Letter to the Editor

Duplex Detection of *Vibrio Cholerae* and *Vibrio Parahaemolyticus* by Real-time Recombinase Polymerase Amplification*

LIAO Lei[&], SHI Wen[&], MA Chao[&], TANG Wen Lian, QIAN Qi Lan, WANG Yu, LI Jiao Jiao, SHEN Jin Yang, JI Jing[#], MA Jin Ming[#], and GAO Song[#]

Vibrio cholerae and *Vibrio parahaemolyticus* are the two most commonly reported pathogens in seafood[1]. Consuming raw or undercooked seafood contaminated with these two *Vibrio* species can cause food poisoning, posing the risk of severe gastrointestinal illness and death[2-3]. Therefore, precise and reliable methods for detecting *V. cholerae* and *V. parahaemolyticus* contamination in seafood are essential for controlling food safety.

Many molecular detection methods have been developed for *V. parahaemolyticus* and *V. cholerae*, including polymerase chain reaction (PCR)-based methods, such as PCR and qPCR, and isothermal amplification methods, such as loop-mediated isothermal amplification and recombinase polymerase amplification (RPA)[1,4,5]. Among them, RPA-based methods are more suitable for on-site applications due to the short detection time and less instrument dependence. An RPA assay coupled with lateral flow dipsticks (RPA-LFD) targeting the *tlh* gene of *V. parahaemolyticus* has been established[6], and *gyrB* of *V. parahaemolyticus* and *lolB* of *V. cholerae* have been selected as targets for detection by real-time RPA assays[4,5]. The real-time RPA assay reads fluorescence signals along with amplification, which avoids opening the reaction containers as in the RPA-LFD assay; thus, there is less risk of carry-over contamination in the assay operating environment.

However, previous studies focused on detecting one pathogen at a time. However, multiplex assays to simultaneously detect *V. cholerae* and *V. parahaemolyticus* are needed for complex food samples. Current multiplex detection assays available for *Vibrio* species have used PCR, qPCR, and RPA-LFD technologies[1,7], but a real-time RPA assay for duplex detection of *V. cholerae* and *V. parahaemolyticus* has not been developed. In this study, we describe a duplex real-time RPA assay to simultaneously detect *V. cholerae* and *V. parahaemolyticus*. This assay will provide added convenience for on-site detection of these two *Vibrio* species in food supply chains, and the principle can be applied to multiplex detection of other foodborne pathogens.

Twenty bacterial strains were used in this study. The reference strains, including *V. cholerae* (ATCC 14100), *V. parahaemolyticus* (ATCC 17802), *Vibrio vulnificus* (ATCC 27562), *Vibrio alginolyticus* (ATCC 17749), *Vibrio harveyi* (ATCC 43516), *Vibrio mimicus* (MCCC 1A02602), *Vibrio splendidus* (MCCC 1A04096), *Vibrio ichthyoenteri* (MCCC 1A00057), *Aeromonas caviae* (ATCC 15468), *Aeromonas hydrophila* (ATCC 43414), *Aeromonas veronii* (ATCC 35622), *Bacillus cereus* (ATCC 14579), *Staphylococcus aureus* (ATCC 6538), *Salmonella enteritidis* (ATCC 14028), and *Yersinia enterocolitica* (ATCC 9610) were purchased from the American Type Culture Collection (Manassas, VA, USA) or the Marine Culture Collection of China (Xiamen, China). Two

doi: 10.3967/bes2022.148

*This study was supported by grants from the National Natural Science Foundation of China [No. 82104174]; the Key Natural Science Research Project of the Jiangsu Higher Education Institutions of China [No. 20KJA416002]; the Natural Science Foundation of Jiangsu Higher Education Institutions of China [No. 20KJB350008]; the Research Program of the "521 Project" of Lianyungang City of China [No. LYG06521202133]; the Open-end Funds of the Jiangsu Key Laboratory of Marine Pharmaceutical Compound Screening [Nos. HY202004 and HY202103]; the Lianyungang Key Laboratory of Marine Biomedicine and Products [5507018035]; the Postgraduate Research & Practice Innovation Program of Jiangsu Province [SJCX22_1649 and SJCX22_1650]; the "Blue Project" of Jiangsu Higher Education Institutions of China, and the Priority Academic Program Development of Jiangsu Higher Education Institutions of China.

Jiangsu Key Laboratory of Marine Pharmaceutical Compound Screening, Jiangsu Key Laboratory of Marine Biological Resources and Environment, Co-Innovation Center of Jiangsu Marine Bio-industry Technology, School of Pharmacy, Jiangsu Ocean University, Lianyungang 222005, Jiangsu , China

environmental strains of *V. cholerae* and three environmental strains of *V. parahaemolyticus* were provided by the Jiangsu Institute of Oceanology and Marine Fisheries (Nantong, China). All strains were confirmed by 16S rRNA sequencing and grown in an Alkaline Peptone Water medium at 37 °C when activated. Genomic DNAs were released by boiling the bacterial cultures at 100 °C for 10 min and used directly as reaction templates.

The primer and probe sequences used in the real-time RPA reactions were derived from previously reported individual real-time RPA assays for *V. cholerae* and *V. parahaemolyticus*[4,5]. The probes for the real-time RPA reaction were modified as described by the manufacturer's instructions in the TwistAmp DNA Amplification Exo kit (TwistDx Inc, Maidenhead, UK). A base at the middle of the probe was substituted with a tetrahydrofuran (THF) group, and two *T* bases adjacent to THF were labeled with a fluorophore and a quencher (BHQ1 in this study), respectively. The fluorophore probes for *V. cholerae* and *V. parahaemolyticus* were FAM (detection wavelength 465–510 nm) and HEX (detection wavelength 533–580 nm), respectively. The sequences of the primers and probes are listed in Table 1. The primers and probes were synthesized by General Biology Co. Ltd. (Anhui, China). The target genes, *gyrB* and *lolB*, if present, were amplified by RPA with their corresponding forward and reverse primers in the duplex real-time RPA assay. For each target, the respective probe pairs for the amplified strand and the exonuclease III were cut at THF to release the fluorophore for signaling; therefore, the DNA template from either bacterium would be specifically amplified. SpC3 was used to block unnecessary extension of the strand at the 3' end of the probe (Figure 1).

The real-time RPA reactions followed the manufacturer's instructions for the TwistAmp DNA Amplification Exo kit. A 15-μL real-time RPA reaction mixture was prepared as follows. To the lyophilized enzyme pellet, 35.4 μL of rehydration buffer, 11.9 μL of nuclease-free water, 2.5 μL of primer (s), and 0.7 μL of the probe (s) were added and mixed uniformly to prepare the premix. Then, 13.25 μL of the premix was removed, and 1 μL of the template and 0.75 μL of MgOAc (280 mmol/L) were added. After brief centrifugation, the reaction mixture was incubated at 39 °C for 4 min, gently mixed, and the fluorescence signal was recorded on the Roche LightCycler 480 II qPCR machine (Basel, Switzerland) with the FAM and HEX channels at 39 °C. The signal was read at 1-min intervals for 26 min.

The optimal concentration ranges of the primers and the probe for *V. cholerae* in the single reaction were examined to determine the primer and probe concentrations in the duplex real-time RPA reaction. Using a ten-fold serially diluted template from 1.0×10^0 to 1.0×10^4 colony forming units (CFU)/μL, the detection limit for *V. cholerae* in the single real-time RPA reaction was 1.0×10^1 CFU/μL (Figure 2A). Using this amount of the template, the concentrations of the primers and probe were decreased proportionally, and the fluorescence signal diminished with the decrease in concentration (Figure 2B). Once the concentration was reduced to 25% of the original, a significant drop in the fluorescence signal was observed. Thus, the primer and probe concentrations were used at 50% of their original concentrations (208 nmol/L final for each

Table 1. Primer and probe sequences

Assay	Pathogen	Name	Sequence (5'–3')	Amplicon size (bp)
Real-time RPA	*V. cholerae*	Forward primer	ATCTTCAAGCTGTTCAACGGGAATATCTAA	218
		Reverse primer	ATCAGCGACAATCGTTCAACTTTCAATGGC	
		Probe	ATCAGGCTTTGTGCATCTTGGTCGCGGTAGA[FAM-dT] [THF] [BHQ1-dT]GATCATCATAAGTTTCG-SpC3	
	V. parahaemolyticus	Forward primer	CGAAGAAAGCGAAAACGGCAACGTCAGGCGA	168
		Reverse primer	CAGATAATTTCTCACCCATCGCCGATTCAACC	
		Probe	GGTTTGACAGCCGTTGTTTCAGTAAAAGTGCC[HEX-dT] [THF] [BHQ1-dT]TCCAAAATTCTCGAGCC-SpC3	
qPCR	*V. cholerae*	Forward primer	CCGTTGAGGCGAGTTTGGTGAGA	195
		Reverse primer	GTGCGCGGGTCGAAACTTATGAT	
	V. parahaemolyticus	Forward primer	CGGTAGTAAACGCACTGTCAGAA	77
		Reverse primer	ACGGTAAGTTTGCGTGTGGAT	

primer and 58 nmol/L final for the probe) for detecting *V. cholerae* in the real-time RPA reaction.

Similarly, the optimal concentration ranges of the primers and probe for *V. parahaemolyticus* were examined in a single reaction. The detection limit of the single reaction was 1.0×10^2 CFU/μL (Figure 2C). As the maximum fluorescence value was moderate at the detection limit, 1.0×10^3 CFU/μL was used to test the primer and probe concentration ranges. A significant decrease in the signal at a concentration of 25% of the original was observed (Figure 2D), suggesting that the primer and probe concentrations at 50% of the original were acceptable for detecting *V. parahaemolyticus*.

As the single reactions of the primer and probe concentrations could be reduced to 50% for both pathogens, the primers and probes at concentrations of 50% of the original (208 nmol/L final for each primer and 58 nmol/L final for each probe) were assembled into the duplex real-time RPA reaction to reach the total concentration of 100%. The sensitivity of the duplex assay for each pathogen was determined. The sensitivity for *V. cholerae* was 1.0×10^1 CFU/μL (Figure 2E). A probit regression analysis (SPSS software; IBM Corp., Armonk, NY, USA) of the results of eight independent repeats showed that the detection limit was 37 CFU/μL in 95% of the cases (Figure 2F). The sensitivity for *V. parahaemolyticus* was 1.0×10^2 CFU/μL, and the limit of detection was 99 CFU/μL in 95% of the cases (Figure 2G–H).

The specificity of the duplex assay was confirmed with a series of *Vibrio* species and other commonly seen zoonotic and foodborne pathogens, including the 15 reference strains and 5 environmental strains described above. In the specificity test, bacterial DNA was extracted using the TIANamp Bacterial DNA Kit (Tiangen Biotech Co., Ltd., Beijing, China). DNA templates were quantified by Qubit 4 (ThermoFisher Scientific Inc., Wilmington, MA, USA) and normalized to 25 ng/μL. Only the reference and environmental strains of *V. cholerae* and *V. parahaemolyticus* produced positive signals on the respective fluorescence channels (Figure 2I–J), suggesting good specificity. The specificity of the primers and probes used in this study was established previously[4,5]. Here we further confirmed no cross-reaction between the 2 primer-probe sets in the duplex system.

The duplex real-time RPA assay was validated with 40 clinical samples, including 20 shrimp samples (*Litopenaeus vannamei*), 15 fish samples (*Trichiurus lepturus*), and 5 shellfish samples (*Pectinidae*). The detection results were compared to the qPCR results (Supplementary Table S1 available in www.besjournal.com). The qPCR primer sequences were obtained from previous reports and are listed in Table 1[8,9]. Among the 40 samples, 5 were positive

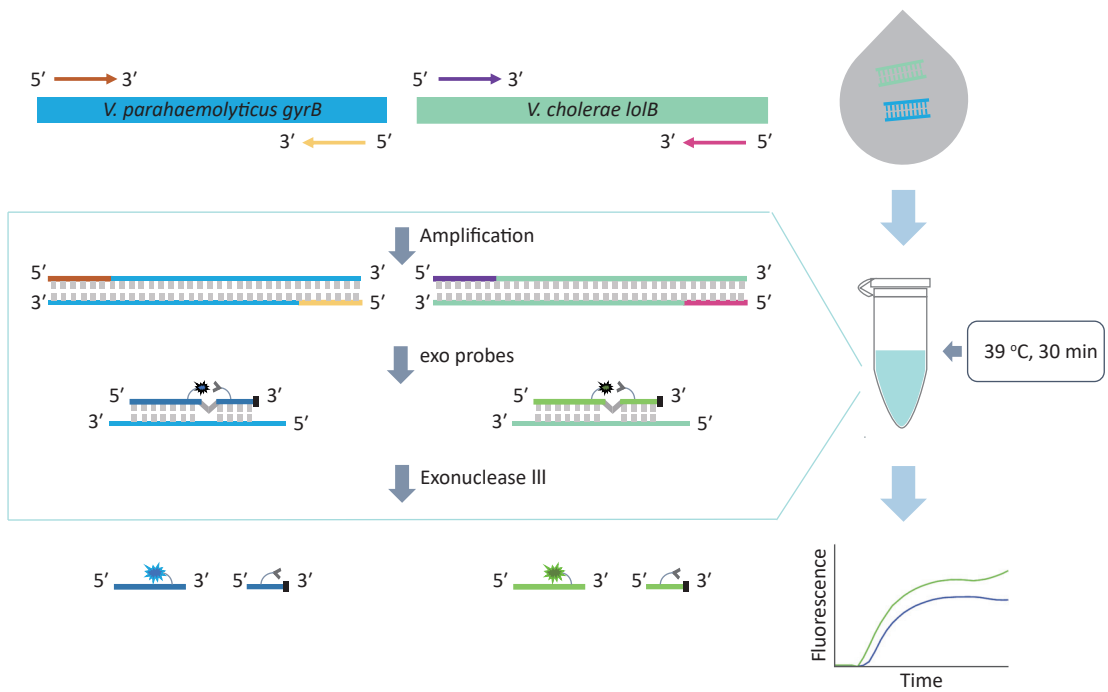

Figure 1. Schematic diagram of the duplex real-time RPA assay.

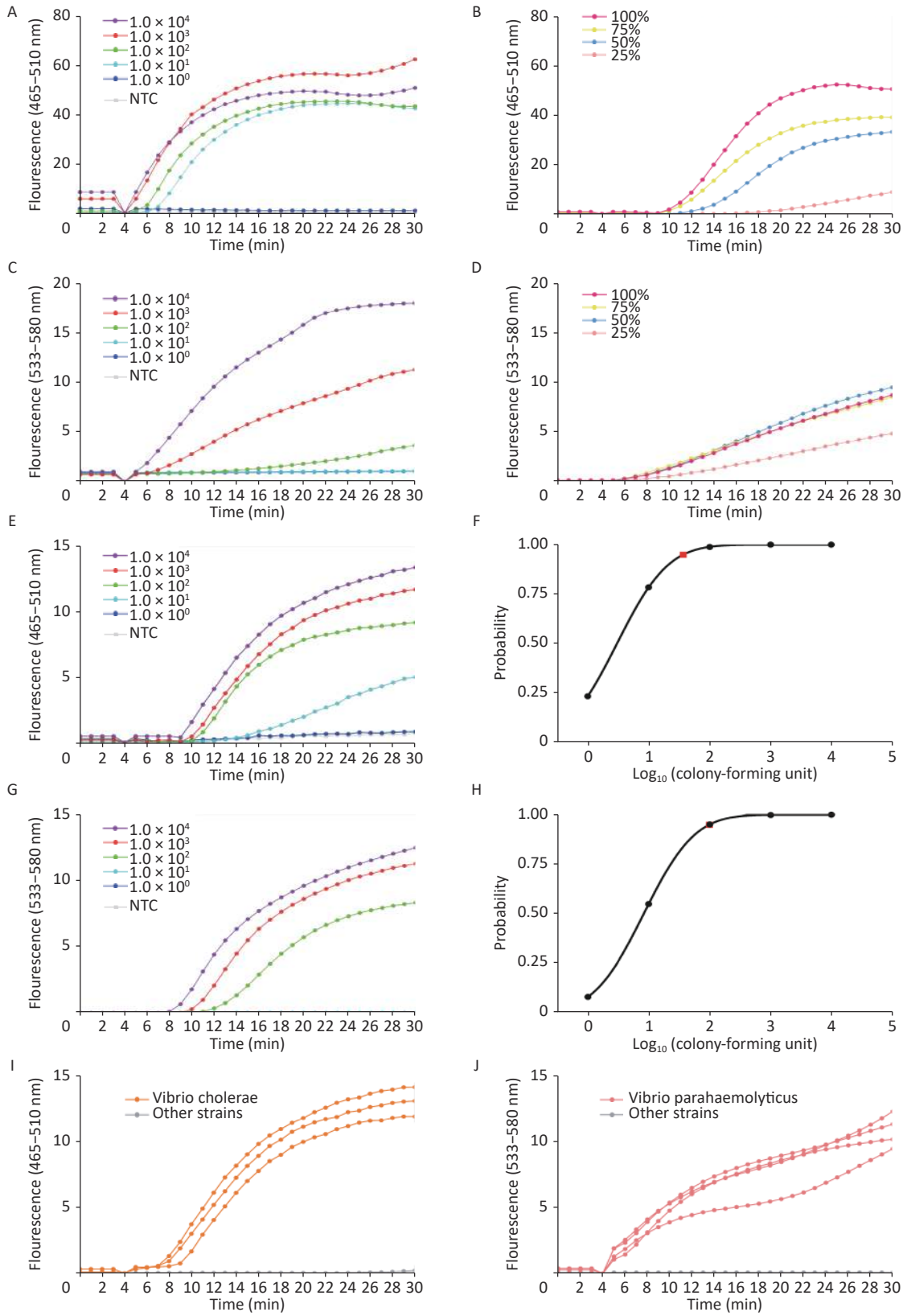

Figure 2. (A–D) Optimizing the primer and probe concentrations. (E–H) Optimizing the duplex real-time RPA reaction. (I–J) Specificity of the duplex real-time RPA assay.

for *V*. *cholerae*, 7 were positive for *V*. *parahaemolyticus*, and 1 was positive for both bacteria. The results were 100% consistent with the qPCR. The duplex real-time RPA assay was accurate and reliable for simultaneously detecting *V*. *cholerae* and *V*. *parahaemolyticus*. This assay is ready for on-site detection, as the qPCR instrument used in this study can be replaced by a portable fluorescence detector, e.g., the Genie III Scanner from Suntrap Science & Technology Co. Ltd. (Beijing, China).

Amplification was an important issue to solve when establishing the duplex RPA reaction, as biased amplification of one target would consume more reaction resources and affect the other[10]. Here, we designed the reaction so that the amplicon sizes of the two targets were similar (218 bp *vs.* 168 bp) and carefully optimized the primer and probe concentrations. We first determined that the concentrations of the primers and probes could be decreased to 50% of the initial for both *V*. *cholerae* and *V*. *parahaemolyticus*. Based on this result, a molar ratio of 1:1 of the *V*. *cholerae* primer-probe set to the *V*. *parahaemolyticus* primer-probe set was selected to compensate for the potential biased amplification. The duplex real-time RPA assay had the same sensitivity as the single reactions for both pathogens, suggesting that the issue of amplification preference was overcome.

In conclusion, a duplex real-time RPA assay to simultaneously detect *V*. *cholerae* and *V*. *parahaemolyticus* was established in this study. The assay exhibited good specificity and sensitivity that reached 37 CFU/μL for *V*. *cholerae* and 99 CFU/μL for *V*. *parahaemolyticus*. These results were comparable to the sensitivity reported for previous singleplex real-time RPA assays[4,5] (*V*. *cholerae*: 5 copies/μL standard plasmid; *V*. *parahaemolyticus*: 1.0×10^2 copies/μL genomic DNA). Validation with clinical samples was accurate and reliable. Importantly, the duplex assay improved detection efficiency. This is the first real-time RPA-based multiplex detection assay available for *Vibrio* species. This assay provides a convenient choice for on-site detection of *V*. *cholerae* and *V*. *parahaemolyticus*, and will guide the development of multiplex detection assays for other foodborne pathogens.

No potential conflicts of interest are disclosed.

We thank Dr. HUI Shen of the Jiangsu Institute of Oceanology and Marine Fisheries (Nantong, China) for providing the bacterial strains and clinical samples.

[&]These authors contributed equally to this work.

[#]Correspondence should be addressed to JI Jing, E-mail: jijing@jou.edu.cn; MA Jin Ming, E-mail: majinming @outlook.com; GAO Song, E-mail: gaos@jou.edu.cn Tel/Fax: 86-518-85895781.

Biographical notes of the first authors: LIAO Lei, male, born in 1995, Master Degree, majoring in Pharmacy; SHI Wen, female, born in 1997, Graduate Student, majoring in Pharmacy; MA Chao, male, born in 1996, Master Degree, majoring in Pharmacy.

Received: September 14, 2022;

Accepted: October 8, 2022

REFERENCES

1. Bonnin-Jusserand M, Copin S, Le Bris C, et al. *Vibrio* species involved in seafood-borne outbreaks (*Vibrio cholerae, V. parahaemolyticus and V. vulnificus*): review of microbiological *versus* recent molecular detection methods in seafood products. Crit Rev Food Sci Nutr, 2019; 59, 597−610.
2. Lee JH, Park SK, Khan F, et al. Simultaneous isolation and enumeration of virulent *Vibrio cholerae* and *Vibrio vulnificus* using an advanced MPN-PCR method. Arch Microbiol, 2022; 204, 5.
3. Barrera-Escorcia G, Wong-Chang I, Fernández-Rendón CL, et al. Quantification of *Vibrio* species in oysters from the Gulf of Mexico with two procedures based on MPN and PCR. Environ Monit Assess, 2016; 188, 602.
4. Geng YY, Tan K, Liu LB, et al. Development and evaluation of a rapid and sensitive RPA assay for specific detection of *Vibrio parahaemolyticus* in seafood. BMC Microbiol, 2019; 19, 186.
5. Tang YY, Cao YQ, Yu YX, et al. Real-time recombinase polymerase amplification assay for the detection of *Vibrio cholerae* in seafood. Food Anal Method, 2017; 10, 2657−66.
6. Yang XH, Zhao PP, Dong Y, et al. An improved recombinase polymerase amplification assay for visual detection of *Vibrio parahaemolyticus* with lateral flow strips. J Food Sci, 2020; 85, 1834−44.
7. Wang P, Liao L, Ma C, et al. Duplex on-site detection of *Vibrio cholerae* and *Vibrio vulnificus* by recombinase polymerase amplification and three-segment lateral flow strips. Biosensors, 2021; 11, 151.
8. Geng YY, Liu GH, Liu LB, et al. Real-time recombinase polymerase amplification assay for the rapid and sensitive detection of *Campylobacter jejuni* in food samples. J Microbiol Methods, 2019; 157, 31−6.
9. Cho MS, Ahn TY, Joh K, et al. A novel marker for the species-specific detection and quantitation of *Vibrio cholerae* by targeting an outer membrane lipoprotein *lolB* gene. J Microbiol Biotechnol, 2013; 23, 555−9.
10. Lobato IM, O'Sullivan CK. Recombinase polymerase amplification: basics, applications and recent advances. TrAC Trends Anal Chem, 2018; 98, 19−35.

Supplementary Table S1. Detection results of clinical samples by real-time RPA and qPCR

No.	Food type	Sample source	Detection results for *Vibrio cholerae*		Detection results for *Vibrio parahaemolyticus*	
			Real-time RPA	qPCR	Real-time RPA	qPCR
1	Shrimp	Lianyungang, China	−	−	−	−
2	Shrimp	Lianyungang, China	−	−	−	−
3	Shrimp	Lianyungang, China	−	−	+	+
4	Shrimp	Lianyungang, China	+	+	−	−
5	Shrimp	Lianyungang, China	−	−	−	−
6	Shrimp	Lianyungang, China	−	−	+	+
7	Shrimp	Lianyungang, China	−	−	−	−
8	Shrimp	Lianyungang, China	−	−	−	−
9	Shrimp	Lianyungang, China	−	−	−	−
10	Shrimp	Lianyungang, China	−	−	−	−
11	Shrimp	Qingdao, China	−	−	−	−
12	Shrimp	Qingdao, China	−	−	−	−
13	Shrimp	Qingdao, China	−	−	−	−
14	Shrimp	Qingdao, China	+	+	+	+
15	Shrimp	Qingdao, China	−	−	−	−
16	Shrimp	Qingdao, China	−	−	−	−
17	Shrimp	Qingdao, China	−	−	−	−
18	Shrimp	Qingdao, China	+	+	−	−
19	Shrimp	Qingdao, China	−	−	−	−
20	Shrimp	Qingdao, China	−	−	+	+
21	Fish	Yancheng, China	−	−	−	−
22	Fish	Yancheng, China	+	+	−	−
23	Fish	Yancheng, China	−	−	−	−
24	Fish	Yancheng, China	−	−	−	−
25	Fish	Yancheng, China	−	−	−	−
26	Fish	Yancheng, China	−	−	−	−
27	Fish	Qingdao, China	−	−	−	−
28	Fish	Qingdao, China	−	−	−	−
29	Fish	Qingdao, China	−	−	−	−
30	Fish	Qingdao, China	−	−	+	+
31	Fish	Qingdao, China	+	+	−	−
32	Fish	Qingdao, China	−	−	−	−
33	Fish	Qingdao, China	−	−	+	+
34	Fish	Qingdao, China	−	−	−	−
35	Fish	Qingdao, China	−	−	−	−
36	Shellfish	Qingdao, China	−	−	−	−
37	Shellfish	Qingdao, China	−	−	+	+
38	Shellfish	Qingdao, China	−	−	−	−
39	Shellfish	Qingdao, China	−	−	−	−
40	Shellfish	Qingdao, China	−	−	−	−

Note. +: positive result; −: negative result.