Evaluation of an Alternative Method for Detection of *Vibrio cholera*, *V. parahaemolyticus* and *V. vulnificus* in Seafood Products Using Real-Time PCR

Laurent Jain[1], André Quintanar[2], Fanny Margotteau[1], Olivier Pradillon[1], Frédéric Pastori[1], Christophe Quiring[3] and Sophie Pierre[1]
Food Science Division, (1) Bio-Rad, Marnes-la-Coquette, France, (2) Bio-Rad, Hercules, CA, (3) Bio-Rad, Steenvoorde, France

Introduction

Vibrio spp. are a leading cause of seafood-borne bacterial infections causing gastrointestinal illness and septicemia when ingested by humans. The primary route of foodborne infection is typically associated with the consumption of raw or undercooked fish and shellfish. The occurrence of outbreaks caused by *Vibrio* highlight the need for rapid detection and accurate identification of three main associated strains: *V. cholera* (VC), *V. parahaemolyticus* (VP) and *V. vulnificus* (VV).

Gold standard methods for *Vibrio* testing, such as the FDA BAM (Chapter 9) or the ISO 21872-1:2017, are labor intensive and rely on microbiological/biochemical identification. For seafood processors and inspections, current methods require at least 3–5 days for results and subjective interpretation for the screening of negative samples. Bio-Rad has designed an alternative *Vibrio* solution based on a rapid single-step enrichment using the proprietary *Vibrio* Enrichment Broth (VEB) and multiplex detection of VC, VP and VV using the real-time PCR iQ-Check *Vibrio* method which not only allows rapid qualitative detection but also enables differentiation of all three strains in seafood products. An optional treatment with iQ-Check Free DNA Removal Solution (FDRS) was also evaluated to address ambiguity caused by dead cell DNA. The iQ-Check *Vibrio* assay is a rapid alternative to the classical Thiosulfate Citrate Bile Saccharose (TCBS) agar plate confirmation method. A *Vibrio* Chromogenic Agar (VCA) plate was also tested.

Methods

Design of the method

As shown in Figure 1, the *Vibrio* method includes a few steps: enrichment of the seafood matrix in *Vibrio* Enrichment Broth, facultative treatment with FDRS, DNA extraction, real-time PCR, and automated data interpretation. The challenge was to develop an efficient enrichment broth and a novel quadruplex PCR assay capable of simultaneously detecting four unique genetic targets in a single tube reaction. Each strain of VP, VV and VC was screened with primers and a patented double stranded probe in combination with FAM, Cy5 and Atto590 fluorophores respectively. An internal control was also designed consisting of a heterologous target sequence (double stranded probe coupled with HEX fluorophore) that was co-amplified with primers identical to one *Vibrio* target.

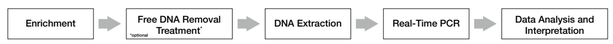


Fig. 1.

Inclusivity — Exclusivity

The specificity of the real-time PCR assay was validated with a comprehensive panel of inclusivity and exclusivity strains. Certified and well-known bacteria, yeasts and molds were tested: 14 VP, 11 VC, 10 VV, and 16 non-VP, VV or VC strains. In addition, 101 closely related and potentially cross-reactive non-*Vibrio* strains, 8 yeast and 8 mold species were chosen for the exclusivity study. Five microliters of culture (10^5 CFU/ml) or DNA extracted from 100 µl of TCS, VEB or Sabouraud cultured media, were tested directly using the novel iQ-Check *Vibrio* real-time PCR assay.

Sensitivity

The analytical sensitivity of a PCR method can be expressed as the limit of detection (LOD_{PCR}), defined as the lowest amount of genome units consistently detectable in at least 95% of all samples tested. In this study, 10 replicates of a dilution series were analyzed to determine assay sensitivity. Each VP, VV and VC (or all three together) were grown at 37°C overnight on TCBS agar and one typical colony was transferred into VEB and incubated at 35°C for 8 hr. Tenfold serial dilutions were prepared from these cultures with a starting concentration equivalent to 8.25 x 10^6, 7.85 x 10^6 and 9.20 x 10^6 CFU/ml of VP, VV and VC respectively. Each serial dilution underwent 10 DNA extractions followed by target amplification using the iQ-Check *Vibrio* real-time PCR kit.

Performance on seafood samples

To evaluate the method on food samples, 5 different matrices (cooked shrimp, fresh shrimp, fresh codfish, fresh oysters, and fresh scallops) were used. Briefly, 25 g per sample was enriched in 225 ml VEB at 35 ±1°C for 8 ± 1 hr. Samples were artificially spiked with an inoculum of VP (CIP 70.63), VV (CIP 109783), or VC (CIP 68.10) individually and with a cocktail of all three strains, homogenized in a stomacher, and incubated. The inoculum concentrations were confirmed by plating on TCBS agar. DNA was subsequently extracted from a 100 µL aliquot of the enriched sample, treated or untreated with FDRS, and 5µL of the DNA extraction was tested with the novel iQ-Check *Vibrio* real-time PCR assay. The Cq values for each target- VP, VV, VC and internal control- were analyzed. The same aliquot of VEB was utilized to confirm results from all samples onto VCA and with the ISO 21872-1:2017 method after a primary isolation on TCBS. The characteristic colony from TCBS was used for a direct confirmation by PCR and a sub-culture onto SNA (Saline Nutritive Agar), followed by complementary identification by PCR.

Results

Inclusivity — Exclusivity

All the 117 non-targeted *Vibrio* strains, molds and yeasts, yielded negative results with a mean value of IC Cq target of 31.56 (95%CI: 31.34 – 31.49). Conversely all the 14 VC, 11 VP and 10 VV strains showed positive results as expected.

Sensitivity

The LOD_{PCR} of the *Vibrio* assay was tested on 10 independent replicates of serial dilutions prepared from overnight cultures of each VP, VV and VC strain with 2 manufacturing builds of the iQ-Check *Vibrio* real-time PCR kit and a cocktail of all three strains together with another PCR build. A 100% detection was repeatedly obtained up to the dilution point -5 from the bacterial culture in exponential phase. The limit of detection of the test as determined by the 95% threshold was similar for the 3 manufacturing builds (Table 1). The regressions are shown in Figure 2 with the second PCR build as an example for each strain. The overall sensitivity is consistent with an LOD close to 72 CFU/ml. With a maximum ratio of 3.3 for the VC target in favor of the mono vs. triple contamination, there is no significant difference between the 3 targets present individually or together within the same sample. Furthermore, no impact was observed between/within replicates or PCR manufacturing builds, demonstrating good repeatability and reproducibility of results.

A known limitation of PCR based assays is often the amplification of free DNA from dead cells. The detection of viable cells enriched in the VEB was not impacted by treatment with FDRS. The proportional bias for *Vibrio* Cq values (VC+VP+VV) is 0.98 (95% CI: 0.89 to 1.09). Conversely, when free *Vibrio* DNA was added to the enrichment and subjected to the extraction, it was possible to remove up to 6.04 Cq value of VC, VP or VV free DNA with 20 µl of the activated FDRS reagent.

Performance on seafood samples

VEB supported efficient growth of the VC, VV and VP strains on different types and grades of seafood matrices. Of the 47 samples tested (Table 2, Figures 2 and 3), all positive and negative PCR results on VEB were confirmed using one of the confirmation methods described above. In the context of the ISO or FDA BAM method, PCR on a single colony or on SNA from that characteristic TCBS colony is recommended due to interfering flora which can cause inaccurate identification of VP/VV and/or VC strains. The use of VCA in conjunction with PCR eliminates ambiguous interpretation of results and generates good overall agreement between the reference method and Bio-Rad's *Vibrio* method (VEB + iQ-Check *Vibrio* + VCA). No false negative results were observed.

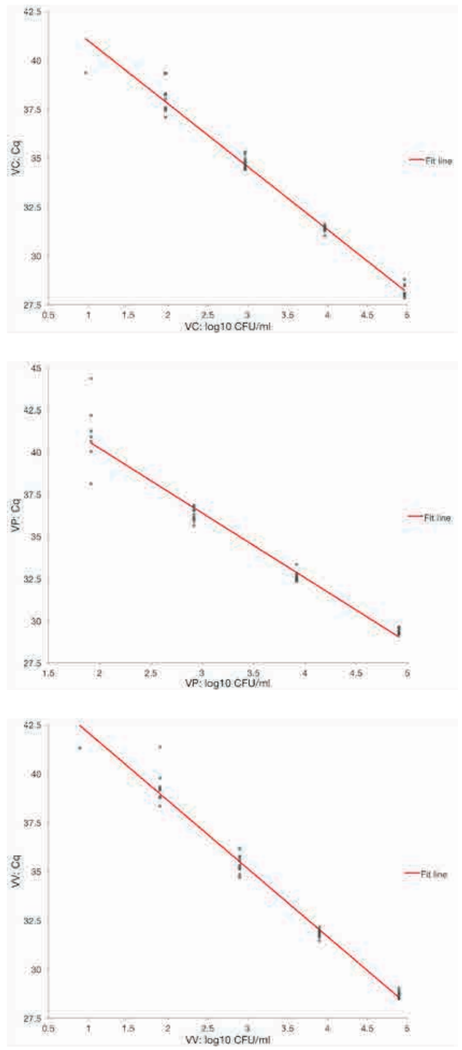


Fig. 2. Regression plots for the coded concentration (log10 CFU/ml) vs. Cq values for each target VC, VP or VV with build #2 of the iQ-Check *Vibrio* real-time PCR kit.

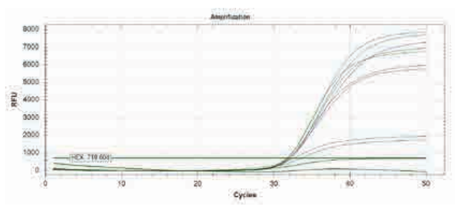
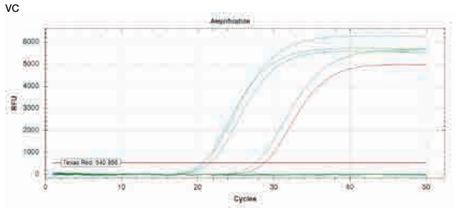
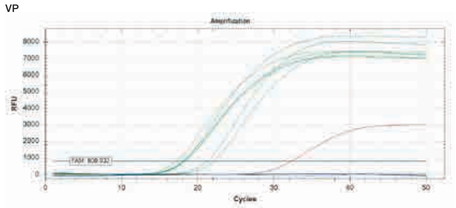


Fig. 3. Detection of *Vibrio* targets in fresh shrimp and oysters with the iQ-Check *Vibrio* real-time PCR assay.
Typical IC amplification curves on samples spiked with VC, VP or VV enriched in VEB. NC in blue and PC in red.

VC



VP



VV

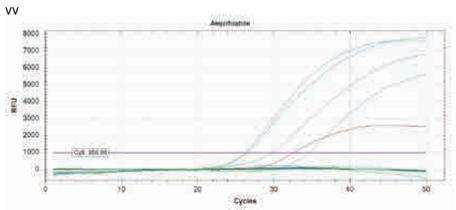


Fig. 4. Detection of *Vibrio* targets in fresh shrimp and oysters with the iQ-Check *Vibrio* real-time PCR assay.
Typical VC, VP and VV amplification curves on samples spiked with VC, VP or VV enriched in VEB. NC in blue and PC in red.

Table 1. Sensitivity of the iQ-Check *Vibrio* real-time PCR assay.
Limit of Detection (95%) in CFU/ml of each target tested in single contamination with iQ-Check *Vibrio* builds #1 and 2 and in triple contamination with the iQ-Check *Vibrio* build #3. ND: not detected.

	iQ-Check *Vibrio* PCR Builds	1			2			3
	contamination	mono VC	mono VP	mono VV	mono VC	mono VP	mono VV	triple
Target(s)	VC	**29**	ND	ND	**22**	ND	ND	**72**
	VP	ND	**14**	ND	ND	**14**	ND	**22**
	VV	ND	ND	**15**	ND	ND	**14**	**9**

Table 2. Performance on seafood products.
Forty seven samples were unspiked or artificially spiked individually or with a cocktail of all VC, VP and VV strains. PCR results are shown with the iQ-Check *Vibrio* PCR kit on the VEB, TCBS and SNA. VCA results are also described as alternative confirmation method. Discrepant results are indicated in red vs. PCR results on VEB.

	TCBS from VEB				VCA from VEB		
PCR Result on VEB	TCBS - VP/VV	TCBS - VC	PCR Result on POS TCBS colonies	PCR Result on SNA	VCA - VP	VCA - VV	VCA - VC
VP positive	POS	NEG	VP positive	VP positive	POS	NEG	NEG
VC positive	NEG	POS	VC positive	VC positive	NEG	NEG	POS
VV positive	POS	NEG	VV positive	VV positive	NEG	POS	NEG
VP and VV and VC positive	POS	NEG	VP and VV positive	VP and VV positive	POS	POS	POS
Negative	NEG	NEG	Negative	Negative	NEG	NEG	NEG
VP positive	NEG	POS	Negative	Negative	POS	NEG	NEG
VC positive	NEG	POS	VC positive	VC positive	NEG	NEG	POS
VV positive	POS	NEG	VV positive	VV positive	NEG	POS	NEG
VP and VV and VC positive	POS	NEG	VV positive	VV positive	POS	POS	POS
VP positive	NEG	POS	Negative	Negative	POS	NEG	NEG
VP and VV and VC positive	NEG	POS	VC positive	VC positive	POS	NEG	NEG
VP and VV positive	NEG	POS	Negative	Negative	POS	POS	NEG
VP and VV and VC positive	POS	NEG	VV positive	VV positive	POS	POS	NEG
VV positive	NEG	POS	VV positive	VV positive	NEG	POS	NEG
VV and VC positive	POS[1]	POS[2]	1: VV positive / 2: VC positive	1: VV positive / 2: VC positive	NEG	NEG	POS
VV and VC positive	POS[1]	POS[2]	1: VV positive / 2: VC positive	1: VV positive / 2: VC positive	NEG	NEG	POS
VC positive	NEG	POS	VC positive	VC positive	NEG	NEG	POS
VV positive	POS	NEG	VV positive	VV positive	NEG	POS	NEG
VV and VC positive	POS[1]	POS[2]	1: VV positive / 2: VC positive	1: VV positive / 2: VC positive	NEG	POS	NEG
VV and VC positive	POS[1]	POS[2]	1: VV positive / 2: VC positive	1: VV positive / 2: VC positive	NEG	POS	NEG
VC positive	NEG	POS	VC positive	VC positive	NEG	NEG	POS
VV positive	POS	NEG	VV positive	VV positive	NEG	POS	NEG
VP and VV and VC positive	POS	NEG	VP and VV positive	VP and VV positive	POS	POS	POS
VP positive	POS[1]	POS[2]	1: VP positive / 2: yellow colonies are PCR Negative	1: VP positive / 2: Negative	POS	NEG	NEG
VP positive	NEG	POS	Negative	Negative	POS	NEG	NEG
VP and VC positive	NEG	POS	VC positive	VC positive	POS	NEG	NEG
VP and VV positive	NEG	POS	VP positive	VP positive	POS	POS	NEG
VP and VV and VC positive	POS[1]	POS[2]	1: VP and VV positive / 2: VC positive	1: VP or VV positive / 2: VC positive	POS	POS	POS
Negative	POS	NEG	Negative	Negative	NEG	NEG	NEG
VP positive	POS	NEG	VP positive	VP positive	POS	NEG	NEG
VC positive	NEG	POS	VC positive	VC positive	NEG	NEG	POS
VV positive	POS	NEG	VV positive	VV positive	NEG	NEG	POS
VP and VV and VC positive	POS[1]	POS[2]	1: VP and VV positive / 2: VC positive	1: VP or VV positive / 2: VC positive	POS	NEG	POS

CONCLUSION

We have developed a novel solution with a simplified real-time PCR assay to qualitatively detect *Vibrio* strains from seafood matrices in 8 hr using the proprietary *Vibrio* Enrichment Broth. Preliminary results demonstrate that iQ-Check *Vibrio* is an effective method for the single or multiplex detection of *V. cholera*, *V. parahaemolyticus* and *V. vulnificus* in seafood. Furthermore, application of the Free DNA Removal Solution enables removal of free DNA without any impact on the detection of viable *Vibrio* cells. The inclusion of an internal control confirmed the absence of false positive and false negative results. The iQ-Check *Vibrio* method, in conjunction with PCR on colony from SNA and *Vibrio* Chromogenic Agar demonstrated good overall agreement with the reference method to correctly distinguish the 3 strains, while reducing the risk of ambiguous results that may occur from the use of TCBS, an agar that has been shown to have low selectivity. This novel method also drastically reduces the turnaround time to 1 day for the screening and release of negative samples. Its integration with the iQ-Check Prep automation system with the optional iQ-Check Free DNA Removal Solution, has also been validated to ensure more flexibility for the customer. This method will be submitted for AOAC PTM status in 2018.

REFERENCES

1) ISO 21872-1:2017: Microbiology of the food chain — Horizontal method for the determination of *Vibrio* spp. — Part 1: Detection of potentially enteropathogenic *Vibrio parahaemolyticus*, *Vibrio cholerae* and *Vibrio vulnificus*.
2) United States Food and Drug Administration. Bacteriological Analytical Manual. May 2004. Chapter 9- *Vibrio*.

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