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Rapid identification and detection of *Vibrio parahaemolyticus* via different types of modus operandi with LAMP method in vivo

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Abstract

Purpose: *Vibrio parahaemolyticus*, an easy-ignored food-borne pathogen, can cause bacterial outbreaks and human disease during early-stage infection. In this study, we aimed to evaluate the detection efficiency of loop-mediated isothermal amplification (LAMP) as an emerging technique to directly detect *V. parahaemolyticus* infection in mammalian hosts and assess its potential in clinical applications.

Methods: A LAMP assay was used for rapid identification of *V. parahaemolyticus* in a variety of mouse models in which animals were infected via the digestive tract, wounds, or through general infection, and the results were compared with routine analytical methods.

Results: Our results confirmed that the LAMP assay was capable of detecting *V. parahaemolyticus* in different mouse organs independent of the source of bacteria, although its sensitivity depended on the route of infection and the organ affected. Foodborne-derived *V. parahaemolyticus* was the most sensitive route, with the small intestine being the most sensitive organ. The LAMP assay indicated that *V. parahaemolyticus* that spread through the blood stream had the most serious consequences during early-stage infection. Positive LAMP results were identified in all blood samples from i.v. injected mice. Furthermore, the LAMP method could directly detect trace quantities of *V. parahaemolyticus* in fresh peripheral blood while conventional methods failed to do so, thereby shortening the time-to-result from days to minutes.

Conclusions: In this study, we demonstrated that the LAMP assay was effective in speeding up the detection of *V. parahaemolyticus*. Instead of being a secondary method to assist in the clinic, the LAMP assay has potential for use as the primary technique for rapid detection of *V. parahaemolyticus* in the future.

Keywords: Detection in vivo, LAMP, Mouse model, *Vibrio parahaemolyticus*

Background

Vibrio parahaemolyticus is a common gram-negative (G⁻) halophilic bacterial species that is principally distributed within sea water (DePaola et al. 1990), coastal areas, and river-sea junctures (Yang et al. 2017a). It can also be found in seafood (Twedt 1989; Rince et al. 2018). In addition to its presence in marine environments, *V. parahaemolyticus* can also be found in livestock and poultry meat, freshwater fish, preserved eggs, and pickles (Barker 1974). Therefore, despite the sources of marine infection, such as seafood and marine salts, a variety of animal species can also be carriers of *V. parahaemolyticus*, rendering it an important pathogenic bacterium that is highly infectious.

Human infection with *V. parahaemolyticus* is an important public health problem (Baker-Austin et al. 2018). *V. parahaemolyticus* is the principal pathogen causing acute gastroenteritis in humans via consumption of infected seafood, for which the symptoms include stomachache (Liu et al. 2015), diarrhea (Gong et al.

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2018), vomiting, dehydration, chills, and fever (Raszl et al. 2016). In the USA, *V. parahaemolyticus* is the leading cause of human gastroenteritis and associated with seafood consumption (Park et al. 2018). In coastal Asian countries, including Indonesia, *V. parahaemolyticus* is the leading cause of bacterial foodborne infections (Lesmana et al. 2001; Lee et al. 2018). In Japan, food poisoning caused by *V. parahaemolyticus* accounts for 20~30% of all cases of human food poisoning (Alam et al. 2002). In Europe and North America, many outbreaks of *V. parahaemolyticus* food poisoning have been reported (McLaughlin et al. 2005; Martinez-Urtaza et al. 2013; Martinez-Urtaza et al. 2016). Coincidentally, over the past two decades, published reports have indicated that *V. parahaemolyticus* has been the principal cause of bacterial foodborne infections in China (Li et al. 2014). According to the continuously monitoring data from 2013 mainland China from the National Foodborne Disease Outbreaks Surveillance System (NFDOSS), *V. parahaemolyticus* was the leading factor causing 27.8% of all the microbial-driven foodborne disease outbreaks, making it the only marine bacterial species among the 5 most common pathogens, and the 5th most common species found in food (Li et al. 2018).

In southern China, there is a strong seasonal pattern in the outbreaks of human infection by *V. parahaemolyticus*, which partially attributes to the different carriage rate of marine products throughout the year. In a study by Yang et al., investigating 504 seafood samples from 11 provinces of China, it was shown that the prevalence of *V. parahaemolyticus* peaked during the summer (33%) and reduced by half during the winter (14%) in fish, oyster, and shrimp (Yang et al. 2017b). The disease caused by *V. parahaemolyticus* can spread widely but with generally mild symptoms, which are usually self-limiting (Rizvi and Bej 2010), possibly causing people to pay little attention to it. Due to symptoms resulting from *V. parahaemolyticus*, infections similar to that by other common food-borne pathogens make the prevention of *V. parahaemolyticus* infection and its early-stage medical treatment easily be neglected.

With the development of technology, methods of *V. parahaemolyticus* detection have increased. To meet improved diagnostic accuracy, culture medium for conventional phenotypes and biochemical identification methods has undergone a process of constant improvement from standard alkaline peptone water (APW) to ST medium, thiosulfate citrate bile salts sucrose agar culture medium (TCBS) or Wagatsuma agar (Martinez-Urtaza et al. 2006; Croci et al. 2007; Letchumanan et al. 2014). The CHROMagar *Vibrio* medium is gradually being applied in the clinic and significantly improves interpretation. Nevertheless, conventional methods still face the obstacles of time- and labor-intensive methodology.

With the advent of nucleic acid-based methods, such as multiplex PCR, real-time PCR, RAPD-PCR, REP-PCR, ERIC-PCR, GS-PCR, nucleic acid sequence-based amplification (NASBA), fluorescence in situ hybridization (FISH), and micro arrays (Di Pinto et al. 2012; Letchumanan et al. 2014; Nordin et al. 2016; Cho et al. 2017; Xu et al. 2018), the detection of *V. parahaemolyticus* is becoming more convenient. In addition, biosensor and immunological-based methods have also become available (Kumar et al. 2011). However, these methods are too complex for the detection of *V. parahaemolyticus* in a large number of clinical samples.

Since 2000, loop-mediated isothermal amplification (LAMP) (Notomi et al. 2000) has played a significant role in medical diagnostics, including the diagnosis of clinical diseases (Dea-Ayuela et al. 2018; Waterfield et al. 2018), qualitative detection of epidemic bacteria or viruses, and gender identification of animal embryos (Poon et al. 2004; Fujita et al. 2017; Wong et al. 2018). As an emerging method for the detection of *V. parahaemolyticus* and the rapid diagnosis of infections, LAMP has greatly boosted the capability of detection of early-stage infections. Chen and Ge developed a *toxR*-based LAMP assay in 2010 (Chen and Ge 2010), and Zhou et al. extended the LAMP assay for *V. parahaemolyticus* identification into field-based detection in 2016 (Zhou et al. 2016). Moreover, Wang et al. optimized the assay in 2016 by developing a multiple endonuclease restriction LAMP method (Wang et al. 2016). However, few reports or systematic studies focused on the rapid detection of early-stage *V. parahaemolyticus* infection in humans using LAMP, and therefore, detailed characteristics of the LAMP assay, such as detection efficiency, sensitivity, and duration, remain unknown.

Based on the rationale described above, in the present study, the LAMP assay was optimized for rapid diagnosis of *V. parahaemolyticus*. Detection efficiency was determined by the detection of *V. parahaemolyticus* infections in a variety of mouse infection models under various conditions. By comparing this LAMP assay with current detection standards, we aimed to establish a more accurate and rapid technique for the detection of *V. parahaemolyticus* infections and provided experimental evidence in support of its use in such diagnostics.

Methods
Bacteria and growth conditions
In this study, a total of 71 *V. parahaemolyticus* strains were used, including 69 food isolates (hereafter referred to as strains *Vp* 1463~1528, *Vp* 4213, *Vp* 4215, *Vp* 11577), a marine isolate (*Vp* 1A10122), and a type strain (*Vp* ATCC 17802, Table 1). *V. parahaemolyticus* food isolates were kindly provided by Professor Shenghui Cui and were isolated from various food samples; *V.*

Li *et al. Annals of Microbiology*   (2020) 70:40

Page 3 of 15

Table 1 *V. parahaemolyticus* used in this study

Bacterial genus	Strain ID	Source	Type strain
V. parahaemolyticus	*Vp* 1463–1528 (*n* = 66)	Food samples	No
V. parahaemolyticus	*Vp* 4213	Food sample	No
V. parahaemolyticus	*Vp* 4215	Food sample	No
V. parahaemolyticus	*Vp* 11577	Food sample	No
V. parahaemolyticus	*Vp* 1A10122	Seawater isolates	No
V. parahaemolyticus	*Vp* ATCC 17802	Clinical isolates	Yes

parahaemolyticus strain 1A10122 and 17802 were purchased from the Marine Culture Collection of China (MCCC, Xiamen, China) and American Type Culture Collection (ATCC, VA, USA), respectively. The *V. parahaemolyticus* strains were recovered on 2216E agar plates (BD Biosciences, NJ, USA) at 35 °C for 12 h, and routinely cultured in 2216E broth (BD Biosciences, NJ, USA) at 35 °C with shaking at 180 rpm for 12 h. Dilution or enrichment of cultured *V. parahaemolyticus* strains was performed in 3% NaCl APW (Land Bridge Technology, Beijing, China).

A growth curve of *V. parahaemolyticus* was plotted based on the Gompertz model using *Vp* ATCC 17802 as the reference strain, which calculated and indicated the relationship between OD_{600} and fresh culture with an isochronous continuous measurement method. After a 9-h culture process (data from 0 to 9 h), an equation was established with the OD_{600} value on the X-axis and the CFU on the Y-axis. The growth curve was fitted based on the optimized Gompertz equation as follows: $Y = 3.42 \times 10^{14} \times e^{-e^{-3.26 \times (x-1.53)}}$. A suspension of fresh *V. parahaemolyticus* strain was prepared for the animal experiments based on the above-mentioned equation.

PCR assay for detection of *V. parahaemolyticus* hemolysin

The density of the 71 *V. parahaemolyticus* strains was adjusted to 3×10^5 CFU/mL, then cultured in 3% NaCl APW at 35 °C with shaking at 180 rpm. After culturing for 6 h, 1 mL of the cultures was centrifuged (10510 × g) to obtain a bacterial pellet, from which genomic DNA was extracted using a DNA extraction kit (Bacterial genomic DNA extraction kit, DP302-02, TIANGEN, Beijing, China). Concentrations of genomic DNA were measured using a NanoDrop 8000 (Thermo Fisher, MA, USA).

Three pairs of primers targeting *tdh* (GenBank accession No. M10069), *trh* (GenBank accession No. KP836472.1), and *tlh* (GenBank accession No. AY289609) genes were designed for PCR based on the DNA sequences (Table 2) using the Primer premier 5.0 software (PREMIER Biosoft, CA, USA). Each PCR mixture consisted of a final volume of 20 μL containing 200 ng of *V. parahaemolyticus* genomic DNA, 400 nM of each primer pair, 10 μL of 2 × GoTaq DNA polymerase mix (Promega, WI, USA) and water. PCR

was performed as follows: initial denaturation at 94 °C for 3 min, followed by 30 cycles of denaturation at 94 °C for 40 s, annealing at 58 °C for 30 s and extension at 72 °C for 1 min, followed by a final extension at 72 °C for 5 min, then storage at 4 °C. The amplified PCR products were separated on Tris-boric acid gels in Tris-boric acid buffer to visualize the band sizes.

Mouse source and feeding conditions

Balb/C female mice (5~6 week old, 17~18 g) used in this study were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China) and were bred under specific-pathogen-free (SPF) conditions.

Mouse infection model of *V. parahaemolyticus* utilizing various routes of administration

Based on the growth curve, *Vp* 1474, *Vp*1496, *Vp*1513, *Vp*1A10122, and *Vp*ATCC 17802 were incubated at 35 °C with shaking at 180 rpm until the culture had reached an OD_{600} value of 0.35 (approximately 1.5×10^6CFU). The cell pellet was rinsed once with pre-cooled PBS, then centrifuged for 5 min at 950 × g and 4 °C, after which the supernatant was discarded. The cell pellet was resuspended in 100 μL of pre-cooled PBS, then stored on ice before their immediate use following the next procedure.

The *V. parahaemolyticus*-localized infection model consisted of the intradermal (i.d.) injection of 5 strains of *V. parahaemolyticus* (approximately 1.5×10^6 CFU for each strain, 100 μL) into 2 cm² of skin on the back of each mouse adjacent to the point of attachment of the right leg to the dorsal line following sterilization. Infected mice were then observed for 4 h for signs of localized inflammation resulting from *V. parahaemolyticus* infection. For the *V. parahaemolyticus* foodborne infection model, 5 strains of *V. parahaemolyticus* (approximately 1.5×10^6 CFU for each strain, 100 μL) were administered into the stomach of each mouse via intragastric gavage (i.g.), then infected mice were observed for 8 h to determine the extent of the intestinal inflammation caused by *V. parahaemolyticus*. The wound infection model consisted of the intravenous (i.v.) injection of 5 strains of *V. parahaemolyticus* (approximately 1.5×10^6 CFU for each strain, 100 μL) into the caudal vein of

Li *et al. Annals of Microbiology*   (2020) 70:40

Page 4 of 15

Table 2 Pairs of primers targeting on gene *tdh*, *trh*, and *tlh*

Target	Gene source	Primer name	Sequence (5′ → 3′)	Product length
tdh	GenBank accession No. M10069	tdh-F	TTATTGTTGATGTTTACATT	570 bp
		tdh-R	ATGAAACACCAATATTTTGC	
trh	GenBank accession No. KP836472.1	trh-F	CATTTCCGCTCTCATATGC	250 bp
		trh-R	GGCTCAAAATGGTTAAGCG	
tlh	GenBank accession No. AY289609	tlh-F	GTTGTTGCTACTTTCTAGCATTTTC	1233 bp
		tlh-R	GATGAAAAAAACAATCACAC	

the sterilized tail of each mouse. Infected mice were observed for 8 h to ascertain the response to *V. parahaemolyticus* blood infection. In the *V. parahaemolyticus* general infection model, the left abdomen of each mouse was sterilized under the diaphragm, and 5 strains of *V. parahaemolyticus* (approximately 1.5 × 10^6 CFU for each strain, 100 μL) were injected intraperitoneally (i.p.) into the abdominal cavity. Infected mice were observed for 8 h for secondary infection caused by *V. parahaemolyticus* that had spread from the initial site.

Treatment of mouse peripheral blood (MPB) and major organs

After the period of observation, the mice were anesthetized i.p. using pentobarbital sodium (50 mg/kg, 2% PBS v/v solution). MPB was obtained from the right eye of each mouse and preserved in acid citrate-dextrose (ACD) solution. Mice were then euthanized by cervical dislocation. The whole body of each mouse was sterilized in 75% ethyl alcohol for 5 min. The target digestive organs, including the stomach, small intestine, and liver, in addition to the kidney (urinary tract), and spleen (immune organ) were removed. Each organ in every group was homogenized using a tissue homogenizer (Servicebio, Wuhan, China).

Subculture of *V. parahaemolyticus* and extraction of genomic DNA

Ten microliters of homogenates or 10 μL of MPB was mixed in 10 mL of sterile 3% NaCl APW and incubated at 35°C for 12 h with shaking at 180 rpm, respectively. A portion of each suspension (100 μL) was plated on a TCBS plate. The plates were incubated at 35 °C for 18 h to determine *V. parahaemolyticus* colonies.

The remaining MPB was diluted with PBS to 1 mL and 1 mL of the enriched suspension from each organ used for DNA extraction using a DNA extraction kit (Genomic DNA extraction kit for blood/cell/tissue, DP304-03, TIANGEN, Beijing, China).

LAMP assay

The LAMP-based primer pairs as referred to by Yi et al. are listed in Table 3 (Yi et al. 2014). All primers used in this study were targeted to the *tlh/ldh* gene of *V. parahaemolyticus*. The LAMP assay was performed according to the method of Fu et al. (Fu et al. 2016), using Bst WarmStart DNA polymerase (New England Biolabs, Herts, UK; 1 μL) mixed in 22 μL buffer solution, which included 0.2 μM F3, 0.2 μM B3, 1.6 μM FIP, 1.6 μM BIP, 0.8 μM FLP, 0.8 μM BIP, 1 M betaine, 8 μM MgSO$_4$, 1.6 μM dNTP, 2.5 μL 10 × Thermo pol buffer (New England Biolabs, Herts, UK), 5 μM SYTO®-9 (Invitrogen, Life Technology, NY, USA), and ddH$_2$O. Two microliters of DNA template from the enriched culture and fresh MPB was used for each assay, in a total of 25 μL.

Statistics

The results were analyzed using one-way analysis of variance (ANOVA) and Dunnett's test using IBM Statistics SPSS v22 (Chicago, IL, USA). *P* values were two-tailed, and the threshold for statistical significance was set at 0.05. Results are presented as the mean ± standard error of the mean (SEM) for all independent experiments at each time point. All animal injection experiments were performed in triplicate. Three duplicate test results of each LAMP assay were used only when the triplicate results were consistent.

Results
Hemolysin detection in *V. parahaemolyticus* strains

To explore the presence or absence of different hemolysins in *V. parahaemolyticus* strains from various sources, expression of the *tdh*, *trh*, and *tlh* genes was evaluated to determine *V. parahaemolyticus* hemolysin diversity. Gel electrophoresis results indicated that all 71 strains expressed the *tlh* gene, including foodborne, marine, and type strains, apparently, a general hemolysin in *V. parahaemolyticus*. However, only 4 strains possessed the *tdh*

Table 3 LAMP primers used to detect *V. parahaemolyticus*

Primer number	Sequence (5′ → 3′)
F3	GACAGCTTGTCTGATACAGG
B3	GTTCTTCGCCAGTTTTGC
FIP	GCGGAAGGTTCTTCGCTTTGGCTGGTTCTTAGGTCACTTC
BIP	TCTACAACTGGGCAGTTGGCCTTGATCACCAACCCCTG
LoopF	GTCCACACAAAACCGTTGG
LoopB	GGCTGGTGAGAACCAATACA

Li *et al. Annals of Microbiology*   (2020) 70:40

Page 5 of 15

gene, all belonging to foodborne *V. parahaemolyticus* isolates. Of the 71 *V. parahaemolyticus* strains, 5 strains expressed the *trh* gene, including 4 foodborne isolates and the *V. parahaemolyticus* type strain. The hemolysin features of the strains described above are listed in Table 4.

Based on the presence of the hemolysin regulatory genes, 5 strains were selected for use in animal experiments. *Vp* 1474 expressed TDH and TLH encoded by the *tdh* and *tlh* genes; *Vp* 1496 expressed only TLH encoded by the *tlh* gene; *Vp* 1513 expressed TRH and TLH encoded by the *trh* and *tlh* genes. In comparison to the virulence of foodborne *V. parahaemolyticus* strains, *Vp* 1A10122 was chosen as a marine isolate, which expressed only TLH encoded by the *tlh* gene. *Vp* ATCC 17802 was also chosen as the type strain expressing TRH and TLH encoded by *trh* and *tlh*.

Identification of *V. parahaemolyticus* colonies on TCBS plates

Among localized infection models (Fig. 1a, Table 5), TCBS plates spread with skin samples from mice infected with *Vp*1474 and MPB samples from mice infected with *Vp*1513 and *Vp*1A10122 developed scattered *V. parahaemolyticus* colonies. On TCBS plates spread with skin samples from mice infected with *Vp*1496 and *Vp*1513 and MPB samples from mice infected with *Vp*1474, *Vp*1496, and *Vp* ATCC 17802, the presence of *V. parahaemolyticus* was ambiguous due to the presence of other bacteria on the plates. No *V. parahaemolyticus* colonies were observed on plates spread with skin samples from mice infected with *Vp* 1A10122 and *Vp* ATCC 17802.

Among foodborne infection models (Fig. 2a, Fig. S1, and Table 5), TCBS plates spread with stomach tissue samples from mice infected with *Vp*1474 and *Vp* ATCC 17802; small intestine samples from mice infected with all experimental foodborne strains; liver tissue samples from mice infected with *Vp*1474 and *Vp*1513; kidney tissue samples from mice infected with *Vp*1474, *Vp*1513, *Vp*1A10122, and *Vp* ATCC 17802; spleen samples from mice infected with *Vp*1513 and *Vp* ATCC 17802; and MPB samples from mice infected with *Vp*1496 showed recognizable *V. parahaemolyticus* colonies. Moreover, on TCBS plates spread with stomach tissue samples from mice infected with *Vp*1A10122, small intestine

samples from mice infected with *Vp*1A10122, liver tissue samples from mice infected with *Vp*1496 and *Vp*1A10122, and MPB samples from mice infected with *Vp*1513 and *Vp* ATCC 17802, the presence of *V. parahaemolyticus* was ambiguous as other bacteria competitively grew on the plates. No *V. parahaemolyticus* colony developed on plates spread with stomach tissue samples from mice infected with *Vp*1496 and *Vp*1513; small intestine and liver samples from mice infected with *Vp* ATCC 17802; kidney tissue samples from mice infected with *Vp*1496; spleen samples from mice infected with *Vp*1474, *Vp*1496, and *Vp*1A10122; and MPB samples from mice infected with *Vp*1474 and *Vp*1A10122.

Among wound infection models (Fig. 3a, Fig. S2, and Table 5), TCBS plates spread with stomach, small intestine, liver, kidney, spleen, and MPB samples of mice infected with all experimental foodborne strains and *Vp* ATCC 17802 developed apparent *V. parahaemolyticus* colonies, whereas on TCBS plates spread with stomach, small intestine, liver, kidney, spleen, and MPB samples from mice infected with *Vp*1A10122, the presence of *V. parahaemolyticus* was ambiguous due to the proliferation of other bacteria on the plates.

In general infection models (Fig. 4a, Fig. S3, and Table 5), TCBS plates spread with stomach samples from mice infected with *Vp*1474; small intestine samples from mice infected with *Vp*1474 and *Vp*1496; liver, kidney, and spleen samples from mice infected with all experimental foodborne strains; and kidney samples from mice infected with *Vp* ATCC 17802 developed distinct *V. parahaemolyticus* colonies. In addition, TCBS plates spread with small intestine samples from mice infected with *Vp*1A10122, liver samples from mice infected with *Vp* ATCC 17802, and spleen samples from mice infected with *Vp*1A10122, the development of *V. parahaemolyticus* was ambiguous as other bacteria competitively proliferated on the plates. No *V. parahaemolyticus* colony developed on the plates spread with stomach samples from mice infected with *Vp*1496, *Vp*1513, *Vp*1A10122, and *Vp* ATCC 17802; small intestine samples from mice infected with *Vp* 1513 and *Vp* ATCC 17802; liver and kidney samples from mice infected with *Vp* 1A10122; and spleen samples from mice infected with *Vp* ATCC 17802.

Table 4 Hemolysin features of *V. parahaemolyticus* strains

Hemolysin gene expression	Strain ID
tdh^+, trh^-, tlh^+	*Vp* 1470, *Vp* 1474, *Vp* 1507, *Vp* 4215
tdh^-, trh^+, tlh^+	*Vp* 1511, *Vp* 1513, *Vp* 4213, *Vp* 11577, *Vp* ATCC 17802
tdh^-, trh^-, tlh^+	*Vp* 1463, *Vp* 1464, *Vp* 1465, *Vp* 1466, *Vp* 1467, *Vp* 1468, *Vp* 1469, *Vp* 1471, *Vp* 1472, *Vp* 1473, *Vp* 1475, *Vp* 1476, *Vp* 1477, *Vp* 1478, *Vp* 1479, *Vp* 1480, *Vp* 1481, *Vp* 1520, *Vp* 1482, *Vp* 1483, *Vp* 1484, *Vp* 1485, *Vp* 1486, *Vp* 1487, *Vp* 1488, *Vp* 1489, *Vp* 1490, *Vp* 1491, *Vp* 1492, *Vp* 1493, *Vp* 1494, *Vp* 1495, *Vp* 1496, *Vp* 1497, *Vp* 1498, *Vp* 1499, *Vp* 1500, *Vp* 1525, *Vp* 1501, *Vp* 1502, *Vp* 1503, *Vp* 1504, *Vp* 1505, *Vp* 1506, *Vp* 1508, *Vp* 1509, *Vp* 1510, *Vp* 1512, *Vp* 1514, *Vp* 1515, *Vp* 1516, *Vp* 1517, *Vp* 1518, *Vp* 1519, *Vp* 1521, *Vp* 1522, *Vp* 1523, *Vp* 1524, *Vp* 1526, *Vp* 1527, *Vp* 1528, *Vp* 1A10122
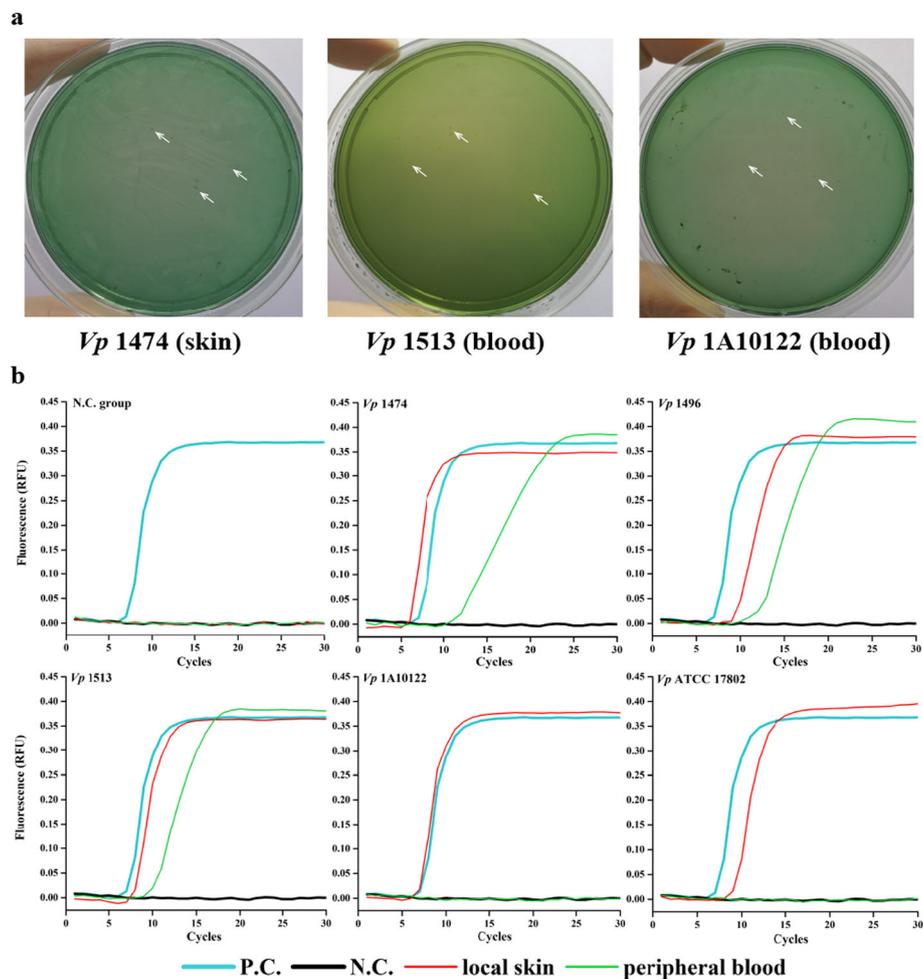
Fig. 1 *V. parahaemolyticus* detection in the localized infection mouse models. **a** The representative of *V. parahaemolyticus* proliferation identified on the specific TCBS plates from skin and MPB of localized infected mice. **b** The representative of *V. parahaemolyticus* detection using rapid LAMP assay from skin and MPB of localized infected mice. White arrowhead, representative sparse colonies of *V. parahaemolyticus*; P.C., positive control using plasmid containing target gene of *V. parahaemolyticus* as the template of LAMP reaction; N.C., negative control using ddH$_2$O as the template of LAMP reaction; N.C. group, mice injected with PBS as the negative control of localized infection mouse models

MPB samples from mice in the wound infection model were also directly plated for the detection of *V. parahaemolyticus* (Fig. 5a, Table 5). The results indicated that *V. parahaemolyticus* developed on the plates spread with MPB samples from mice infected with *Vp*1496, *Vp*1513, *Vp*1A10122, and *Vp* ATCC 17802, while MPB samples from mice infected with *Vp*1474 were interfered by other bacteria.

LAMP detection of *V. parahaemolyticus*
Results of the LAMP assay demonstrated that after *V. parahaemolyticus* infection of mice through localized inflammation (Fig. 1b, Table 5), the skin from mice infected with all experimental groups, in addition to MPB from mice infected with all experimental foodborne strains, exhibited "S"-shaped curves, confirming the presence of *V. parahaemolyticus*.

LAMP assay results of *V. parahaemolyticus* digestive system infection (Fig. 2b, Table 5) indicated that the stomach of mice infected with *Vp*1496, *Vp*1513, and *Vp* ATCC 17802 exhibited "S"-shaped curves, as did the small intestine from mice infected with all experimental foodborne strains and *Vp*1A10122; the liver from mice infected with *Vp*1496, *Vp*1513, and *Vp* ATCC 17802; the kidney and spleen from mice infected with *Vp* 1513, in addition to the MPB from mice infected with *Vp* 1496.

The LAMP assay indicated that, after infection with *V. parahaemolyticus* through wounds (Fig. 3b, Table 5), the MPB from mice infected with all experimental foodborne strains, the marine isolate, and type strain exhibited "S"-shaped curves. In addition, the stomach, small intestine, liver, kidney, and spleen samples from mice in all experimental groups were positive for *V. parahaemolyticus*.

Li *et al. Annals of Microbiology*   (2020) 70:40

Page 7 of 15

Table 5 Results interpretation for *V. parahaemolyticus* infection in target organs of mice

Strain ID	Injection mode							
	i.d.		i.g.		i.v.		i.p.	
	Proliferation	LAMP	Proliferation	LAMP	Proliferation	LAMP	Proliferation	LAMP
Vp 1474	+[a] +/−[b] N/A[c, d, e, f, g, *]	+[a, b] N/A[c, d, e, f, g, *]	+[c, d, e, f] −[b, g] N/A[a, *]	+[d] −[b, c, e, f, g, *] N/A[a]	+[b, c, d, e, f, g] +/−[*] N/A[a]	+[b, c, d, e, f, g, *] N/A[a]	+[c, d, e, f, g] N/A[a, b, *]	+[c, d, e, f, g] N/A[a, b, *]
Vp 1496	+/−[a, b] N/A[c, d, e, f, g, *]	+[a, b] N/A[c, d, e, f, g, *]	+[b, d] +/−[e] −[c, f, g] N/A[a, *]	+[b, c, d, e, *] −[f, g] N/A[a]	+[b, c, d, e, f, g, *] N/A[a]	+[b, c, d, e, f, g, *] N/A[a]	+[d, e, f, g] −[c] N/A[a, b, *]	+[c, d, e, f, g] N/A[a, b, *]
Vp 1513	+[b] +/−[a] N/A[c, d, e, f, g, *]	+[a, b] N/A[c, d, e, f, g, *]	+[d, e, f, g] +/−[b] −[c] N/A[a, *]	+[c, d, e, f, g] −[b, *] N/A[a]	+[b, c, d, e, f, g, *] N/A[a]	+[b, c, d, e, f, g, *] N/A[a]	+[e, f, g] −[c, d] N/A[a, b, *]	+[c, d, e, f, g] N/A[a, b, *]
Vp 1A10122	+[b] −[a] N/A[c, d, e, f, g, *]	+[a] −[b] N/A[c, d, e, f, g, *]	+[f] +/−[c, d, e] −[b, g] N/A[a, *]	+[d] −[b, c, e, f, g, *] N/A[a]	+[*] +/−[b, c, d, e, f, g]	+[b, c, d, e, f, g, *] N/A[a]	+/−[d, g] −[c, e, f] N/A[a, b, *]	+[d, g] −[c, e, f] N/A[a, b, *]
Vp ATCC 17802	+/−[b] −[a] N/A[c, d, e, f, g, *]	+[a] −[b] N/A[c, d, e, f, g, *]	+[c, f, g] +/−[b] −[d, e] N/A[a, *]	+[c, e] −[b, d, f, g, *] N/A[a]	+[b, c, d, e, f, g, *] N/A[a]	+[b, c, d, e, f, g] −[*] N/A[a]	+[f] +/−[e] −[c, d, g] N/A[a, b, *]	+[d, g] −[c, e, f] N/A[a, b, *]

N/A not applicable
[a]Local skin; [b]MPB; [c]stomach; [d]small intestine; [e]liver; [f]kidney; [g]spleen; [*]directly detection from MPB without proliferation; +, V. parahaemolyticus proliferation observed on the plate or LAMP positive; +/−, bacteria unidentified proliferation observed on the plate; −, no bacteria proliferation observed on the plate or LAMP negative

The LAMP assay indicated that the major organs from mice infected with all experimental foodborne strains exhibited "S"-shaped curves when mice were infected with *V. parahaemolyticus* through general infection (Fig. 4b, Table 5), while the small intestine and spleen were the most sensitive target organs in mice infected with the marine strain *Vp* 1A10122 or type strain *Vp* ATCC 17802.

The LAMP assay also demonstrated that *Vp*1496 could be successfully identified even in fresh MPB without proliferation from mice in the foodborne infection model (Fig. 5b, Table 5). When mice were infected with *V. parahaemolyticus* in the wound model, the LAMP assay successfully detected most experimental strains, including *Vp*1474, *Vp*1496, *Vp*1513, and *Vp*1A10122.

Discussion

Seafoods contaminated with *V. parahaemolyticus*, a species of seafood-derived *Vibrio*, are common in coastal cities. Combined with the high rates of exposure to marine or brackish environments, the abundance of *V. parahaemolyticus* results in many aquatic-derived human infections. Because its symptoms of infection are similar to those of other bacteria, such as *Escherichia coli* and *Salmonella* spp. (Park et al. 2018), little attention is generally paid to *V. parahaemolyticus*. Therefore, studies on the epidemiology of *V. parahaemolyticus* are limited, although there are considerably greater numbers of vulnerable populations than reported internationally.

Before the 1960s, *V. parahaemolyticus* outbreaks were limited to Japan (Fujino et al. 1953), but the very first large outbreak of *V. parahaemolyticus* was reported in Maryland, USA early in 1971, due to the ingestion of contaminated crab meat (Center for Food Safety and Applied Nutrition (CFSAN) 2000). From then on, *V. parahaemolyticus* infection has become a global problem according to data from the Center for Disease Control and Prevention (CDC) (CDC 1998), including areas around the Pacific, Atlantic, and Indian oceans.

Virulence specificity vs. *V. parahaemolyticus* infection

When *V. parahaemolyticus* invades a host, multiple toxins are released, and these accelerate deterioration of the site of infection. TDH, TRH, and TLH are the three major toxins produced during *V. parahaemolyticus* proliferation (DePaola et al. 2003). Among these, TDH and TRH are reported to be the two main pathogenic factors (Honda et al. 1988; Nishibuchi and Kaper 1995).

TDH is the primary toxin produced by *V. parahaemolyticus* and is responsible for damage to erythrocytes and it also exhibits multi-cell type cytotoxicity. Sequence variations of the *tdh* gene that encodes for TDH exist in *V. parahaemolyticus* strains. The *tdh* gene family has formed as a result of phylogenetic evolution and gene mutation (Tsunasawa et al. 1987). TRH was purified and characterized in 1988 as a *V. parahaemolyticus* hemolysin related to TDH by Honda et al. (Honda et al. 1988) also identified in 1989 by Nishibuchi et al. (Nishibuchi
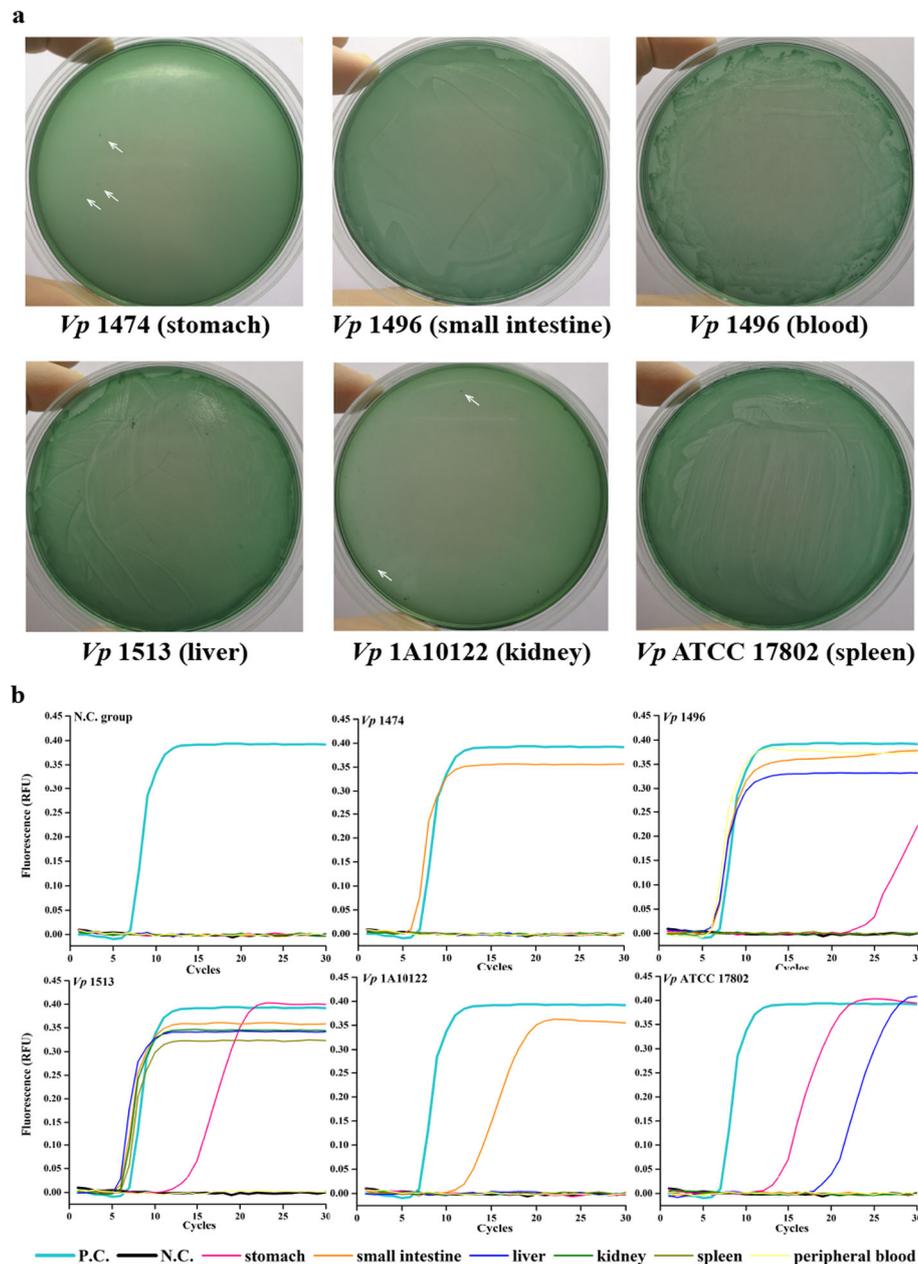
Fig. 2 *V. parahaemolyticus* detection in the foodborne infection mouse models. **a** The representative of *V. parahaemolyticus* proliferation identified on the specific TCBS plates from the stomach, small intestine, liver, kidney, spleen, and MPB of foodborne infected mice. **b** The representative of *V. parahaemolyticus* detection using rapid LAMP assay from the stomach, small intestine, liver, kidney, spleen, and MPB of foodborne-infected mice. White arrowhead, representative sparse colonies of *V. parahaemolyticus*; P.C., positive control using plasmid-containing target gene of *V. parahaemolyticus* as the template of LAMP reaction; N.C., negative control using ddH$_2$O as the template of LAMP reaction; N.C. group, mice injected with PBS as the negative control of foodborne infection mouse models

et al. 1989). TRH is another important hemolysin and is a recognized virulence factor. It is encoded by the *trh* gene, which is closely related to the *tdh* gene, having up to 68% sequence homology (Bej et al. 1999). Therefore, TRH has the same intestinal toxicity but a different hemocytocatheresis as TDH (Park et al. 2004), also often detected in clinical strains of *V. parahaemolyticus*.

TLH is a separate virulence protein and encoded by the *tlh* gene. It does not produce hemolysis on Wagatsuma agar and is not responsible for human infection by *V. parahaemolyticus tdh$^+$* or *trh$^+$* isolates (McCarthy et al. 1999). Different from the *tdh* and *trh* gene families, *tlh* is a single gene and exists in all subtypes of *V. parahaemolyticus* including clinical and environmental
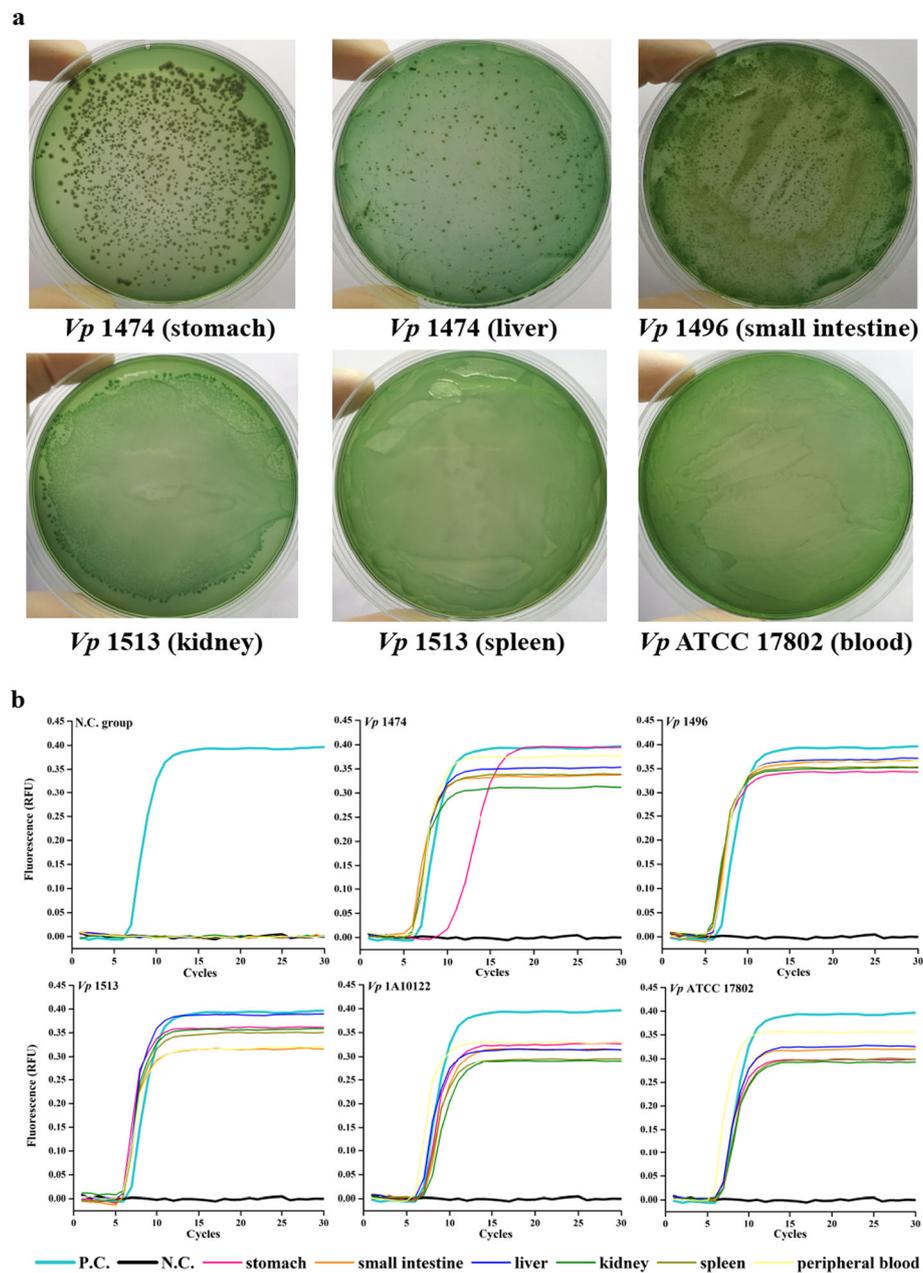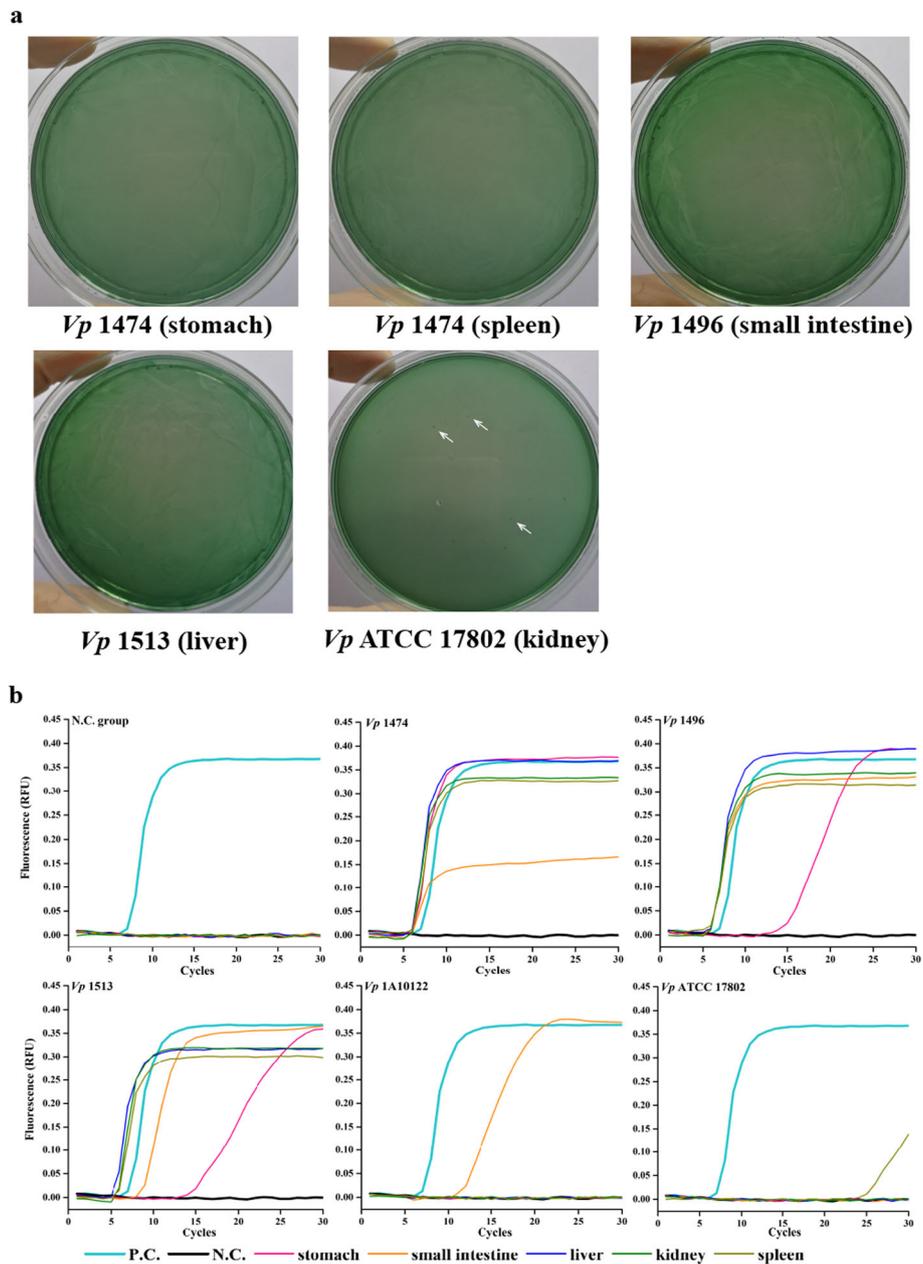
Fig. 3 *V. parahaemolyticus* detection in the wound infection mouse models. **a** The representative of *V. parahaemolyticus* proliferation identified on the specific TCBS plates from the stomach, small intestine, liver, kidney, spleen, and MPB of wound-infected mice; **b** The representative of *V. parahaemolyticus* detection using rapid LAMP assay from the stomach, small intestine, liver, kidney, spleen, and MPB of wound-infected mice. P.C., positive control using plasmid-containing target gene of *V. parahaemolyticus* as the template of LAMP reaction; N.C., negative control using ddH$_2$O as the template of LAMP reaction; N.C. group, mice injected with PBS as the negative control of wound infection mouse models

isolates (Taniguchi et al. 1990). Therefore, TLH is a *V. parahaemolyticus*-specific virulence factor (Taniguchi et al. 1985; Taniguchi et al. 1990). However, the mechanism of action of TLH remains unclear.

Although TDH and TRH are the two major virulence factors of *V. parahaemolyticus*, the Kanagawa reaction is not necessarily linked to these two factors (Tada et al. 1992). Bej et al. confirmed that *tdh*⁻ and *trh*⁻ *V.*

parahaemolyticus still cause a positive Kanagawa reaction. Therefore, the clinical result of that specific assay (Kanagawa) is not 100% correct for the identification of *V. parahaemolyticus* (Ottaviani et al. 2012). Thus, in our study, we detected expression of all 3 types of hemolysin produced by *V. parahaemolyticus*. The hemolysin assay indicated that all 71 *V. parahaemolyticus* strains produced TLH, consistent with previous studies. Of these

Li *et al. Annals of Microbiology*   (2020) 70:40

Page 10 of 15

Fig. 4 *V. parahaemolyticus* detection in the general infection mouse models. **a** The representative of *V. parahaemolyticus* proliferation identified on the specific TCBS plates from the stomach, small intestine, liver, kidney, and spleen of general infected mice; **b** The representative of *V. parahaemolyticus* detection using rapid LAMP assay from the stomach, small intestine, liver, kidney, and spleen of general infected mice. White arrowhead, representative sparse colonies of *V. parahaemolyticus*; P.C., positive control using plasmid-containing target gene of *V. parahaemolyticus* as the template of LAMP reaction; N.C., negative control using ddH₂O as the template of LAMP reaction; N.C. group, mice injected with PBS as the negative control of general infection mouse models

strains, 3 foodborne *V. parahaemolyticus* isolates had genes for the expression of TDH, TRH, and TLH. To ensure all *V. parahaemolyticus* subtypes from different sources were included in the animal experiments, 5 strains (considering both the source and virulence factors) were chosen to comprehensively represent *V. parahaemolyticus* lineages.

In previous studies, Tsai et al. suggested that there were fewer than 5% prevalent *V. parahaemolyticus* strains in environmental or marine food isolates (Tsai et al. 2013), and so, it was hypothesized that the pathogenicity of *V. parahaemolyticus* environmental isolates was weaker than clinical isolates. However, the continuously increasing proportion of pathogenic *V. parahaemolyticus* has drawn

Li *et al. Annals of Microbiology*   (2020) 70:40

Page 11 of 15
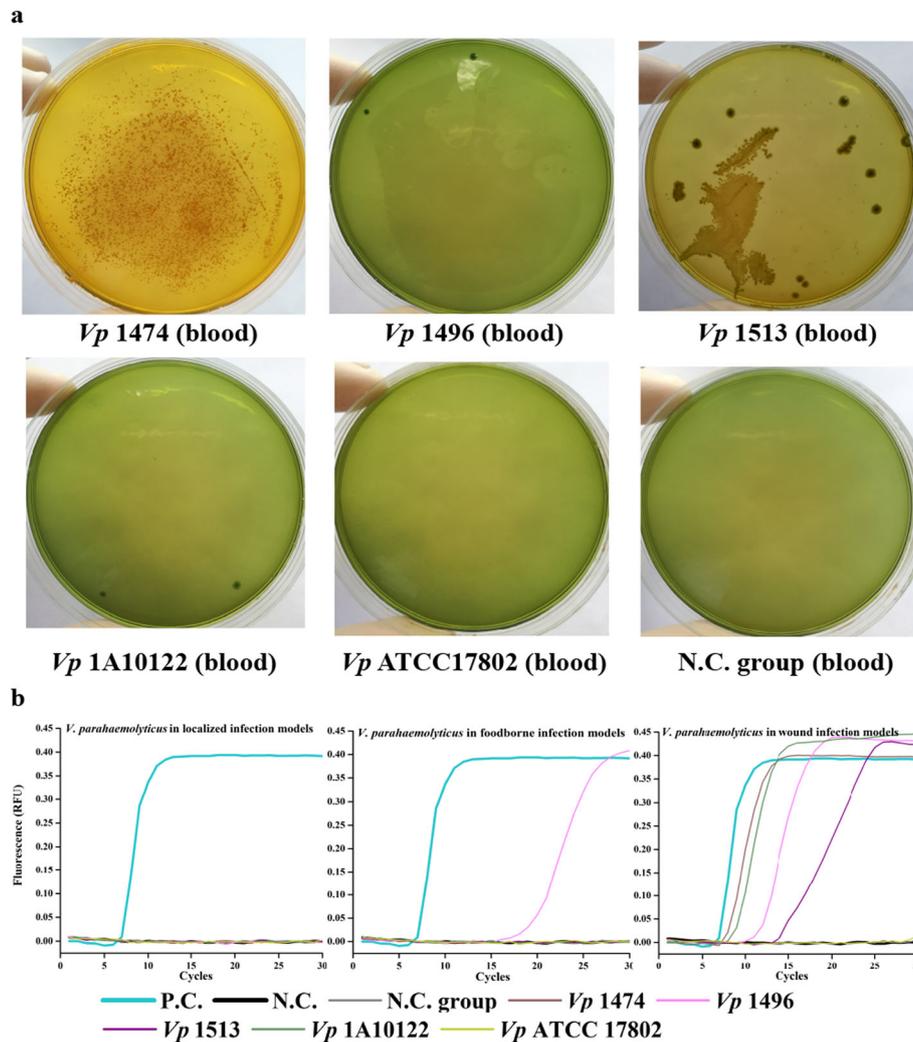
Fig. 5 *V. parahaemolyticus* direct detection from fresh MPB of infected mice via different infection routes. **a** The representative of *V. parahaemolyticus* proliferation identified on the specific TCBS plates from fresh MPB of wound-infected mice; **b** The representative of *V. parahaemolyticus* detection using rapid LAMP assay from fresh MPB of localized, foodborne-derived, and wound infection mice. P.C., positive control using plasmid containing target gene of *V. parahaemolyticus* as the template of LAMP reaction; N.C., negative control using ddH₂O as the template of LAMP reaction; N.C. group, mice injected with PBS as the negative control of general infection mouse models

attention to food safety. Up to the year 2000, a maximum of 6% of *V. parahaemolyticus* environmental isolates were reported to express *tdh/trh* in Europe and Asia (Letchumanan et al. 2014), leaping to more than 8% (*tdh*[+]) and 12% (*trh*[+]) (Yang et al. 2017b). In the present study, we co-incidently found that 5.88% and 7.04% of our tested *V. parahaemolyticus* strains expressed *tdh/trh* genes. In the future, this significantly increasing trend is very likely to be a factor influencing the outbreak of human infections caused by *V. parahaemolyticus*.

Source specificity vs. *V. parahaemolyticus* infection

Apart from virulence gene expression, there is a close relationship between the source of a *Vibrio* isolate and the severity of any resulting infection. Baker-Austin et al.

reviewed the differences in dissemination pathways and infection gradients due to *Vibrio* source diversity (Baker-Austin et al. 2018), which also have different infection risks. The most important source of *V. parahaemolyticus* in human infection is marine food products (Zhang et al. 2017). *V. parahaemolyticus* is a pathogen that exists in multiple water sources. Despite its lack of pathogenicity in fresh water, several studies have confirmed human infection by *V. parahaemolyticus* isolated from sea water. Another infection route that is easily ignored is the cross contamination of cooked foods transferred to humans through food or wounds (Brennan-Krohn et al. 2016).

The correlation between the source of *Vibrio* and type of infection may be reflected in genotype (Jones and

Li *et al. Annals of Microbiology*   (2020) 70:40

Page 12 of 15

Oliver 2009), which has previously been reported by Rosche et al. for *V. vulnificus* (Rosche et al. 2005). In addition, their infection data concluded that 90% of C-genotype *V. vulnificus* strains was clinical isolates while 85~90% of E-genotype strains were environmental isolates (Rosche et al. 2005). This data on strain heterogeneity has reinforced the correlation between *Vibrio* genotype and pathogenicity. The information described above raises a warning for the optimization of *Vibrio* source identification and the updating of related data. The *Vibrio* source can be confirmed by whole genome sequencing (Orata et al. 2014), therefore an understanding of the *Vibrio* source may provide an accurate reference to answer future research questions.

For *V. parahaemolyticus*, different sources typical of specific serotypes can lead to particular outbreaks of human infections. The *V. parahaemolyticus* strains responsible for the majority of outbreaks in the USA have different serotypes from those responsible for outbreaks in Japan. Although a *V. parahaemolyticus* outbreak usually happens within a limited region caused by strains with similar genotypes, a close relationship between strain heterogeneity and pathogenicity remains (Baker-Austin et al. 2018), e.g., the pandemic O3:K6 *V. parahaemolyticus* strain is generally present in the Bay of Bangladesh region (Nair et al. 2007). However, the mechanism by which a bacterial strain source affects a *Vibrio* outbreak is still not well understood.

Comparative research on pathogenic *V. parahaemolyticus* strains has shown that there is a diversity of pathogenicity when clinical *V. parahaemolyticus* are isolated from different areas of water. Considering both foodborne infections and regional proliferation of *V. parahaemolyticus*, the *V. parahaemolyticus* strains were from both foodborne and seawater isolates, with ATCC type strain as the standard for the experiments. Foodborne *V. parahaemolyticus* strains were all isolated from domestic consumption of seafood, including seashells, crabs, and oysters, and the seawater strain was isolated from the south China sea. The results obtained in our study mirror the *V. parahaemolyticus* infections seen in China and our LAMP assay provides a possible solution for the rapid detection of *V. parahaemolyticus* in current circumstances.

LAMP detection sensitivity

Based on Nucleic acid-PCR technology, the greatest advantage of the LAMP assay lies in its high sensitivity and rapid speed of amplification. With specific pairs of primers, the LAMP assay minimized the template to 2 CFUs at most during its reaction, while other PCR methods require $10~10^3$-fold additional templates compared with the LAMP assay (Law et al. 2015). Other than these advantages, the LAMP assay is simple to operate and is of low cost. Thus, the LAMP assay has fewer disadvantages than other methods.

Among the different routes of infection, *V. parahaemolyticus* wound infections were the most easily detected by the LAMP assay. Wound infections caused by *V. parahaemolyticus* are not common, but they are serious. Although there are few such reports, it can be life-threatening. In this study, we demonstrated that early stages of *V. parahaemolyticus* infection can easily be detected using a LAMP assay with visual interpretation of the results.

Data using the LAMP assay to detect *V. parahaemolyticus* strains in various organs indicated that foodborne strains were most sensitive to the assay, and *V. parahaemolyticus* was detected in all organs tested in localized, wound, and general infection models. Although *V. parahaemolyticus* could not be detected in all mice in the foodborne infection model, bacteria that spread to the small intestine could be detected. Our results indicated that the small intestine was a good target organ to detect foodborne *V. parahaemolyticus* infection. On the other hand, combined with different virulence values for each isolate, infectivity varied. In the foodborne infection model, the stomach was the first organ to encounter *V. parahaemolyticus*. The LAMP assay, on the contrary showed false positive results. In all tested groups, *Vp* 1513 was detected with the highest efficiency, thereby demonstrating its high-level infectivity. In combination with the results of virulence gene expression, our findings demonstrated that the LAMP assay responded differently to various *V. parahaemolyticus* strains.

When using the LAMP assay to detect *V. parahaemolyticus* in different organs, the sensitivity of the LAMP assay varied in the different infection models. The results implied that the infection route affected how *V. parahaemolyticus* invaded the host, and this confirms the concern and suggestion of Baker-Austin et al., who highlighted in their research the importance of epidemiology data updates, such as source of *Vibrio* and route of transmission (Baker-Austin et al. 2018). Combined with the LAMP results of different routes to infection, our results offer a personalized method for tracing back to the original explanation for human infection by *V. parahaemolyticus*.

Conclusions and future perspectives

Based on previous reports, only a limited number of *V. parahaemolyticus* infection cases resulted in death. Despite this, *V. parahaemolyticus* can still cause human infections in a wide range of populations. The increasing antibiotic resistance of pathogenic bacteria has encouraged the marine culture industry to raise the concentrations of antibiotics added to sea water (Letchumanan et al. 2014), causing inevitable antibiotic tolerance in *V. parahaemolyticus*, especially to carbapenems resistance

(Lee et al. 2018). This situation indirectly leads to the excessive proliferation of *V. parahaemolyticus*. Therefore, conducting early screening of potential susceptible populations plays a role in pre-warning for the limited prevention measure of its infection.

Therefore, in the early stages of *V. parahaemolyticus* infection, non-invasive and rapid detection of the bacteria is essential to prevent it from spreading further. Under this circumstance, our LAMP results demonstrated a possible method to directly detect *V. parahaemolyticus* in peripheral blood (PB). In this study, although the results showed different sensitivity when the LAMP assay was applied to specific infection models, the assay still has potential. When using the LAMP assay in the general infection model, positive results were obtained from all target organs, indicating that the LAMP assay could be used for *V. parahaemolyticus* screening.

Thus, the LAMP assay makes it possible to directly detect *V. parahaemolyticus* without the need for ex vivo studies. Although there are some drawbacks to the application of this method, the rapid detection speed, intuitive results, and high efficiency are promising for further investigation for use in clinical cases. In addition, optimization of the LAMP assay requires further improvement.

Supplementary information

Supplementary information accompanies this paper at https://doi.org/10.1186/s13213-020-01585-6.

Additional file 1: Fig. S1. *V. parahaemolyticus* identification on TCBS plates in the foodborne infection mouse models. The representative of *V. parahaemolyticus* development on the specific TCBS plates from stomach, small intestine, liver, kidney and spleen of foodborne infected mice; white arrowhead, representative sparse colonies of *V. parahaemolyticus*.

Additional file 2: Fig. S2. *V. parahaemolyticus* identification on TCBS plates in the wound infection mouse models.The representative of *V. parahaemolyticus* development on the specific TCBS plates from stomach, small intestine, liver, kidney, spleen and MPB of wound infected mice..

Additional file 3: Fig. S3. *V. parahaemolyticus* identification on TCBS plates in the general infection mouse models. The representative of *V. parahaemolyticus* development on the specific TCBS plates from small intestine, liver, kidney and spleen of general infected mice.

Abbreviations

G⁻: Gram-negative; NFDOSS: National Foodborne Disease Outbreaks Surveillance System; NASBA: Nuclear acid sequence-based amplification; FISH: Fluorescence in situ hybridization; LAMP: Loop-mediated isothermal amplification; TCBS: Thiosulfate citrate bile salts sucrose; SPF: Specific-pathogen-free; i.d.: Intradermal injection; i.g.: Intragastric gavage; i.v.: Intravenous injection; i.p.: Intraperitoneal injection; MPB: Mouse peripheral blood; ACD: Acid citrate-dextrose; ANOVA: One-way analysis of variance; SEM: Standard error of the mean; CDC: Center for Disease Control and Prevention; PB: Peripheral blood

Authors' contributions

SZ and LjZ conceived this study; KfF and LjZ designed the research; JL, JfL and KwQ performed the experiments; JfL and CIW analyzed experimental data; JL, JfL, KfF, and XjY wrote the paper. JL and JfL contributed equally to this work. All authors discussed the results, agreed on the interpretation and contributed in the finalization of the manuscript.

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Ethics approval and consent to participate

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. All animal procedures complied with the institutional and national guidelines prescribed by the International Council for Laboratory Animal Science (ICLAS) from the Ministry of Health of the People's Republic of China. All procedures performed in studies involving animals were in accordance with the ethical standards of Animal Ethics Committee of Navy General Hospital of Chinese PLA at which the studies were conducted.

Consent for publication

N/A.

Competing interests

The authors declare that they have no competing interests.

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Li *et al. Annals of Microbiology*   (2020) 70:40

Page 15 of 15

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