. However, this gene was not detected in any of the environmental isolates identified as non-O1. Our findings are consistent with the results obtained by Singh et al. [14], whose analysis also did not confirm the presence of the *ctxA* gene in any of the tested non-O1/non-O139 *V. cholerae* strains [14]. Additionally, the study by Teh et al. showed that the *ctxA* gene did not occur in environmental non-O1/non-O139 isolates, but it was present in the reference strains of O1 El Tor and Classical, as well as in clinical isolates of O1 [18]. This was further demonstrated by Kotetishvili et al. [20], Chomvarin et al. [9], Rivera et al. [21], Roy et al. [22], and Khuntia et al. [13]. Recent studies showed the presence of the *ctxA* gene in 65 clinical *V. cholerae* O1 strains and its absence in 30 environmental and eight clinical non-O1/non-O139 strains. Interesting results were obtained by Huang et al. [16], who showed the presence of the *ctxA* gene only in 4 out of 25 tested *V. cholerae* O1 strains, whereas 11 reference strains of non-O1 and non-O139 were negative in this respect. Begum et. al. [5] did not detect the presence of the *ctx* gene in any of the environmental isolates examined in their study, which is consistent with our observations. Additionally, complementary results were obtained by Bag et al. [4], who also did not identify the *ctxA* gene in the non-O1/non-O139 clinical strains. Some especially noteworthy results were reported by Chakraborty et al. [23], demonstrating the presence of the *ctxA* gene in three environmental O1 isolates and two non-O1 isolates, which indicates the possibility of horizontal transfer genes by environmental strains of *V. cholerae*.

Ctx and *zot* genes [5, 6, 24-26], which occur in the majority of virulent strains of *V. cholerae* serotype O1 or O139 [16, 25, 27-29], are mainly responsible for the toxicity of *V. cholerae*. This justifies the choice of these genes as markers identifying their pathogenicity. However, in recent years the presence of *ctxAB* genes in non-O1/non-O139 strains has also been observed. Ghosh et al. [6] showed the

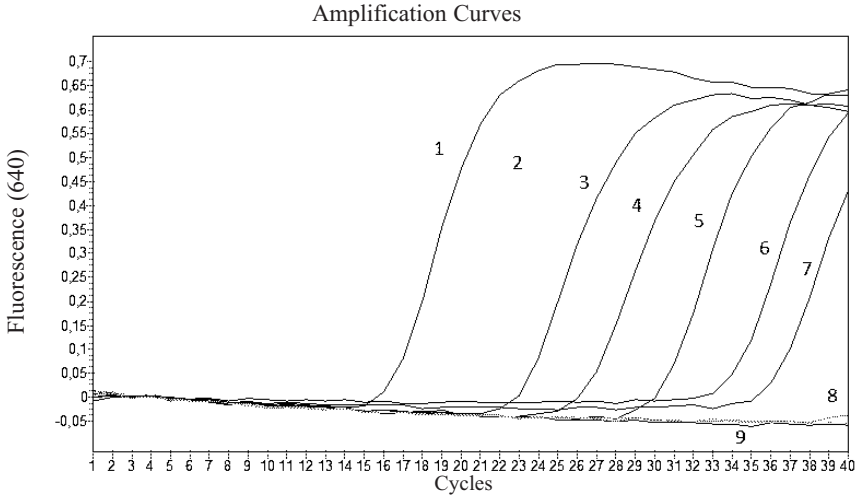

Fig. 4. Detection limit of real-time PCR reaction for *ctxA* gene in spiked environmental water samples with *V. cholerae* O139 strain CIP 104152 (Pasteur Institute): 1 – $1.4 \cdot 10^{6}$ CFU/ml, 2 – $1.4 \cdot 10^{5}$ CFU/ml, 3 – $1.4 \cdot 10^{4}$ CFU/ml, 4 – $1.4 \cdot 10^{3}$ CFU/ml, 5 – $1.4 \cdot 10^{2}$ CFU/ml, 6 – $1.4 \cdot 10^{1}$ CFU/ml, 7 – $1.4 \cdot 10^{0}$ CFU/ml, 8 – $1.4 \cdot 10^{-1}$ CFU/ml, 9 – negative control.

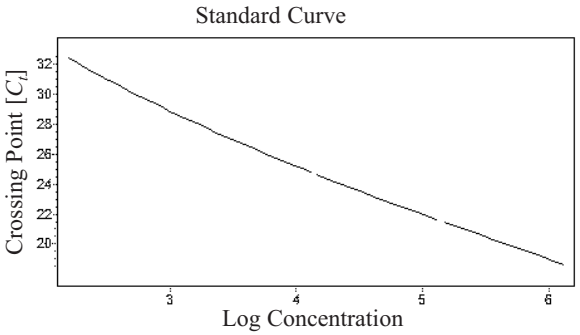

Fig. 5. The linear correlation between the C_t values and the number of *V. cholerae* cells in spiked water samples.

presence of the *ctxA* gene in 9 of 24 selected non-O1/non-O139 strains (including five clinical and four environmental isolates). Similar results were obtained by Diogo et al. [24]. Sharma et al. [19], Fykse et al. [15], Lee et al. [11], and Chakraborty et al. [23] in their studies showed the presence of *ctxAB* genes in three environmental O1 isolates and two non-O1 isolates. These results suggest that *ctxA* may also occur in non-toxigenic *V. cholerae* strains, defined as not causing cholera [24]. This is why this gene is appropriate for surveillance of toxin genes circulating natural reservoirs.

The function of the cholera toxin (CT) is assisted by ZOT (zonula occludens toxin) in cholera pathogenesis. Since both of these genes are located on the CTX genetic element, it can be assumed that they occur simultaneously. Actually, these genes can be found together or independently. Johnson et al. [30] demonstrated that in 167 isolates of *V. cholerae*, the *zot* gene occurred in conjunction with the *ctx* gene in 100% of clinical O1 isolates. Similar results were also obtained by Singh et al. [14], Kumar et al. [10], and Rivera et al. [21]. In our study, we found the presence of both the *ctx* and *zot* genes in all tested reference strains of the O1 serogroup. Different results were obtained by Pourshafie et al. [31], who reported that the frequency of the *ctx* gene within serotype O1 *V. cholerae* ranged from 81 to 97%, while in the case of the *zot* gene from 67 to 92%.

Among the environmental non-O1/non-O139 strains, independent existence of the *ctx* and *zot* genes may be a common occurrence. In our study the presence of the *zot* gene was demonstrated in three environmental non-O1 isolates, which has not been previously described in respect to the expression of the *ctx* gene. Similarly, Jiang et al. [26] reported the presence of *zot* gene sequences in 12 non-O1/non-O139 isolates, in which the *ctx* gene was not detected, while the other 74 analyzed samples were negative for both of the genes. Diogo et al. [24] also detected the presence of the *zot* gene in 11 non-O1/non-O139 isolates, and in 10 of the isolates both genes did not occur. In the study by Jiang et al. [26] 17% of the strains were characterized by the presence of *ctx* gene sequences, of which one third also contained the *zot* gene, and six isolates possessed both genes. Stypułkowka et al. [32] isolated the strain at the reference laboratory identified as *V. cholerae* non-O1 and non-O139, which was negative for cholera toxin, but positive for genes coding ACE (accessory cholera toxin), ZOT (zonula occludens toxin) and haemolysin. Diogo et al. [24] found the presence of *zot* and *ctxA* in four isolates and Johnson et al. [30] found the presence of both genes in 3% of the non-O1 strains. Rivera et al. [21], in their study on 39 non-O1/non-O139 *V. cholerae* isolates, demonstrated the presence of the *ctx* gene in four strains, in which the *zot* gene was absent. Other studies have not shown the presence of the *zot* gene in non-O1/non-O139 strains [8, 14].

The cited results suggest that the *ctx* and *zot* genes are characteristic only for the toxigenic, virulent *V. cholerae* strains that cause cholera in humans. The emergence of virulent cholera cells capable of inducing the disease is an effect of a horizontal gene transfer, which may occur in several ways. One of them is to acquire mobile elements of VPI (virulence pathogenic island), which contain the TCP (toxin-coregulated pilus) operon required to initiate colonization of the intestine by the bacterial cells. *V. cholerae* cells, containing the VPI, initiate expression of the *tcpA* gene, the product of which is used by the CTXΦ phage as a receptor [33]. The second method of gene transfer is based on a transduction process, in which the previously mentioned CTXΦ phage plays an important role, since its genetic material contains the genes encoding cholera toxin, causing symptoms of cholera. The VPI elements were detected in both environmental and clinical *V. cholerae* isolates [34, 35]. Fragments derived from phage CTXΦ have also been identified in environmental *V. cholerae* isolates (non-O1), as well as isolates belonging to *Vibrio mimicus* [19]. This may explain the randomness of the transfer of only some of the genes to the environmental non-O1 strains. Although the presence of genes encoding CT, ZOT and ACE (accessory cholera enterotoxin) has been demonstrated previously in a few clinical and environmental non-O1/non-O139 *V. cholerae* strains, there is no information regarding their position on the CTX genetic element. The presence of these and other genes associated with the so-called virulence cassette in the CTX genetic element, has also been shown by Ghosh et al. [6]. His study demonstrated that two of the six strains producing CT contained at least two complete copies of this element on the chromosome, and one other strain had three or more copies. In addition, two complete copies of the CTX genetic element, found in one of the strains, were found in different loci. These results also demonstrated the presence of extra copies of the incomplete core region in two other strains, containing *zot* and *ace* genes, and not containing the *ctxA* gene. Additionally, the recent report of Kurazono et al. [27] has indicated that *V. cholerae* O1 El Tor strains contain *zot* and ace genes, but not the *ctxA* gene in 1% of tested clinical strains and approximately 8.6% of tested environmental strains. However, the pathogenic potential of strains possessing incomplete copies of virulence cassette remains to be elucidated. Other studies suggest the existance of an adaptive system for acquiring genes from other organisms [7]. Spread of the *ctx* gene in the environment can be facilitated by exposure of CTXΦ-positive strains to sunlight. It is currently unclear whether the CT genes, present among the environmental isolates, are expressed and what their biological and ecological role is in the aquatic environment.

The multiplex PCR method has been used in some studies for the detection of *V. cholerae* [7, 12, 13, 16]. The detection of the *wbe* (O1), *wbf* (O139), and *ctxA* genes by PCR enables the differentiation of O1 and O139 strains, and their toxin-producing capability. Huang et al [16] developed a quadruplex PCR method that allows simultaneous detection of genes specific for *V. cholerae* O1 and/or O139 (*wbe* and/or *wbf*), cholera toxin subunit A (*ctxA*), toxin coregulated pilus (*tcpA*) and the central regulating protein ToxR (*toxR*) in a single-tube reaction. The *ctxA* gene, which encodes cholera toxin, is the most important determinant of *V. cholerae* toxigenicity.

The combination of four target genes, namely *O1-rfb*, *O139-rfb*, *hlyA*, and *ctxA*, has proven to be suitable for simultaneous detection of the two major serogroups of *V. cholerae*, O1 and O139, and their distinction from other types of *V. cholerae* [16]. The presence of *rfbO1* was found in seven reference strains belonging to serogroup O1, but it was not present in O139. The second gene, *rfbO139*, was present only in strains belonging to O139. These results demonstrate the usefulness of our strategy to design primers and probes for detecting *V. cholerae*, and also shed new light on the presence of various markers among the environmental *V. cholerae* strains, which may be important for appropriate interpretation of the results.

Our study on the sensitivity of the real-time PCR assay, performed with the use of environmental water samples spiked with *V. cholerae* O139, determined that the limit of detection is a 1.4 CFU/ml water sample. The strategy described herein, based on using real-time PCR with different genetic targets, enables the identification of *V. cholerae* and differentiation of serogroups and biovars of this pathogen. This method is very helpful in the rapid monitoring process of the presence of *V. cholerae* in the water sources. It is very important for assessment and surveillance of the public health threat posed by *V. cholerae* [36-40].

Conclusions

1. The diagnostic strategy based on the real-time PCR method, with specific sets of probes and primers, enabled the identification of *V. cholerae* species, and distinction of the toxigenic *V. cholerae* strains from the non-toxigenic strains of this pathogen.
2. The detection of the *zot* and *rfbO1* genes in the environmental *V. cholerae* strains indicates their presence in a natural reservoir and potential threat to human health.
3. The proposed method is specific, fast, and sensitive, and may be useful for environmental monitoring of the presence of *V. cholerae* in water sources.

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