Fast, simple and highly specific molecular detection of Vibrio alginolyticus pathogenic strains using a visualized isothermal amplification method

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Abstract

Background: Vibrio alginolyticus is an important pathogen that has to be closely monitored and controlled in the mariculture industry for its strong pathogenicity, quick onset after infection and high mortality rate to aquatic animals. Fast, simple and specific methods are demanded for its on-site detection to effectively control its outbreaks and prevent economic losses. Detection specificity towards the pathogenic strains has to be emphasized to facilitate pointed treatment and prevention. Polymerase chain reaction (PCR) based molecular approaches have been developed, but their application is limited due to the requirement for complicated thermal cycling machines and trained personnel.

Results: A fast, simple and highly specific detection method for V. alginolyticus pathogenic strains was established based on the isothermal recombinase polymerase amplification (RPA) and lateral flow dipsticks (LFD). The method targeted a virulence gene toxR that was reported to have a good coverage for the V. alginolyticus pathogenic strains. To ensure the specificity, the primer-probe set of the RPA system was carefully designed to recognize regions in gene toxR that were diverged in different Vibrio species but conserved in V. alginolyticus pathogenic strains. The primer-probe set was determined after a systematic screening on amplification performance, primer-dimer formation and false positive signals. The RPA-LFD method was confirmed to have a high specificity to V. alginolyticus pathogenic strains without any cross reaction with other Vibrio species, and was able to detect as low as 1 colony forming unit per microliter of the bacterium without DNA extraction. The method finishes detection within 30 min under the temperature between 35oC and 45oC, and the visual signal on the dipstick was directly read with naked eye. In an application simulation, randomly infected shrimp homogenate samples were 100% accurately detected.

Conclusions: The RPA-LFD method developed in this study is fast, simple, highly specific and independent of complicated equipment. It is well applicable to the on-site detection of V. alginolyticus pathogenic strains for the mariculture industry.

Background

Vibrio alginolyticus belongs to the Vibrio genus, the Vibrionaceae family. It is a Gram-negative, rod-
shaped, flagellar bacterium that shares halophilic feature and widely exists in the ocean and estuarine areas [1–3]. *Vibrio alginolyticus* is considered as one of the most harmful *Vibrio* species that is pathogenic to both humans and aquatic animals [2, 4]. For humans, soft tissues, the ear, and superficial wounds are easily invaded by *V. alginolyticus* when exposed to contaminated seawater [4]. Clinical symptoms include chronic diarrhea, otitis and wound infection [5, 6]. For aquatic animals, *V. alginolyticus* causes a variety of diseases such as septicaemia of *Sparus aurata* [7, 8], exophthalmia and corneal opaqueness of *Epinephelus* spp. [9], melanosis of *Rachycentron canadum* [10], white spot of *Penaeus vannamei* [11], necrosis of *Macrobrachium rosenbergii* larvae [12], and massive mortality of *Tapes decussatus* [13, 14]. Control of Vibro alginolyticus has to be effective in the mariculture industry for its strong pathogenicity, quick onset after infection and high mortality rate [15].

Being a member of *Vibrio* species, *V. alginolyticus* shares a lot of similarities of the family, including morphology, surface antigens, virulence factors, genome sequences, and early symptoms of infection [16–18]. These similarities made specific detection of *V. alginolyticus* and distinguishing it from other *Vibrio* species difficult. Conventional culturing, immunodetection and symptom judgement stayed at the level of identifying a *Vibrio* infection but not to the level of species specificity [19–21]. It has to be noted that, not only the severity of infection was different between *Vibrio* species, but also the treatment methods had different outcomes [22]. As one of the most virulent *Vibrio* species in the mariculture industry, *V. alginolyticus* has to be specifically detected for pointed treatment and prevention [23, 24].

Polymerase chain reaction (PCR) based molecular detection methods had been developed for specific detection of *V. alginolyticus* because they could select and target specific sequences on the *V. alginolyticus* genome [25]. The virulence genes including tlh (thermolabile hemolysin), tdh (thermostable direct hemolysin), toxR (cholera toxin transcriptional activator), sto (heat stable heat-stable enterotoxin), ctxA (Cholera toxin A subunit) and vpi (*V. cholerae* pathogenicity island) were the most frequent targets [26–28]. Due to the complicated mechanism of pathogenicity of *V. alginolyticus*, a pathogenic strain may not have all the virulence genes. To date, only the toxR gene had shown a good coverage for all the pathogenic strains [29, 30].
Though PCR based specific detection methods for *V. alginolyticus* targeting the toxR gene are promising, they cannot fulfill the current requirement for on-site detection by the mariculture industry because they still need complicated thermal cycling devices and trained personnel [31]. Recombinase polymerase amplification (RPA) is an isothermal *in vitro* nucleic acid amplification technology with good rapidness, simplicity and specificity [32]. Combining the recombinase and polymerase activities, it opens DNA strands and amplifies DNA targets isothermally. Chemical labeling enables end-point reading of the amplification product as colored signals from gold nanoparticles (AuNP) with the naked eye on lateral flow dipsticks (LFD) [32]. The RPA-LFD method has been applied to the detection of many pathogenic bacteria including goose parvovirus [33], *Burkholderia mallei* [34], *Trichinella* spp. [35], *Mycoplasma bovis* [36] and *Candidatus Liberibacter asiaticus* [37].

In this study, we developed a RPA-LFD method that highly specifically detected *V. alginolyticus* pathogenic strains. The virulence gene toxR was targeted, and the primer-probe set of the RPA reaction was designed to specifically recognize *V. alginolyticus* pathogenic strains but not any other *Vibrio* species. The RPA-LFD method finished the detection within 30 min under an isothermal temperature between 35°C and 45°C. The rapidness, simplicity and high specificity of this method make it well suited for on-site detection of *V. alginolyticus* pathogenic strains.

**Results**

An RPA-LFD method for visual detection of *V. alginolyticus*

The molecular detection of *V. alginolyticus* is based on recombinase polymerase amplification of a specific DNA fragment on its genome and visualization of the amplification product on a lateral flow dipstick (Fig. 1). The amplification starts with pairing of the forward (F) and reverse (R) primers to the amplification target on the genome (Fig. 1a, step a). With the help of the recombinase, the double strands open, and the extension from the 3’ ends of the primers is conducted by the Bsu DNA polymerase (Fig. 1a, step b). There is no thermal cycling and the exponential amplification is done isothermally (Fig. 1a, step c). A specially designed probe (P) binds to the amplification product and facilitates strand displacement (Fig. 1a, step c). A specially designed probe (P) binds to the amplification product and facilitates strand displacement (Fig. 1a, steps d-g). The probe has a
dideoxycytidine (DDC) at the 3’ end that blocks strand extension, and a THF site in the middle that is cleaved by the Nfo enzyme after pairing. Because the Nfo enzyme only cleaves when the flanking bases of the THF site are paired, and the polymerase only extends the strand when the C3-spacer is cleaved, using this specially designed probe significantly increases the amplification specificity [32]. The reverse primer and probe were modified with biotin and fluorescein isothiocyanate (FITC) at the 5’ ends, respectively. Only the amplification product that has both the labels can give signal on the lateral flow dipstick.

When the amplification product is spotted onto the lateral flow dipstick, it migrates through an area that is preloaded with the anti-FITC antibody functionalized with AuNP, and then is trapped by the biotin-streptavidin affinity at the test line where streptavidin is coated (Fig. 1b). Because of the FITC label, the amplification product binds to the anti-FITC antibody when migrating through and shows red color at the test line where it is trapped. The control line coated with anti-mouse antibody is located farther from the spotting position than the test line. The anti-FITC antibody produced from mouse could bind to the anti-mouse antibody when reaches the control line and shows red color to validate the dipstick test. With this lateral flow dipstick, the specific amplification product of the V. alginolyticus DNA is visually detected by observing the red color lines on the dipstick.

Design of the primer-probe set for specific detection of V. alginolyticus pathogenic strains
The virulence expression regulatory gene toxR of V. alginolyticus was selected as the representative gene for all the pathogenic strains [30]. To specifically targeting V. alginolyticus but not any other Vibrio species, the sequence of toxR gene of V. alginolyticus was compared with 7 other Vibrio species including V. parahaemolyticus, V. anguillarum, V. vulnificus, V. harveyi, V. mediterranei, V. shilonii and V. cholera (Fig. 2). Regions 90 to 316 and 329–362 (base numbers on toxR gene of V. alginolyticus) were found to have good sequence diversity. To ensure the primer-probe set covers the detection of all the pathogenic strains of V. alginolyticus, sequences of toxR gene of all these strains were aligned (Fig. 3). Region 50 to 518 (base numbers on toxR gene of V. alginolyticus) was found to have a good conservation. Combining the sequence diversity and conservation information, 5
candidate regions were selected for the design of primer-probe set (Fig. 4). To avoid the formation of primer-probe complexes, a criteria was set as in a given primer-probe set, the primers and probe should have less than 3 consecutive bases (and less than 1 if located at the 3’ end) pairing each other. Possible sequences 12 forward primers (divided into 2 groups), 1 reverse primer and 2 probes were obtained (Fig. 4 and Table 1).

Screening of primers and probes
The 12 primer pairs composed by the 12 forward primers and the reverse primer, respectively, were screened for RPA amplification performance of the \textit{V. alginolyticus} toxR gene fragments. The results showed that, the primer pair F6/R had the brightest amplification band on the agarose gel, and primer pairs F1/R, F2/R and F3/R were obviously brighter than the rest of the primer pairs (Fig. 5). As the primer pair F3/R produced an obvious primer-dimer band on the gel at the lower position of the lane, F1/R, F2/R and F6/R were selected for further screening.

The 3 primer pairs were confirmed for their specificity to amplify \textit{V. alginolyticus} DNA but not any other \textit{Vibrio} species, nor several commonly seen pathogenic bacteria (Fig. 6). Only with the \textit{V. alginolyticus} DNA that the 3 primer pairs produced amplification bands as seen on the agarose gel. According to the pairing positions of the probes on the toxR gene, they both could work with the 3 primer pairs. To avoid the risk of probe-primer complex to give false positive signal on the dipstick without amplification of the target DNA, the probes and primers were tested in the RPA reaction without the template DNA. With the reverse primer R, Probe 1 but not Probe 2 gave a red color band at the test line, which was a false positive signal (Fig. 7a). Then Probe 2 was tested with the 3 primer pairs, and the results showed that only primer pair F2/R with Probe 2 did not give false positive signal (Fig. 7b). The primer-probe set F2/Probe 2/R was confirmed to give a good signal on the dipstick with the \textit{V. alginolyticus} template, and its specificity towards only \textit{V. alginolyticus} but not any other \textit{Vibrio} species or commonly seen pathogenic bacteria was again confirmed (Fig. 8).

Optimization of the RPA-LFD conditions
The conditions of the RPA reaction were optimized on reaction temperature and time length. The RPA reaction amplifying the \textit{V. alginolyticus} template was set under different temperatures ranging from
15°C to 45°C (Fig. 9a). The red color band at the test line was visible between 25–45°C and achieved the best thickness at temperatures 40°C and 45°C. Under 40°C, the reaction time range was test from 10 min to 45 min (Fig. 9b). The red color band at the test line appeared from 15 min and remained the same thickness from 25 min to 45 min. So it was determined that the best reaction conditions of the RPA-LFD detection was 40°C for 25 min.

Detection limit of the RPA-LFD method for *V. alginolyticus*

A 10-fold series dilution of inactivated *V. alginolyticus* from $10^{-1}$ CFU to $10^3$ CFU/μL was tested with the RPA-LFD method. A weak red color band at the test line was seen with $10^0$ CFU/μL, and the band thickness increased with the higher and higher concentrations of *V. alginolyticus* (Fig. 10). The result indicated that the detection limit of the RPA-LFD method for *V. alginolyticus* was 1 CFU/μL.

Application simulation of the RPA-LFD method for *V. alginolyticus* detection

The RPA-LFD method was applied to *V. alginolyticus* detection in artificially infected shrimp homogenate samples, and the detection accuracy was compared with the traditional quantitative PCR method. 58 shrimp homogenate samples were prepared with 8 of them artificially infected with *V. alginolyticus*. The 58 samples were randomly numbered and were subjects for detection of *V. alginolyticus* with both RPA-LFD and quantitative PCR. All the artificially infected samples were successfully detected and the results of the RPA-LFD method were consistent with those from quantitative PCR (Table 2).

Discussion

Fast and specific detection of *V. alginolyticus* pathogenic strains is important in the mariculture industry to control its outbreaks and prevent economic losses [38]. The detection has to be specific to facilitate pointed treatment and prevention [39]. Rapid and accurate on-site detection is essential because of the fast onset and outbreak of the pathogen [38]. The RPA-LFD combined method should be a promising solution for the rapidness, simplicity and the possibility to target specific gene sequences [34].

Selection of the detection target was important for the specificity. *Vibrio alginolyticus* includes non-pathogenic strains as well, which are beneficial for aquatic animals and should not be detected [3].
The virulence genes were usually selected as biomarkers in the PCR based detections. Among them, the toxR gene was reported to have covered all the pathogenic strains, and was selected as our target in this study [40]. The toxR gene exists not only in *V. alginolyticus* pathogenic strains, but also in other virulent *Vibrio* species. Thus we had to carefully select regions within this gene that was specific in *V. alginolyticus*.

To find regions specific in *V. alginolyticus*, the toxR gene sequence of *V. alginolyticus* was compared with a series of other *Vibrio* species and the diverged regions were selected (Fig. 2). These regions were then compared within the *V. alginolyticus* pathogenic strains (Fig. 3). Only the areas that were diverged in *Vibrio* species and were conserved in *V. alginolyticus* pathogenic strains were considered as possible targeting areas of the primers and probes in the RPA system. This way ensured our RPA system to have the specificity for all the *V. alginolyticus* pathogenic strains. In the complicated RPA reaction, small variations in position and length of the primers and probe could affect the amplification result. Thus multiple primers and probes were designed for a systematic screening (Fig. 4). The screening considered amplification performance, primer-dimer formation and false positive signals, and finally a primer-probe set that was highly specific to *V. alginolyticus* pathogenic strains was obtained (Fig. 8).

After optimization, our RPA-LFD method was able to detect as low as 1 CFU/μL of *V. alginolyticus* in the culture without DNA extraction. This was close to the detection limit of the PCR based approaches, which was $10^2$~$10^3$ CFU/mL [41]. Considering the PCR based approaches were always associated with DNA extraction steps to increase the template concentrations, the detection limit of our RPA-LFD method was actually satisfactory given the simplicity. The detection finished within 30 min under the temperature between 35°C and 45°C, and the visual signal on the dipstick was directly read with the naked eye. In an application simulation, randomly infected shrimp homogenate samples were 100% accurately detected by the RPA-LFD method, and the results were consistent with the quantitative PCR method.

**Conclusions**

A fast and simple RPA-LFD method to detect *V. alginolyticus* pathogenic strains with high specificity
was established in this study. By carefully selecting the target gene and the specific amplification regions, the RPA-LFD method was able to accurately identify the *V. alginolyticus* pathogenic strains but not any other *Vibrio* species. The method is well suited for the on-site detection of *V. alginolyticus* pathogenic strains in the mariculture industry.

**Materials And Methods**

**Bacterial strains**
*Vibrio alginolyticus, Listeria monocytogenes, Staphylococcus aureus,* and *Escherichia coli* O157 in the inactivated form at a concentration of $10^6$ colony forming units (CFU)/mL in LB medium were kindly given from Wuhan Institute for Food and Cosmetic Control (Wuhan, China). *Vibrio parahaemolyticus, Vibrio cholerae, Vibrio vulnificus, Vibrio anguillarum, Vibrio harveyi, Vibrio mirabilis, Vibrio mediterranei, Vibrio shilonii, Vibrio azureus* in the inactivated form at a concentration of colony forming units (CFU)/mL in LB medium were kindly given from Jiangsu Institute of Oceanology and Marine Fisheries (Nantong, China). For RPA or RPA-LFD reactions, the inactivated cultures were treated at 100°C for 10 min before used as the templates; In the detection of contaminated samples, the genome is extracted using the animal tissue gene extraction kit.

**Sequences and alignments**
The nucleic acid sequences of toxR genes were downloaded from GenBank. Accession nos: *V. parahaemolyticus*, AB029907.1; *V. anguillarum*, AB042547.1; *V. vulnificus*, AB175481.1; *V. harveyi*, DQ640258.1; *V. mediterranei*, EU727207.1; *V. shilonii*, EU727208.1; *V. cholera*, MF100077.1. *V. alginolyticus* pathogenic strains, EU15543.1; JN188462.1; EU155556.1; KT194118.1; EU155576.1; JN188451.1. The alignments were done with the clustalX software with default parameter settings [42]. The alignment images were produced with the ESPript software (http://escript.ibcp.fr).

**Design of primers and probes**
The design was done with the Primer Premier 5.0 software (Premier Biosoft International, CA, USA). For primers, after the sequence of a particular targeting region was input, the parameters were set as follows: PCR product size, 100bp to 300 bp; Primer length, 30bp to 35 bp. For probes, the parameters were set as follows: PCR product size, 100bp to 300 bp; Primer length, 46bp to 51 bp. The possibilities of pairing between forward and reverse primers and probe and reverse primers were manually
checked. Sequences pairing for more than 3 consecutive bases (and more than 1 base if at the 3’ end) were abandoned. The sequences of the primers and probes were then confirmed for species specificity with Primer BLAST on the NCBI website.

RPA procedure

RPA reactions were setup according to the manufacturer’s instructions of TwistAmp® Liquid DNA Amplification Kit (TwistDx Inc., Maidenhead, UK). The reaction contained 25 μL of 2 × Reaction buffer, 5 μL of 10 × Basic e-mix, 2.5 μL of 20 × core mix, 2.1 μL of each primer (10 μM), 9.8 μL of distilled water, 1 μL (the concentration is 10^6 CFU/mL) of the template and a dried enzyme pellet. The primers were commercially synthesized with the 5’ end of the reverse primer labeled with Biotin (General Biosystems, Anhui, China). To initiate the reaction, 2.5 μL of magnesium acetate (280 mM) was added into the mixture. After brief centrifugation, the reaction mixture was immediately incubated at 37°C for 30 min. The RPA amplification products were purified using PCR Cleaning Kit (Monad Biotech Co. Ltd, Wuhan, China) and electrophoresed on a 1.5% agarose gel.

RPA-LFD procedure

The reverse primers and probes were modified with biotin and fluorescein isothiocyanate (FITC) at the 5’ ends, respectively (General Biosystems). The 3’ end of each probe was blocked with a dideoxycytidine (DDC), and a base “C” at the middle of the probe that was at least 30 bases away from the 5’ end and 15 bases away from the 3’ end was replaced with a THF group. RPA reactions were setup according to the manufacturer’s instructions of TwistAmp® DNA Amplification nfo Kit (TwistDx). The reaction contained 29.5 μL of Rehydration Buffer, 2.1 μL of each primer (10 μM), 0.6 μL of probe (10 μM), 12.2 μL of distilled water, 1 μL of template and a dried enzyme pellet. To initiate the reaction, 2.5 μL of magnesium acetate (280 mM) was added into the mixture. After brief centrifugation, the reaction mixture was immediately incubated at 15–45°C for 10–45 min. 5 μL of the amplification products were used for LFD (Ustar Biotechnologies Ltd, Hangzhou, China) detection. The amplification products were added to the sample pad of LFD, and the stick of LFD was inserted into 100 μL of the sample buffer (Ustar Biotechnologies) for 5 min and then for visual reading.

Preparation of artificially infected shrimp homogenate samples

For each sample, fresh shrimps were cut into small pieces and 300 mg pieces of tissue were ground in
liquid nitrogen. Into several randomly selected homogenate samples, 3 μL of the 10^6 CFU/mL inactivated *V. alginolyticus* culture was mixed. DNA was extracted from the homogenate samples by the TIANamp Genomic DNA kit (Tiangen Biotech Co. Ltd, Beijing, China) into a 50 μL volume. 1 μL of the extracted DNA was used for RPA-LFD or quantitative PCR detection.

**Quantitative PCR**

A pair of specific primers (forward: 5’-ATTGAGAACCCGACAGAAGCGAAG–3’; reverse: 5’-CCTAATGCGGTGATCAGTGTTACT-3’) targeting the gyrB gene (GenBank accession no. AF007288) of *V. alginolyticus* was used for quantitative PCR [41]. The qPCR reaction mixture contained 25 μL of MonAmp™ SYBR Green qPCR Mix (Monad Biotech), 0.4 μL of a solution containing each of the oligonucleotide primers at a concentration of 10 μmol/L, and 1 μL of the DNA sample. The cycling program was 95°C 60 s followed by 45 cycles of 95°C 15 s, 60°C 15 s and 72°C 45 s on a Roche LightCycler 480 qPCR machine.

**Availability of data and materials**

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

**Abbreviations**

*PCR*: Polymerase chain reaction  
*RPA*: Recombinase polymerase amplification  
*LFD*: Lateral flow dipsticks  
*V. alginolyticus*: *Vibrio alginolyticus*  
*toxR*: Toxin transcriptional activator  
*AuNP*: Gold nanoparticles  
*DDC*: Dideoxycytidine  
*FITC*: Fluorescein isothiocyanate  
*CFU*: Colony forming units  
*qPCR*: Quantitative PCR

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Declarations

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Contributions
KZ, JD and SG designed the research. YD, PZ, HW, XS, HS, JL, YQ, QC and WJ conducted the research. PZ, JD and SG wrote the manuscript. JD directed the project.

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Ethics declarations
Ethics approval and consent to participate
The artificial infected samples used in the study were obtained from fresh shrimps during routine processing at the market. No animal was sacrificed for the purpose of this study. No separate ethical approval was required for the study. All efforts were made to minimise the discomfort of the animals during sampling.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

Tables
Table 1 Primer and probe sequences designed for specific detection of *V. alginolyticus*
| Name | Sequence (5' to 3') |
|------|---------------------|
| F1   | 5'-GAGCCCATTTTCTACTGACTCTAACAACAGTACG |
| F2   | 5'-AGCCCATTTTCTACTGACTCTAACAACAGTACG |
| F3   | 5'-GCCCATTCTTCTACTGACTCTAACAACAGTACG |
| F4   | 5'-CCCATTCTTCTACTGACTCTAACAACAGTACG |
| F5   | 5'-TTCTACTGACTCTAACAACAGTACGTTGAAGAACC |
| F6   | 5'-TTCTACTGACTCTAACAACAGTACGTTGAAGA |
| F7   | 5'-ACTGACTTAAACTAATAACTGACGTGTTGAAGAAACCG |
| F8   | 5'-ACTGACTTAAACTAATAACTGACGTGTTGAAGAAACCG |
| F9   | 5'-GAGATAGTGACCGATACTAATCTGACTTGGAG |
| F10  | 5'-GAGATAGTGACCGATACTAATCTGACTTGG |
| F11  | 5'-GAGATAGTGACCGATACTAATCTGACTTGG |
| F12  | 5'-GAGATAGTGACCGATACTAATCTGACTTGG |
| R    | 5'-Biotin –GTTCGTGAATAACATAACGCAACAGGAAG |
| Probe 1 | 5'-FITC- AAGCGCCAGCAGTGGAGTTGAGGACGAGCG[THF]TACA CCACCAACAGA-DDC |
| Probe 2 | 5'-FITC- TCAAGTAGAGCGACTAACAATCTAGCCGAA[THF]CCAGC ATCTAACACAG-DDC |

(F: forward primers 1-12. R: reverse primer.)

Table 2 Detection accuracy of RPA-LFD and qPCR for *V. alginolyticus*
| Sample number | Detection methods | Ct Value (n=3) |
|---------------|------------------|---------------|
|               | RPA-LFD | qPCR |               |
| 1             | -       | -     | 35.17±0.23    |
| 2             | +       | +     | 25.63±0.24    |
| 3             | -       | -     | 36.02±0.20    |
| 4             | -       | -     | 35.10±0.15    |
| 5             | -       | -     | 36.76±0.11    |
| 6             | -       | -     | 34.83±0.04    |
| 7             | +       | +     | 29.16±0.21    |
| 8             | -       | -     | 33.79±0.21    |
| 9             | -       | -     | 35.55±0.15    |
| 10            | -       | -     | 34.78±0.06    |
| 11            | -       | -     | 34.98±0.15    |
| 12            | -       | -     | 36.46±0.04    |
| 13            | -       | -     | 35.80±0.10    |
| 14            | -       | -     | 36.24±0.08    |
| 15            | -       | -     | 35.14±0.24    |
| 16            | -       | -     | 34.79±0.15    |
| 17            | -       | -     | 36.31±0.19    |
| 18            | -       | -     | 34.03±0.10    |
| 19            | -       | -     | 36.62±0.12    |
| 20            | -       | -     | 35.17±0.23    |
| 21            | -       | -     | 35.63±0.08    |
| 22            | -       | -     | 35.83±0.25    |
| 23            | -       | -     | 36.63±0.17    |
| 24            | -       | -     | 34.92±0.01    |
| 25            | -       | -     | 36.11±0.18    |
| 26            | -       | -     | 34.44±0.38    |
| 27            | -       | -     | 34.92±0.26    |
| 28            | -       | -     | 35.66±0.15    |
| 29            | +       | +     | 30.41±0.27    |
| 30            | -       | -     | 35.49±0.08    |
| 31            | -       | -     | 34.47±0.13    |
| 32            | -       | -     | 36.36±0.06    |
| 33            | -       | -     | 34.40±0.11    |
| 34            | +       | +     | 25.31±0.01    |
| 35            | -       | -     | 35.14±0.27    |
| 36            | -       | -     | 35.51±0.06    |
| 37            | -       | -     | 34.89±0.10    |
| 38            | -       | -     | 36.52±0.31    |
| 39            | -       | -     | 34.43±0.33    |
| 40            | -       | -     | 36.42±0.27    |

(+: positive result. -: negative result.)

Figures
Figure 1

Schematic diagram of the RPA-LFD method. (a) Principle of RPA amplification. DNA strands are presented as horizontal lines and the base pairings are indicated as short vertical lines between the DNA strands. Forward primer (F), reverse primer (R), probe (P), Nfo, Bsu and modifications on DNA are indicated with different shapes and colors. The legends are given at the bottom of the image. (b) Schematic diagram of the working principle of the lateral flow dipstick. The sample pad is indicated by the gray parallelogram on the left, the absorbent pad is indicated by the gray parallelogram on the right, and the conjugate pad is
shown in pink. The liquid migration direction is indicated by an arrow. Molecules could be trapped by the materials on the Test line and the Control line were indicated by different shapes. Shapes and their representing molecules were listed at the bottom of the image.

Figure 2
Sequence alignment of the toxR genes of Vibrio species. GenBank numbers are indicated on the left of each sequence. Diverged regions are indicated by the black boxes.
Figure 3

Sequence alignment of the toxR genes of V. alginolyticus pathogenic strains. GenBank numbers are indicated on the left of each sequence. The conserved region is indicated by the black box.
Figure 4
Relative positions of the primer and probe sequences. The upper horizontal line represents the toxR gene and the relative position on the genome. The blue arrows indicate the forward primer positions and the amplification direction. The red arrows indicate the probe positions and the amplification direction. The black arrow indicates the reverse primer position and the amplification direction. The forward primer sequences are written under their respective positions.

Figure 5
Screening for primer pairs. The agarose gel image shows the amplification results of the 12 primer pairs. The primer pair name was indicated at the top of each lane. The NTC lane immediately after is the no-template control of the respective primer pair. The band sizes of the DNA ladder are shown on the right.
Detection specificity of the primer-pairs. The agarose gel images show the RPA amplification results of different bacterial templates by F1/R, F2/R and F6/R primer-pairs. The species of the bacteria are indicated on top of each lane. The NTC lane is the no-template control. The size of each band of the DNA ladder is indicated on the right of the gel image.
Screening for primer-probe sets without false positive signal. (a, b) The images of LFD show the detection results of primer-probe sets without any DNA template. The primer-probe set names are indicated on top of each dipstick. The positions of the Control and Test lines are indicated on the right of the image.
Confirmation of the RPA-LFD method for specificity. The image of LFD shows the results of RPA amplification of different bacterial templates. The species of the bacteria are indicated on top of each dipstick. The NTC dipstick is the no-template control. The positions of the Control and Test lines are indicated on the right of the image.
Figure 9

Optimization of the RPA-LFD method. (a) The image shows the LFD results of RPA amplifications under different temperatures. The temperatures under which the RPA reactions were performed are indicated on top of each dipstick. The amplification template was V. alginolyticus. The NTC dipstick is the no-template control that performed at 40°C. The positions of the Control and Test lines are indicated on the right of the image. (b) The image shows the LFD results of RPA amplifications with different time lengths. The time lengths for which the RPA reactions were performed are indicated on top of each dipstick. The amplification template was V. alginolyticus. The NTC dipstick is the no-template control that performed for 45 min. The positions of the Control and Test lines are indicated on the right of the image.
Detection limit of the RPA-LFD method. The image shows the LFD results of RPA amplifications with different concentrations of *V. alginolyticus* culture. The concentrations tested in the RPA reactions are indicated on top of each dipstick. The NTC dipstick is the no-template control. The reactions were performed at 40°C for 25 min. The positions of the Control and Test lines are indicated on the right of the image.