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ORIGINAL PAPER

Detection of enteropathogenic *Vibrio parahaemolyticus, Vibrio cholerae* and *Vibrio vulnificus*: performance of real-time PCR kits in an interlaboratory study

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Abstract Bacteria belonging to the genus *Vibrio* are very common to marine and estuarine environments and are found in association with marine plants and animals. *Vibrio parahaemolyticus* strains that produce thermostable direct hemolysin (TDH) and TDH-related hemolysin (TRH), toxigenic strains of *Vibrio cholerae* belonging to the serogroups O1 and O139, and *Vibrio vulnificus* are regarded as important food-borne pathogens, which represent a serious and growing public health hazard. In this study, we established and validated real-time PCR assays for the detection of enteropathogenic *Vibrio* strains. In a first step, seafood is investigated for the presence of the three *Vibrio* species. In case of detection of *V. cholerae* or *V. parahaemolyticus*, samples are tested for the presence of the cholera toxin gene (*ctxA*) or *tdh*/*trh* genes, respectively, in a second step. All PCR analyses were performed with the same cycling program. Primer/probe sets were thoroughly tested for limit of detection, inclusivity, exclusivity and performance in the matrix. In an interlaboratory study, kits based on these primer/probe sets were successfully tested with cultural and DNA samples.

Keywords Enteropathogenic *Vibrio* · Interlaboratory study · Real-time PCR · *Vibrio cholerae* · *Vibrio parahaemolyticus* · *Vibrio vulnificus*

Introduction

Vibrionaceae are Gram-negative, halophilic bacteria found worldwide in marine and estuarine environments. The occurrences of *Vibrio* bacteria rise during warmer months of the year when the seawater temperature increases [15, 16]. Several pathogenic species are known to lead to diseases by ingestion of contaminated seafood or water or through wound infections after contact with contaminated seawater [8, 13]. The majority of food-borne illness is caused by *Vibrio cholerae, Vibrio parahaemolyticus or Vibrio vulnificus* (http://www.fao.org/food/food-safety-quality/scientific-advice/jemra/risk-assessments/vibrio0/en/). In addition, it is predicted that the number of infections caused by pathogenic *Vibrio* spp. will increase due to global warming [8, 13, 20].

The most frequent *Vibrio* species involved in gastrointestinal infections worldwide is *V. parahaemolyticus*, which is taken up by consumption of raw or undercooked seafood. *V. parahaemolyticus* strains, can be found in all types of seafood [9]. Strains producing thermostable hemolysin (TDH) or TDH-related hemolysin (TRH), encoded by *tdh* and *trh*, respectively, constitute most of the strains with clinical significance [16, 22]. Hence, techniques that are able to detect *tdh* and *trh* genes, and therefore enteropathogenic *V. parahaemolyticus*, would greatly improve quality control of seafood [26].

Vibrio cholerae strains can be divided into two groups. The first group encompasses toxigenic strains of the serogroups O1 and O139, which cause cholera outbreaks and

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which affect millions of people in developing countries [21, 25]. The second group, designated as non-O1/non-O139, comprises strains belonging to more than 200 different serogroups that are responsible for sporadic diseases [28]. Food safety measures aim to discriminate between toxigenic and harmless strains using PCR assays that detect the cholera toxin gene *ctxA*. CTX is the key virulence factor of toxigenic strains and is only rarely present in non-O1 and non-O139 strains [25].

Infections due to *V. vulnificus* are low in number, however, often result in severe outcomes. In the USA, *V. vulnificus* accounts for 95% of all seafood-related deaths [23]. Numerous virulence factors of *V. vulnificus* have been identified of which the *pilF* gene has been suggested to be useful for the detection of human pathogenic strains [27]. In contrast, up to now not a single marker is available for the discrimination between clinical and environmental strains [5]. Thus, PCR detection of *V. vulnificus* in food control targets only species-specific genes.

In this study, we developed five real-time PCR assays to detect enteropathogenic *Vibrio* species in a two-step approach. First, the presence of the enteropathogenic species *V. vulnificus, V. parahaemolyticus* and *V. cholerae* in seafood is investigated by species-specific PCR. In cases in which *V. parahaemolyticus* and *V. cholerae* are detected, an additional PCR is performed to verify the presence of *tdh*/*trh* and *ctx* positive strains, respectively. All PCR conditions are identical and only two fluorescent channels are needed; therefore no specialized PCR machine is required. In addition, the use of only two sets of fluorescent probes per assay increases the sensitivity of the PCR analysis. All PCR assays were characterized with respect to their sensitivity, specificity and performance in seafood matrices. An interlaboratory study was performed to prove the general and reliable application of the developed *Vibrio* PCR assays.

Materials and methods

Culture of *Vibrio* strains and DNA extraction

V. cholerae and *V. parahaemolyticus* were grown on thiosulfate–citrate–bile–sucrose agar (Merck KG, Darmstadt, Germany). *V. vulnificus* was cultured on salt nutrient agar (SNA) containing 0.5% meat extract, 0.3% peptone and 1% NaCl. LB broth medium (SIFIN Diagnostics GmbH, Berlin, Germany) was inoculated with single colonies and incubated at 37 °C without shaking. Alkaline peptone water (APW; Merck KG, Darmstadt, Germany) was used for enrichment of *Vibrio* spp. in the matrix. DNA was extracted with the QuickBlue DNA Extraction and Purification Kit (QB-Ex) according to the manufacturer's instructions (Q-Bioanalytic GmbH, Bremerhaven, Germany).

Primer and probe design

All primers and probes, except primers for *V. vulnificus*, were developed in this study (Table 1) using the NCBI database and the software PrimerQuest (Integrated DNA Technologies, Inc. Coralville, Iowa, USA). Basic settings included an annealing temperature of 60 °C and an amplicon length of 80 to 240 base pairs. Cross-hybridization

Table 1 Primers and probes used for real-time PCR with *Vibrio* spp. All probes are 5´-labeled with 6-FAM and 3´-labeled with TAMRA. All primers and probes, except for *V. vulnifiucs* [7] were designed in this study

Vibrio species	Target gene	Accession number	Forward primer	Reverse primer	Probe	Amplicon size (bp)
V. cholerae	Hemolysin A, hlyA	AY427780	gcaatacggcattatgggttcc	catcggttgaccactcacgga	tcggttatcgtcagtttggagccagt	169
	Cholera enterotoxin A, ctxA	EU487781	ttgctccagcagcagatggttatg	atgatgaatccacggctcttccct	attggcaggtttccctccggagcata	82
V. parahaemolyticus	Thermolabile hemolysin, tlh	AY578148	atgaactacaaccgtggcgtt	tgttgtaaccttgcgctttgtag	tcgtttgacggacgcaggtgcgaagaa	234
	Thermostable direct hemolysin, tdh	JQ047092	catctgcttttgagcttccatc	ccatttagtacctgacrtga	ggtctctgacttttggacaaaccgt	202
	tdh-related hemolysin, trh1	JF730305	taactacacaatggctgctc	ctcatatgcttcgacattgacg	agatggcctttcaacggtcttcac	192
	tdh-related hemolysin, trh2	LM993802	yaactatacratggcwgctc	ctcatatgcctcgacagtaaca	asatggtayttctacggtcttcac	192
V. vulnificus	Hemolysin A, vvhA	FJ222405	tgtttatggtgagaacggtgaca	ttctttatctaggccccaaacttg	ccgttaaccgaaccaccccgcaa	99

of sequences of primers and probes were excluded using nucleotide BLAST [2].

Development of *Vibrio*-specific real-time PCR assays

All real-time PCR analyses were established based on the TaqMan probe principle [14] using a Roche Light Cycler® 480 equipped with an ABI Prism 7700 Sequence Detector and LightCycler® 480 software (Roche Diagnostics Deutschland GmbH, Mannheim, Germany). Uracil *N*-glycosylase (UNG) was employed to avoid cross-contamination from previous runs [19]. Plasmid DNA pUC19 was included as an internal amplification control (IAC). A single PCR program was used for all PCR analyses comprising 2 min UNG activation at 50 °C, 10 min initial denaturation at 95 °C, 45 cycles of 20 s for denaturation at 95 °C, 30 s for annealing at 60 °C and 20 s for extension at 72 °C. Reagents were purchased from Life Technologies Corp [TaqMan® Fast Universal PCR Master Mix (2x)], BioRon GmbH (pUC19), Biomers.net GmbH (unlabeled primers), MWG Biotech AG (labeled probes) and PeqLab Biotechnolgie GmbH (dUTP, $MgCl_2$, UNG).

In-/exclusion and limit of detection

Inclusion testing was performed with 46–53 *Vibrio* target strains with kits for species detection, and with 31 and 13 targets with kits for toxigenic *V. parahaemolyticus* and *V. cholerae* detection, respectively (Table S1). The toxigenic *V. parahaemolyticus* strains tested consisted of nine tdh^+/trh^+ (four *trh1* and five *trh*Ψ; Ψ = pseudo gene), 22 tdh^-/trh^+ (four *trh1*, 17 *trh2* and one *trh*Ψ) and no tdh^+/trh^- genotypes. Exclusion testing was performed with 59 to 108 non-target strains, including 13 non-*Vibrio* species (Table S2). All strains are listed in tables S1 and S2 and described in table S5. Detection limits were determined by extracting DNA from serial dilutions of fresh overnight cultures of representative *Vibrio* strains and subsequent PCR analysis. In parallel, each bacterial dilution was grown on agar to determine cell numbers (colony forming units, CFU). Detection limit was defined as the lowest CFU that could be detected by three independently performed PCR assays.

Performance in food matrix

To test the performance of the PCR assays as well as the efficiency of the enrichment procedure, assays were performed with regular trade ware of a seawater fish (Alaska pollock, *Theragra chalcogramma*), a freshwater fish (striped catfish, *Pangasianodon hypophthalmus*) and a crustacean (Black tiger prawn, *Penaeus monodon*). Approximately, 25 g portions of each species were employed as matrix material and diluted tenfold with APW. Each preparation was inoculated with 100 µL aliquots of a series of diluted *Vibrio* cultures ensuring less than 1 CFU/100 µL in the higher dilutions. After controlled crushing with a paddle blender, samples were incubated overnight at 37 °C without shaking. After DNA extraction, PCR analysis was performed as described above.

Interlaboratory study

In total, five assays comprising three for species-specific (Kit_Vulnificus, Kit_Cholerae and Kit_Parahaemolyticus) and two for toxin gene detection (Kit_Para_Tox for *tdh*/*trh and* Kit_Chol_Tox for *ctxA*), were evaluated. All participating laboratories (Table S3) received their materials from Q-Bioanalytic, including kits, matrix samples (i.e., portions of *Pangasius* filets), *Vibrio* cultures (including a control without any bacteria) and DNA samples.

Vibrio cultures included *V. parahaemolyticus* CH443 (*tdh*/*trh* negative), *V. cholerae* NCTC 4711 (*ctx* negative), *V. vulnificus* VN-0016 and *V. alginolyticus* ATCC 14582. Analysis of cultured samples was initiated by inoculating 10 g of matrix material with 1 mL of *Vibrio* culture (100–200CFU/mL) followed by addition of 90 ml of APW and homogenization. After overnight incubation, DNA was extracted as described from 1 ml of homogenate.

DNA samples (2.4–31.0 ng/µl) provided separately were prepared by Q-Bioanalytic from *V. parahaemolyticus* CH443 (*tdh*/*trh* negative), *V. parahaemolyticus* SZ5367/00 (*tdh* positive, *trh* positive), *V. cholerae* NCTC 4711 (*ctx* negative), *V. cholerae* O1 1376 (*ctx* positive), *V. vulnificus* VN-0016 and *V. alginolyticus* ATCC 14582.

PCR analysis was performed by all laboratories from five cultured samples and nine DNA samples resulting in a total of 55 and 99 samples, respectively. Of the cultured samples, 11 were expected to be positive using Kit_Vulnificus, Kit_Cholerae and Kit_Parahaemolyticus, whereas all were expected to be negative for Kit_Para_Tox and Kit-Chol-Tox (Table S4). 33 of the DNA samples were expected to be positive for Kit_Vulnificus, 66 for Kit_Cholerae, 44 for Kit_Parahaemolyticus, 33 for Kit_Para_Tox and 55 for Kit-Chol-Tox (Table S4). Results were statistically evaluated by the National Reference Laboratory (NRL) of the BfR.

Statistical measures

Performance of the assays was assessed by determining the sensitivity and specificity with confidence intervals [10], as well as negative (NPV) and positive (PPV) predictive values, which is a measure of the ratio of the expected negative (and positive) to all results [6] (Table S4). To measure

the agreement of results obtained by the laboratories participating in the interlaboratory study with the reference results of Q-Bioanalytic, Cohen's kappa statistic value κ was applied [1]. κ is a measure between 0 and 1 and indicates the range of no to complete agreement [29]. Corresponding confidence intervals were calculated according to Fleiss et al. [11]. All statistical evaluations were performed with IBM® SPSS Statistics version 21 and Excel 2010.

Statement of human and animal rights

Non-applicable.

Results

Primer and probe design

To develop PCR assays to detect enteropathogenic *Vibrio* strains, we chose the genes encoding hemolysin/cytolysin as species-specific targets in Kit_Vulnificus (*vvhA*), Kit_Cholerae (*hlyA*) and Kit_Parahaemolyticus (*tlh*). The cholera toxin A gene of *V. cholerae* and the clinically relevant hemolysin genes of *V. parahaemolyticus* were chosen as targets for the detection of toxigenic strains with Kit-Chol-Tox (*ctxA*) and Kit_Para_Tox (*tdh, trh1, trh2*) (Table 1).

Table 2 Optimized PCR reagent compositions for amplification of *Vibrio* target genes

(A) Reagents		Vvul (vvhA)	Vpara (tlh)	Vchol (hlyA)	Vchol (ctxA)
Forward primer (target)	pmol/µl	0.35	0.21	0.41	0.41
Reverse primer (target)	pmol/µl	0.35	0.21	0.41	0.41
Probe (target)	pmol/µl	0.07	0.04	0.08	0.08
IAC (pUC19 plasmid)	pg/µl	0.02	0.02	0.02	0.29
Forward primer (IAC)	pmol/µl	0.21	0.44	0.29	0.29
Reverse primer (IAC)	pmol/µl	0.21	0.44	0.29	0.06
Probe (IAC)	pmol/µl	0.04	0.09	0.06	0.02
dUTP	mM	0.18	0.04	0.74	0.18
MgCl2	mM	0.15	0.74	0.04	0.15
Glycerol	%	0.70	0.70	0.70	0.70
UNG	U/µl	0.01	0.01	0.01	0.01
2× ABI Mastermix	×	0.88	0.88	0.88	0.88
(B) Reagents		Vpara (tdh, trh1, 2)			
Forward primer (tdh)	pmol/µl	0.41			
Reverse primer (tdh)	pmol/µl	0.41			
Probe (tdh)	pmol/µl	0.08			
Forward primer (trh1)	pmol/µl	0.35			
Reverse primer (trh1)	pmol/µl	0.35			
Probe (trh1)	pmol/µl	0.04			
Forward primer (trh2)	pmol/µl	0.47			
Reverse primer (trh2)	pmol/µl	0.47			
Probe (trh2)	pmol/µl	0.09			
IAC (pUC19 plasmid)	fg/µl	2.35			
Forward primer (IAC)	pmol/µl	0.29			
Reverse primer (IAC)	pmol/µl	0.29			
Probe (IAC)	pmol/µl	0.03			
dUTP	mM	0.04			
MgCl2	mM	0.35			
Glycerol	%	0.70			
UNG	U/µl	0.01			
2× ABI Mastermix	x	0.88			

The ABI Mastermix contains *T*aq polymerase; uracil-*N*-glycosylase (UNG) was employed to avoid carryover contaminations from previous PCR runs; *IAC* internal amplification control (= pUC19 plasmid DNA); target genes are indicated in brackets after abbreviated *Vibrio* species designations: *Vvul = Vibrio vulnificus, Vchol = V. cholerae, Vpara = V. parahaemolyticus*. Multiplex PCRs simultaneously amplify one (A) or three (B) *Vibrio* target genes and the IAC. The IAC probe was labeled 5′-HEX/3′-TAMRA, while all *Vibrio* probes were labeled 5′-6-FAM/3′-TAMRA

Table 3 Results of inclusivity and exclusivity tests. Target and non-target strains are specified in tables S1, S2 and S5 included in the supplementary material

Vibrio PCR Kits	Target strains			Non-target strains		
	Total no.	Detected	Not det.	Total No.	Detected	Not det.
Kit_Vulnificus	53	53	0	63	0	63
Kit_Parahaemolyticus	52	52	0	63	0	63
Kit_Cholerae	46	45	1[a]	59	0	59
Kit_Para_Tox	31	30	1[b]	84	2[c]	82
Kit_Chol_Tox	13	13	0	108	0	108

[a]Non-toxigenic strain V166/12 without *hlyA* gene

[b]Non-toxigenic strain VN0070 with a pseudo-*trh* gene

[c]*Grimontia hollisae* strains M106 and M107 with a *tdh* gene

For the simultaneous detection of all clinically relevant hemolysin genes of *V. parahaemolyticus*, we included all primers and probes in the Kit_Para_Tox (Table 2b). To control for PCR inhibition, we adjusted individually the concentration of PCR reagents for all PCR kits and included an IAC (Table 2).

The expected length of 80–240 base pairs for the PCR amplicons was validated using high-percentage agarose gel electrophoresis (data not shown). Real-time PCR with serial dilutions of DNA from selected *Vibrio* strains consistently resulted in the amplification of the expected PCR products. Standard curves depicted a strong linear correlation between C_T values and the DNA concentrations, with a correlation coefficient R^2 of at least 0.98 (Fig. S1).

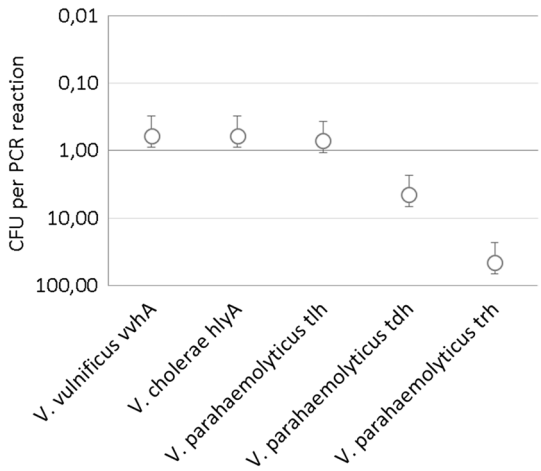

Fig. 1 Detection limits are expressed as colony forming units (CFU) per PCR reaction. Serial dilutions of *V. cholerae, V. parahaemolyticus* (with and without *tdh* or *trh* genes) and *V. vulnificus* cultures were plated on nutrient agar and analyzed by real-time PCR

In-/exclusion and limit of detection

The five different real-time PCR systems detected all but two target strains (Table 3). The two exceptions were strains *V. parahaemolyticus* VN-0070, which contains a pseudo-*trh* gene that was not detected by Kit_Para_Tox, and *V. cholerae* V166/12 (non-O1/non-O139), which does not contain a *hylA* gene, and therefore could not be detected by Kit_Cholerae. On the other hand, two non-target strains, *Grimontia hollisae* M106 and M107, which contain a *tdh* gene, were detected with Kit_Para_Tox (Table 3).

Limits of detection were 0.6 ± 0.3 CFU for *V. vulnificus* and *V. cholerae* and 0.7 ± 0.4 CFU for *V. parahaemolyticus* (Fig. 1). Depending on the presence of either the *tdh* or a *trh* gene, 4.5 ± 2.2 or 45.0 ± 22.1 CFU of the toxigenic strains of *V. parahaemolyticus* was detected (Fig. 1). We did not determine the detection limits for the toxigenic *V. cholerae* strains.

Internal amplification control (IAC) and performance in food matrix

An IAC allows the identification of false-negative results due to inhibiting substances impairing *T*aq-polymerase activity. Different labels used for *Vibrio* (6-FAM) and IAC-specific (HEX) probes enabled parallel monitoring of co-amplified PCR products by employing different filter sets (Fig. S2). Analysis of fish and shellfish obtained from customary trade ware inoculated with serial dilutions of *Vibrio* cultures, revealed that, in principle, one CFU was sufficient for the detection of *Vibrio* using enrichment in APW followed by real-time PCR (data not shown). No inhibitory effects, as indicated by complete PCR failure (drop out of the IAC) or shifts in C_T values, were observed.

Interlaboratory study

Evaluation of the results from all participating laboratories by the NRL showed a success rate between 89 and 98% for the different *Vibrio* PCR kits (Fig. 2). Sensitivity and specificity were above 85% in all but one case. NPV and PPV were above 90% with cultural samples generally exhibiting a lower PPV, presumably because of the low

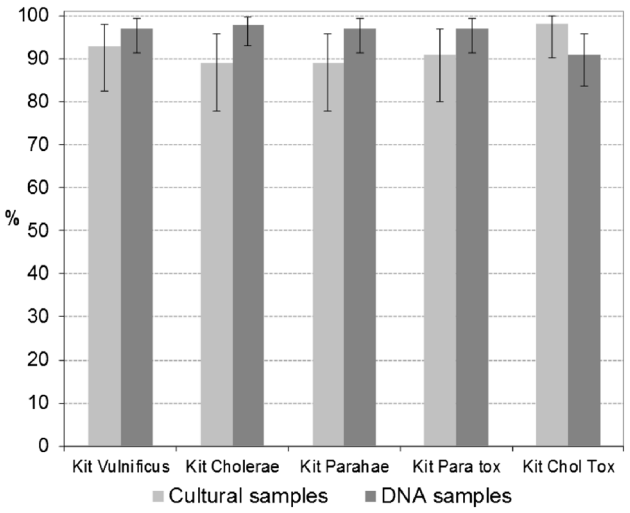

Fig. 2 Percentages of correctly determined samples by each kit (95% confidence interval is depicted) in an interlaboratory study. *Light gray bars* indicate examined DNA preparations from cultural samples (55 samples tested per kit), and *dark gray bars* indicate DNA samples (99 samples tested per kit)

(20%) prevalence (Table 4 and Table S4). All κ coefficients calculated for the combined experimental results obtained with cultured and DNA samples were in the range of 0.858–0.898 (Fig. 3), showing an almost perfect concordance with the reference results of Q-Bioanalytic.

Discussion

The aim of this study was to develop a system of real-time PCR assays that enables the detection of enteropathogenic *V. cholerae, V. parahaemolyticus* and *V. vulnificus* in seafood in a two-step process using the same cycling conditions for all assays. The first step detects strains of the three species with a limit of detection of approximately one CFU per reaction. If *V. cholerae* or *V. parahaemolyticus* is present, putative pathogenic strains are identified by performing a second PCR targeting the *ctxA* gene and *tdh*/*trh* genes, respectively. The detection limits for *V. parahaemolyticus* strains harboring the *trh* gene were slightly higher. However, CFU numbers should be sufficient as PCR is performed from enrichment cultures.

The selection of genes to identify different species was based on previously published PCR assays and targeted hemolysin/cytolysin genes of all three species. Application of the *tlh* gene for the identification of *V. parahaemolyticus, vvhA* for *V.vulnificus* and *hlyA* for *V. cholera*e had been described by Panicker et al. [24] and the

Table 4 Performance of the *Vibrio* real-time PCR kits calculated independently for cultural (A) and DNA (B) samples. Sensitivity, specificity, negative and positive predictive values were calculated according to [6], κ coefficients were determined according to [1] and the prevalence calculated as the ratio of the expected positive results to all results

	Kit Vulnificus	Kit Cholerae	Kit Parahae-molyticus	Kit Para_Tox	Kit Chol_Tox
(a) cultural samples (N = 55; 11 labs, 5 samples per lab)					
Sensitivity *N*, (%)	100 (11)[a]	100 (11)[a]	90.9 (11)[a]	–[b]	–[b]
Specificity *N*, (%)	90.9 (44)[a]	86.4 (44)[a]	88.6 (44)[a]	90.9 (55)[a]	98.2 (55)[a]
NPV (%)	100	100	97.5	–[b]	–[b]
PPV (%)	73.3	64.7	66.7	–[b]	–[b]
κ coefficient	0.8	0.717	0.7	–[b]	–[b]
Prevalence (%)	20	20	20	0	0
(b) DNA samples (N = 99; 11 labs, 9 samples per lab)					
Sensitivity *N*, (%)	100 (33)[a]	100 (66)[a]	95.5 (44)[a]	100 (33)[a]	100 (55)[a]
Specificity *N*, (%)	95.5 (66)[a]	93.9 (33)[a]	98.2 (55)[a]	95.5 (66)[a]	79.5 (44)[a]
NPV (%)	100	100	96.4	100	100
PPV (%)	91.7	97.1	97.7	91.7	85.9
κ coefficient	0.933	0.954	0.938	0.933	0.812
Prevalence (%)	33.3	66.7	44.4	33.3	55.5

Definitions for calculation:

Sensitivity (%) = 100 × no. of truly positive tested samples/no. of truly positive tested samples + no. of falsely negative tested samples

Specificity (%) = 100 × no. of truly negative tested samples/no. of truly negative tested samples + no. of falsely positive tested samples

PPV = 100 × no. of truly positive tested samples/no. of truly positive tested samples + no. of falsely positive tested samples

NPV = 100 × no. of truly negative tested samples/no. of truly negative tested samples + no. of falsely negative tested samples

[a]Number of expected positive results are shown in brackets

[b]No calculation possible as all cultural samples were negative for Kit_Chol-Tox and Kit_Para_Tox

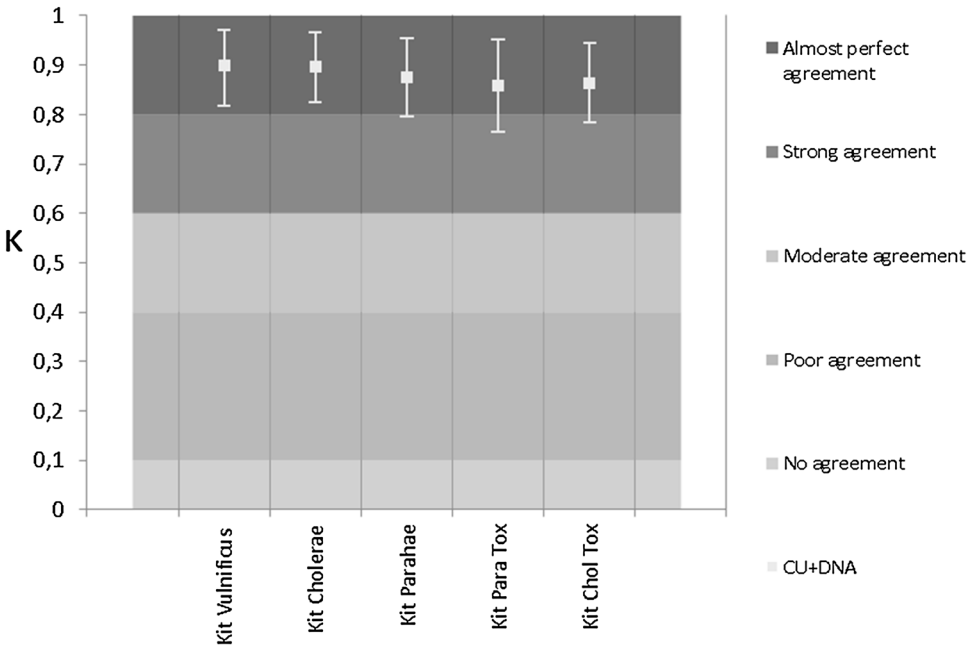

Fig. 3 Degree of agreement between the expected results (reference results of Q-Bioanalytic GmbH) and the observed results of the laboratories. The degree and strength of agreement is quantified by the κ coefficient [1, 29]. *Error bars* indicate a confidence interval of 95% [11]. The results of the cultural and of the DNA samples were combined for calculation of the *κ* coefficient

same target genes were used for species identification in several other published approaches [12, 17, 26]. We used the *V. vulnificus* primers and probe described by Campbell and Wright [7] without any further modifications in our protocol as they work with the same cycling profile. Inclusivity and exclusivity confirmed the suitability of the selected target genes for species identification. In the case of *V. cholerae*, we detected one environmental strain lacking the hemolysin gene; however, all toxigenic strains possess this gene [24, 25] and thus will be detected.

Nucleotide BLAST [2] analysis revealed sequence similarities of the *V. cholerae ctxA* gene and the *V. parahaemolyticus tdh*/*trh* genes to related virulence genes of other bacterial species. The primer and probe sequences targeting the *tdh* toxin gene of *V. parahaemolyticus* also hybridize to *tdh* genes of strains of *V. mimicus and V. hollisae*. Similarly, the *trh* primer and probe sequences of *V. parahaemolyticus* were detected in sequences of the *trh* genes of strains of *Aeromonas veronii, V. anguillarum* (formerly *Listonella anguillarum*) and *V. alginolyticus*. The *trh2* primer and probe sequences, however, are present only in a strain of *V. alginolyticus*. Few strains of *V. alginolyticus* can also harbor the *ctxA* gene, and hence are likely to be detected with the primer–probe system developed for the detection of toxigenic *V. cholerae*. However, the detection of other bacterial strains harboring *tdh*/*trh* or *ctxA* genes should lead to the same measures concerning contaminated foods as if enteropathogenic *Vibrio* were present. Of all tested target strains of *V. cholerae* and *V. parahaemolyticus* harbouring *ctxA and tdh*/*trh* genes, respectively, only *V. parahaemolyticus* strain VN-0070 was negative, most likely caused by internal nucleotide deletions within the *trh* pseudogene [4].

All five kits developed on the basis of the primer/probe systems performed well in the interlaboratory study. *V. alginolyticus* was included as a non-target *Vibrio* control in this study, as this species is found frequently in seafood. The *κ* coefficients, which were calculated from the reports of the laboratories, demonstrated that the participants' results were in strong agreement with the expected results.

In conclusion, we developed a two-step real-time PCR approach that is suitable for the detection of enteropathogenic *V. cholerae, V. parahaemolyticus* and *V. vulnificus*, with the advantage that all PCR assays are optimized for the use of the same cycling settings. The PCR assays were validated and tested for their sensitivity, specificity and performance in the food matrix. In addition, the interlaboratory study confirmed the general applicability of the *Vibrio* PCR kits.

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Compliance with ethical standards

Conflict of interest BO, J-OA, EE, JH, CS, and SU are employed with the Company Q-Bioanalytic GmbH (Bremerhaven, Germany). RH and J-HS are former employees of Q-Bioanalytic GmbH.

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