Table 3 Isolates of clinical and environmental *V. parahaemolyticus* (Vp) that were susceptible to bacteriophages

Vp strains	Serotypes	Virulence genes		Numbers of bacteriophage that form plaques on Vp strains				Total
		tdh	*trh*	Phages isolated on indicated Vp hosts[a]				
				VP2598	VP3622	VP4118	VP4211	
Clinical Vp								
PSU4251	O3:K6	+	−	8	7	8	8	31
PSU4286	O5:K15	+	+	1	8	4	0	13
PSU4295	O11:K36	−	−	1	2	4	5	12
PSU4325	O3:K6	+	−	8	4	8	8	28
PSU4341	O3:K6	+	−	7	7	7	8	29
PSU4371	O3:K6	+	−	8	6	8	5	27
PSU4388	O3:K6	+	−	8	6	8	8	30
PSU4395	O3:K6	+	−	8	5	8	8	29
PSU4408	O3:K6	+	−	8	6	8	8	30
PSU4472	O1:K20	+	−	8	4	8	6	30
PSU4473	O10:KUT	−	−	1	8	1	1	11
PSU4483	O3:K6	+	−	8	8	7	7	30
PSU4517	O3:K6	+	−	8	6	7	8	29
PSU4532	O4:K13	+	−	8	2	7	3	20
PSU4538	O4:K8	−	−	8	3	5	8	24
PSU4554	O10:KUT	−	+	6	7	1	4	18
PSU4585	O3:K6	+	−	8	8	7	8	31
PSU4605	O8:K22	+	−	0	2	1	0	3
Total								425
Environmental Vp								
PSU4815	O10:KUT	−	−	5	0	5	6	16
PSU4816	O10:KUT	−	−	2	2	6	6	16
PSU4817	O1:KUT	−	−	2	5	0	5	12
PSU4818	O4:KUT	−	−	8	4	5	8	25
PSU4819	O5:KUT	−	−	4	2	3	7	16
PSU4820	O3:KUT	−	−	1	0	2	5	8
PSU4821	O3:KUT	−	−	0	0	0	5	5
PSU4822	O10:KUT	−	−	8	5	4	0	17
PSU4823	O1:KUT	−	−	5	1	3	0	9
PSU4824	O3:KUT	−	−	0	0	0	8	8
PSU4825	O10:KUT	−	−	7	5	3	0	15
PSU4826	O2:KUT	−	−	0	0	0	0	0
PSU4827	O5:KUT	−	−	2	6	3	4	15
PSU4828	O4:KUT	−	−	5	3	4	8	20
PSU4829	O4:KUT	−	−	5	2	5	8	20
PSU4830	O4:KUT	−	−	8	5	8	1	22
PSU4831	O3:K37	−	−	0	0	2	8	10
PSU4832	O5:KUT	−	−	7	4	7	0	18
Total								252

[a] Eight bacteriophages from different samples were propagated with their specific hosts and investigated for their host range specificity

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Serotypes and virulence genes of those clinical and environmental *V. parahaemolyticus* isolates were determined for their correlation with bacteriophage susceptibility. In addition, the host range of bacteriophages that could be propagated on these three pandemic indicator strains was also investigated with ten clinical isolates of pandemic *V. parahaemolyticus* that

Table 4 Susceptibility of *Vibrio* spp. and enteric bacteria to *V. parahaemolyticus* bacteriophages

Bacteria	Numbers of bacteriophage that form plaques on the indicated host bacteria				Total
	Phages derived from				
	VP2598	VP3622	VP4118	VP4211	
V. cholerae O1	0	0	0	0	0
V. cholerae O139	0	0	0	0	0
V. cholerae nonO1	0	0	0	0	0
V. alginolyticus	7	8	2	8	25
V. mimicus	6	6	8	6	26
V. vulnificus	0	0	0	0	0
S. flexneri	0	0	0	0	0
S. typhi	0	0	0	0	0
A. hydrophila	0	0	0	0	0
E. coli O157/H7	0	0	0	0	0
Total	13	14	10	14	51

possessed the same serotype. Moreover, bacteriophages were tested against four pathogenic *Vibrio* spp. and four other enteric bacteria including *Shigella flexneri*, *Salmonella typhi*, *Aeromonas hydrophila*, and *Escherichia coli* O157/H7 using the spotting assay as described above.

Serotyping

To determine somatic (O) and capsular (K) serotypes of *V. parahaemolyticus*, the slide-agglutination technique was performed using anti-O and anti-K antibodies (Denka Seiken, Tokyo, Japan). Briefly, for determination of the O serotype, bacterium grown in tryptic soy agar (TSA) containing 3 % NaCl was washed with 3 % NaCl and 5 % glycerol. The suspension was autoclaved for 1 h. The pellet was obtained by centrifugation and resuspended in 3 % NaCl. A heavy bacterial suspension was subjected to the agglutination test with specific anti-O antibodies. For the K antigen, bacterium grown in TSA was washed with 3 % NaCl solution and was tested first with pooled K antisera (I–IX), and then with each of the monovalent K antisera.

Statistical Analysis

Pearson's product–moment correlation was used for statistical analysis.

Results and Discussion

A total of 139 cockle samples ranging from 7 to 22 samples/month were obtained between June 2009 and May 2010 (Table 1). *V. parahaemolyticus* was isolated from all cockle samples. The average number of this bacterium detected was between 5.9×10^3 and 1.2×10^5 MPN/g of cockle (data not shown). Bacteriophages specific to *V. parahaemolyticus* host strains were detected in 76 out of 139 cockle samples (Table 1). The negative samples might contain bacteriophages that were not specific to the tested *V. parahaemolyticus* strains. Throughout the year, we detected bacteriophages that could form plaques on three to ten strains of our *V. parahaemolyticus* indicator strains. An expanded host range and susceptibility to bacteriophages in these strains were observed more often during February to May with an average of 9.2±0.8, than in the rainy season, June to September, with an average of 8.0±2.3, and October to January with an average of 6.2±2.2. However, we did not observe any strong correlations between the presence of *V. parahaemolyticus* and its bacteriophages in cockles between seasons. This lack of seasonality may be due to the average temperature in the 2 years during which the area was investigation showed little change (28.0 and 28.2 °C in 2009 and 2010, respectively) (www.songkhla.tmd.co.th). The most susceptible serotype for *V. parahaemolyticus* bacteriophages was O3/K6 (Table 2). This is not surprising because it is the most prevalent pandemic serotype continuously isolated from patients in this area ([32]; unpublished data).

In the southern part of Thailand, cockle is a very popular raw seafood item and is frequently contaminated with *V. parahaemolyticus* [33]. We sought to determine if there was any correlation between the frequency of patients infected with *V. parahaemolyticus* and the presence of this bacterium and its bacteriophages in cockles from the same local area. In this study, 97 isolates of *V. parahaemolyticus* were obtained from patients in the Hat Yai hospital during December 2009 and February 2010 (Fig. 1). The following four different categories of clinical isolates based on PCR analysis were: (1) tdh^+ trh^-, (2) tdh^+ trh^+, (3) tdh^- trh^-, and (4) tdh^- trh^+. PCR types 1–4 were identified in 62, 3, 13, and 2 of the isolates, respectively. Seventeen isolates were not examined for virulence genes because of their death during transport to the laboratory. The number of patients infected locally significantly coincided at the 95 % confidence level (p value=0.02; R^2= 0.40) with the increase in the number of *V. parahaemolyticus* in the cockle extracts (Figs. 2 and 3a). Correlation between the decrease in the numbers of *V. parahaemolyticus* bacteriophage in the cockle filtrates and an increase in the level of *V. parahaemolyticus* in the cockle extracts was detected in some months but there was no significant correlation obtained over the whole period of the study time (p value=0.07; R^2= 0.27) (Fig. 3b). The low numbers of bacteriophage in the cockle filtrates seemed to correlate with an increase in the number of *V. parahaemolyticus* in some months (Jun–Aug 2009 and Oct–Dec 2009) (Fig. 2). An increase in the numbers of bacteriophage appeared to follow the high numbers of *V. parahaemolyticus* in the former period of time (Dec

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2009–Feb 2010). However, this inverse correlation was not consistent perhaps because some *V. parahaemolyticus* might develop resistance to the local phages as has been seen with *Listeria* spp. co-cultured with listeriophages [29]. In addition, Middelboe and colleagues [16] demonstrated the temporary effects on dynamics and diversity of the individual bacterial host species after interaction with their specific phages, because phage-resistant bacteria were present after the first lysis of hosts. Thus, we conclude that the levels of *V. parahaemolyticus* present in cockle are useful to assess the relative risk of cockles as a source of *V. parahaemolyticus* infection in southern Thailand.

Bacteriophages obtained in this study were tested against 18 clinical and 18 environmental *V. parahaemolyticus* strains. They were more active against clinical *V. parahaemolyticus* than environmental strains (Table 3). This may be due to the clinical isolates of *V. parahaemolyticus* being used as hosts for screening them. Those clinical and environmental strains were investigated for serotypes, *tdh* and *trh* virulence genes to determine their correlation with phage susceptibility. Most of the clinical isolates possessed either *tdh* or *trh* gene, but none of the environmental isolates harbored those genes. In addition, most of the environmental isolates were K untypable (KUT) because O/K typing scheme has been established using the clinical strains. Although the most susceptible bacterial serotype was O3/K6, correlation between serotypes, virulence genes, and bacteriophage profiles was not observed. The reason may be due to all bacteriophages were isolated from environment, thus they might adapt themselves to wide specific host range.

Each set of the eight bacteriophages isolated from the O4/K68, O1/K25, and O3/K6 pandemic strains (PSU2598, PSU4118, and PSU 4211, respectively) were capable of forming plaque on the same serotypes of each set of ten pandemic strains of *V. parahaemolyticus* (data not shown). In addition, seven bacteriophages that were isolated on the O3/K6 pandemic strain PSU 4211 were able to infect different serotypes of pandemic *V. parahaemolyticus* (O4/K68-PSU2598 and O1/K25-PSU4118) (data not shown). In Hat Yai city, around 60 % of patients in one study were infected with these pandemic strains [32]. Thus, these may be useful both as indicators of the presence of the pandemic strain as well as for use as a possible biological control to suppress *V. parahaemolyticus* in food items that are at high risk for contamination by this organism.

In this study, 25 and 26 of the *V. parahaemolyticus* bacteriophages obtained from the three pandemic (PSU 2598, PSU 4118, and PSU4211) and one non-pandemic (PSU3622) *V. parahaemolyticus* hosts were active against *Vibrio alginolyticus* and *Vibrio mimicus*, respectively (Table 4). This would seem to indicate that these three *Vibrio* species might occupy the same ecological niche. None of the bacteriophages active on *V. parahaemolyticus* grew on other enteric pathogens including *V. cholerae*. Therefore, it is possible that they may participate in the processes of genetic exchange between the *V. parahaemolyticus* and those *V. alginolyticus* and *V. mimicus* [8, 10, 23, 31]. Recently, two bacterial isolates obtained from Alaskan oysters were identified as *V. alginolyticus* and expressed a *trh* gene with 98 % homology to the *trh*2 of *V. parahaemolyticus* [9]. In addition, it has been demonstrated that one clinical isolate of *V. mimicus* harbored a *tdh* with a 97 % homology to *tdh*2 of *V. parahaemolyticus* [22].

In conclusion, we have found that the level of bacteriophages present in cockle extracts was not significantly correlated with the incidence of *V. parahaemolyticus* disease rates in infected patients in Hat Yai, Thailand, but the level of the causative organism in mollusk samples did closely correlate and could be used as an indicator for assessment of possibility of infection.

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References

1. Arias CR, Macián MC, Aznar R, Garay E, Pujalte MJ (1999) Low incidence of *Vibrio vulnificus* among *Vibrio* isolates from sea water and shellfish of the western Mediterranean coast. J Appl Microbiol 86:125–134
2. Baross JA, Liston J, Morita RY (1978) Incidence of *Vibrio parahaemolyticus* bacteriophages and other *Vibrio* bacteriophages in marine samples. Appl Environ Microbiol 36:492–499
3. Benson HJ (2002) Isolation of phage from sewage. Microbiological applications, 8th edn. McGraw-Hill Publishing Co., Boston
4. Comeau AM, Buenaventura E, Suttle CA (2005) A persistent, productive, and seasonally dynamic vibriophage population within pacific oysters (*Crassostrea gigas*). Appl Environ Microbiol 71:5324–5331
5. Farmer JJ, Janda JM, Birkhead K (2003) *Vibrio*. In: Murray PR, Baron EJ, Jorgensen JH, Pfaller MA, Yolken RH (eds) Manual of clinical microbiology, 8th edn. ASM Press, Washington, DC, pp 706–718
6. Faruque SM, Naser IB, Islam MJ, Faruque ASG, Ghosh AN, Nair GB, Sack DA, Mekalanos JJ (2005) Seasonal epidemics of cholera inversely correlate with the prevalence of environmental cholera phages. Proc Natl Acad Sci U S A 102:1702–1707
7. Faruque SM, Naser IB, Islam MJ, Faruque ASG, Ghosh AN, Nair GB, Sack DA, Mekalanos JJ (2005) Self-limiting nature of seasonal cholera epidemics: role of host-mediated amplification of phage. Proc Natl Acad Sci U S A 102:6119–6124
8. Faruque SM, Mekalanos JJ (2012) Phage-bacterial interactions in the evolution of toxigenic *Vibrio cholerae*. Virulence 17:556–565
9. González-Escalona N, Blackstone GM, DePaola A (2006) Characterization of a *Vibrio alginolyticus* strain, isolated from Alaskan oysters, carrying a hemolysin gene similar to the thermostable direct hemolysin-related hemolysin gene (*trh*) of *Vibrio parahaemolyticus*. Appl Environ Microbiol 72:7925–7929
10. Groisman EA, Ochman H (1996) Pathogenicity islands: bacterial evolution in quantum leaps. Cell 87:791–794

11. Hennes KP, Suttle CA, Chan AM (1995) Fluorescently labeled virus probes show that natural virus populations can control the structure of marine microbial communities. Appl Environ Microbiol 61:3623–3627
12. Honda S, Goto I, Minematsu I, Ikeda N, Asano N, Ishibashi M, Kinoshita Y, Nishibuchi M, Honda T, Miwatani T (1987) Gastroenteritis due to Kanagawa negative *Vibrio parahaemolyticus*. Lancet i:331–332
13. Jensen MA, Faruque SM, Mekalanos JJ, Levin BR (2006) Modeling the role of bacteriophage in the control of cholera outbreaks. Proc Natl Acad Sci U S A 103:4652–4657
14. Kim YB, Okuda J, Matsumoto C, Takahashi N, Hashimoto S, Nishibuchi M (1999) Identification of *Vibrio parahaemolyticus* strains at the species level by PCR targeted to the *toxR* gene. J Clin Microbiol 37:1173–1177
15. Lin Z, Kumagai K, Baba K, Mekalanos JJ, Nishibuchi M (1993) *Vibrio parahaemolyticus* has a homolog of the *Vibrio cholerae* toxRS operon that mediates environmentally induced regulation of the thermostable direct hemolysin gene. J Bacteriol 175:3844–3855
16. Middelboe M, Hagstrom A, Blackburn N, Sinn B, Fischer U, Borch NH, Pinhassi J, Simu K, Lorenz MG (2001) Effects of bacteriophages on the population dynamics of four strains of pelagic marine bacteria. Microb Ecol 42:395–406
17. Nakanishi H, Lida Y, Maeshima K, Teramoto T, Hosaka Y, Ozaki M (1966) Isolation and properties of bacteriophages of *Vibrio parahaemolyticus*. Biken J 9:149–157
18. Nasu H, Iida T, Sugahara T, Yamaichi Y, Park KS, Yokoyama K, Makino K, Shinagawa H, Honda T (2000) A filamentous phage associated with recent pandemic *Vibrio parahaemolyticus* O3:K6 strains. J Clin Microbiol 38:2156–2161
19. Nelson EJ, Chowdhury A, Harris JB, Begum YA, Chowdhury F, Khan AI, Larocque RC, Bishop AL, Ryan ET, Camilli A, Qadri F, Calderwood SB (2007) Complexity of rice-water stool from patients with *Vibrio cholerae* plays a role in the transmission of infectious diarrhea. Proc Natl Acad Sci U S A 104:19091–19096
20. Nelson EJ, Chowdhury A, Flynn J, Schild S, Bourassa L, Shao Y, LaRocque RC, Calderwood SB, Qadri F, Camilli A (2008) Transmission of *Vibrio cholerae* is antagonized by lytic phage and entry into the aquatic environment. PLoS Pathog 4:e1000187
21. Nelson EJ, Harris JB, Morris JG Jr, Calderwood SB, Camilli A (2009) Cholera transmission: the host, pathogen and bacteriophage dynamic. Nat Rev Microbiol 7:693–702
22. Nishibuchi M, Kaper JB (1995) Thermostable direct hemolysin gene of *Vibrio parahaemolyticus*: a virulence gene acquired by a marine bacterium. Infect Immun 63:2093–2099
23. Park KS, Ono T, Rokuda M, Jang MH, Okada K, Iida T, Honda T (2004) Functional characterization of two type III secretion systems of *Vibrio parahaemolyticus*. Infect Immun 72:6659–6665
24. Tada J, Ohashi T, Nishimura N, Shirasaki Y, Ozaki H, Fukushima S, Takano J, Nishibuchi M, Takeda Y (1992) Detection of thermostable direct hemolysin gene (*tdh*) and the thermostable direct hemolysin-related hemolysin gene (*trh*) of *Vibrio parahaemolyticus* by polymerase chain reaction. Mol Cell Probes 6:477–487
25. Takahashi A, Sato Y, Shiomi Y, Cantarelli VV, Iida T, Lee M, Honda T (2000) Mechanism of chloride secretion induced by thermostable direct hemolysin of *Vibrio parahaemolyticus* in human colonic tissue and intestinal epithelial cell line. J Med Microbiol 49:801–810
26. Takahashi A, Yamamoto C, Kodama T, Yamashita K, Harada N, Nakano M, Honda T, Nakaya Y (2006) Pore formation of thermostable direct hemolysin secreted from *Vibrio parahaemolyticus* in lipid bilayers. Int J Toxicol 25:409–418
27. Takeda Y (1983) Thermostable direct hemolysin of *Vibrio parahaemolyticus*. Pharmacol Ther 19:123–146
28. Twedt R (1989) *Vibrio parahaemolyticus*. In: Doyle MP (ed) Foodborne bacterial pathogens. Marcel Decker Inc., New York, pp 543–568
29. Vongkamjan K, Roof S, Stasiewicz MJ, Wiedmann M (2013) Persistent *Listeria monocytogenes* subtypes isolated from a smoked fish processing facility included both phage susceptible and resistant isolates. Food Microbiol 35:38–48
30. Vuddhakul V, Soboon S, Sunnghiran S, Kaewpiboon S, Chowdhury A, Ishibashi M, Nakaguchi Y, Nishibuchi M (2006) Distribution of virulent and pandemic strains of *Vibrio parahaemolyticus* in three molluscan shellfish species (*Meretrix meretrix*, *Perna viridis*, and *Anadara granosa*) and their association with foodborne disease in southern Thailand. J Food Prot 69:2615–2620
31. Waldor MK, Mekalanos JJ (1996) Lysogenic conversion by a filamentous phage encoding cholera toxin. Science 272:1910–1914
32. Wootipoom N, Bhoopong P, Pomwised R, Nishibuchi M, Ishibashi M, Vuddhakul V (2007) A decrease in propotion of infections by pandemic *Vibrio parahaemolyticus* in Hat Yai Hospital, southern Thailand. J Med Microbiol 56:1630–1638
33. Yamamoto A, Iwahori J, Vuddhakul V, Charernjiratragul W, Vose D, Osaka K, Shigematsu M, Toyofuku H, Yamamoto S, Nishibuchi M, Kasuga F (2008) Quantitative modeling for risk assessment of *Vibrio parahaemolyticus* in bloody clams in southern Thailand. Int J Food Microbiol 124:70–78
34. Zahid MS, Udden SM, Faruque AS, Calderwood SB, Mekalanos JJ, Faruque SM (2008) Effect of phage on the infectivity of *Vibrio cholerae* and emergence of genetic variants. Infect Immun 76:5266–5273

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1 Running head: Easy and sensitive quantification of *tdh*$^+$ *V. parahaemolyticus* in shellfish

2

3 **An Easy and Sensitive Quantification Procedure for *tdh*$^+$ *Vibrio parahaemolyticus* in Molluscan Shellfish**

4 **Using K antigen-specific Immunomagnetic Separation and LAMP**

5

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15 Key words: *Vibrio parahaemolyticus*, molluscan shellfish, most probable number method, immunomagnetic

16 separation, loop-mediated isothermal amplification

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ABSTRACT

Although thermostable direct hemolysin (TDH)-producing *Vibrio parahaemolyticus* is the leading cause of seafood-borne gastroenteritis, due to its low distribution in the environment, the enumeration of *tdh*$^+$ *V. parahaemolyticus* remains challenging. In this study, we developed a most-probable-number (MPN)-based A-IS^1-LAMP procedure, in which an immunomagnetic separation (IMS) technique targeting as many as sixty-nine K antigens and a loop-mediated isothermal amplification (LAMP) assay targeting the *tdh* gene were applied in an MPN format. The ability of the procedure to quantify a wide range of *tdh*$^+$ *V. parahaemolyticus* levels was evaluated by testing shellfish samples in Japan and southern Thailand, where shellfish products are known to be naturally-contaminated with relatively low and high levels of total *V. parahaemolyticus*, respectively. Examination of the Japanese shellfish samples detected *tdh*$^+$ *V. parahaemolyticus* in four out of twenty-one samples with relatively low MPN values (0.3 ~ 11 MPN/g); whereas all of the nine Thai shellfish samples showed considerably higher levels (93 ~ 11,000 MPN/g) of *tdh*$^+$ *V. parahaemolyticus*, raising concern about the safety of Thai shellfish products sold to domestic consumers at local morning markets. LAMP consistently showed higher performance than conventional PCR, especially in testing cultures containing (i) a small amount of *tdh*$^+$ *V. parahaemolyticus* cells, or (ii) large amounts of both total and *tdh*$^+$ *V. parahaemolyticus* cells. IMS was shown to be effective (~thirty-two-fold) in concentrating *tdh*$^+$ *V. parahaemolyticus* from Japanese shellfish samples. The A-IS^1-LAMP procedure offers a practical means for the measurement and management of the *tdh*$^+$ *V. parahaemolyticus* levels in shellfish products for use by any health authority in the world.

Vibrio parahaemolyticus is a marine bacterium native in estuarine environments, and is potentially pathogenic as some strains carry the *tdh* gene encoding the thermostable direct hemolysin (TDH) and/or the *trh* gene encoding TDH-related hemolysin (TRH), which are considered important pathogenicity markers *(16)*. The incidence of *V. parahaemolyticus* infection has increased worldwide since 1996, and this is attributed to the emergence and pandemic spread of a new O3:K6 clone *(12, 19)*. Pathogenic strains can cause gastroenteritis in humans through consumption of contaminated seafood, especially filter-feeding molluscan shellfish as they concentrate microorganisms from the environment in their digestive tracts *(20)*. This has resulted in efforts to develop methodologies to measure and manage pathogenic *V. parahaemolyticus* levels in shellfish products *(6)*. Nevertheless, due to the low distribution of pathogenic *V. parahaemolyticus*, which typically accounts for less than 1% of the total *V. parahaemolyticus* population in the environment *(4, 23, 24)*, the enumeration of pathogenic *V. parahaemolyticus* remains challenging. Today, it is generally accepted there are very limited cases where pathogenic *V. parahaemolyticus* can successfully be enumerated by directly analyzing shellfish

homogenates, and therefore enrichment is necessary. Consequently, various molecular methods for the detection of total and pathogenic *V. parahaemolyticus* have been applied in a most-probable-number (MPN) format *(2, 11, 13)*. One such MPN-based procedure described by Hara-Kudo *et al.* employed conventional PCR in conjunction with a three-step enrichment procedure *(7)*. This MPN-PCR procedure was later applied in a field setting, the bloody clam risk assessment in Hat Yai, southern Thailand, in which total and pathogenic (*tdh*$^+$ and *trh*$^+$) *V. parahaemolyticus* counts in bloody clams were followed from harvest to the retail stage *(26)*. Nevertheless, pathogenic *V. parahaemolyticus* was detected only in a small portion of the bloody clam samples, with low MPN values close to the lower detection limit (0.3 MPN/g), raising a need for a more sensitive procedure.

As more sensitive, specific, time- and labor- saving alternative DNA detection methods compared to conventional PCR, real-time PCR and loop-mediated isothermal amplification (LAMP) have been applied in an MPN format *(3, 8, 15, 17)*. Particularly LAMP has great advantages being applied in field settings: It can be performed in a simple isothermal chamber without trained personnel, and yield robust results which allow visual judgment *(14, 28)*. A lyophilized reagent which no longer requires a cold chain system is another advantage (M. Nishibuchi, unpublished data). Since our ultimate goal is to offer a practical means for the measurement and management of the total and pathogenic *V. parahaemolyticus* levels in shellfish products to be used by any health authority in the world, we focused on LAMP and previously developed a LAMP assay for sensitive and rapid detection of *tdh*$^+$ and *trh*$^+$ *V. parahaemolyticus (28)*. When applied in an MPN format in conjunction with the three-step enrichment procedure, the *tdh*-LAMP assay can be a powerful tool for the enumeration of *tdh*$^+$ *V. parahaemolyticus* in shellfish samples. In contrast, our subsequent studies suggested the *trh*-LAMP assay can be improved by utilizing new primer sets currently being tested, which can tolerate the *trh* gene sequence variations widely observed in *trh*$^+$ environmental strains (M. Nishibuchi, unpublished data). Therefore, while this study does not deal with *trh*$^+$ *V. parahaemolyticus*, this MPN-LAMP format can potentially be expanded to the enumeration of *trh*$^+$ environmental strains.

Our experience with the isolation of O3:K6 pandemic strains from bloody clams with the aid of an immunomagnetic separation (IMS) technique targeting the K antigen, an outermost structure of *V. parahaemolyticus* cells *(23)*, prompted interest in screening clinically-important *V. parahaemolyticus* populations with all of the sixty-nine K serotypes from shellfish samples. This idea is based on the fact that *V. parahaemolyticus* O:K serotypes have been established based on clinical isolates. In this study, we prepared immunomagnetic beads specific for as many as sixty-nine K antigens, and additionally incorporated IMS into the MPN format (designated as the A-IS^1-IS^2-LAMP procedure) by introducing PickPen, an eight-channel intra-solution magnetic particle separation device *(18)*. The ability of the A-IS^1-IS^2-LAMP procedure to quantify

1 a wide range of *tdh*$^+$ *V. parahaemolyticus* levels was evaluated by testing shellfish samples in Japan and southern
2 Thailand, where shellfish products are known to be naturally-contaminated with relatively low and high levels of
3 total *V. parahaemolyticus*, respectively.
4
5 ## MATERIALS AND METHODS
6
7 **Preparation of immunomagnetic beads.** Commercially available *V. parahaemolyticus* polyvalent K
8 antiserum I ~ IX (Denka Seiken Co. Ltd., Tokyo, Japan) which was raised against a mixture of K antigens, as
9 polyvalent I: K1 and K3 ~ K8; II: K9 ~ K13, K15 and K17; III: K18 ~ K24; IV: K25, K26 and K28 ~ K32; V:
10 K33, K34 and K36 ~ K40; VI: K41 ~ K47; VII: K48 ~ K54; VIII: K55 ~ K61 and IX: K63 ~ K69, respectively,
11 were physically-absorbed to ~1 μm diameter magnetic beads separately. Since polyvalent K antiserum specific
12 for the rest of the K antigens, K70 ~ K75, is not available, the corresponding *V. parahaemolyticus* monovalent K
13 antisera (Denka Seiken Co. Ltd., Tokyo, Japan) were preliminarily mixed together in equal proportion in terms
14 of total protein, and physically-absorbed to the same magnetic beads. All antisera were purified by ammonium
15 sulfate fractionation before absorption. The resulting ten immunomagnetic bead sets were mixed together in
16 equal proportion, and used for PickPen-IMS. The specificity of the immunomagnetic beads was confirmed as
17 follows: Two K serotypes were selected from each group of serotypes, and *V. parahaemolyticus* laboratory
18 strains possessing the corresponding K antigens were subjected to agglutination tests with the ten
19 immunomagnetic bead sets (data not shown).
20
21 **Processing of Japanese shellfish samples.** Twenty-one Japanese shellfish samples consisting of thirteen
22 short-necked clams (*Tapes japonica*), six Japanese hard clams (*Meretrix lusoria*) and two freshwater clams
23 (*Cyrenidae*) were purchased at a supermarket in Mie, Japan at 2- to 4-week intervals from May 2012 to Oct
24 2012; transported at temperatures below 10 °C to the Mie Prefecture Health and Environment Research Institute;
25 and processed within an hour of purchase. The Japanese shellfish sample was shucked and homogenized in a
26 plastic bag. A three-tube MPN dilution series was prepared as described in the FDA's Bacteriological Analytical
27 Manual *(5)* with slight modifications. Briefly, a 25 g portion of the homogenate was weighed into 225 ml
28 alkaline peptone water (APW, Nissui Pharmaceutical Co., Ltd., Tokyo, Japan), and used for qualitative and
29 quantitative analyses. For the quantitative analysis, 10 ml aliquots of the shellfish homogenate diluted 1:10 in
30 APW were transferred to three empty tubes, and subsequent 10-fold dilutions were prepared by transferring 1 ml
31 aliquots of each one-log higher dilution to three tubes containing 9 ml APW. The rest of the shellfish

homogenate diluted 1:10 in APW (~220 ml) was stored for the qualitative analysis. Only shellfish samples shown to be positive for the *tdh* gene in the qualitative analysis were further examined in the quantitative analysis.

Enrichment procedures for qualitative analysis of Japanese shellfish samples. The procedures are schematically shown in Fig. 1. The A-S^1-S^2 procedure was performed as previously described *(7)* except the incubation times of the APW pre-enrichment and 2nd SPB enrichment were changed to 6 h and 18 h, respectively. The A-IS^1-IS^2 procedure was performed as follows: The shellfish homogenates diluted 1:10 in APW (~220 ml) were incubated at 37 °C for 6 h (A culture). A 1 ml aliquot from the A culture was used for PickPen-IMS as described below. The resulting 1 ml bead suspension was inoculated into 9 ml salt polymyxin broth (SPB, Nissui Pharmaceutical Co., Ltd., Tokyo, Japan), and incubated at 37 °C for 18 h (IS^1 culture). A 1 ml aliquot from the IS^1 culture was used for PickPen-IMS. The resulting 1 ml bead suspension was inoculated into 9 ml SPB, and incubated at 37 °C for 18 h (IS^2 culture). One ml aliquots from each culture (the shellfish homogenate diluted 1:10 in APW prior to incubation, and the A, S^1, S^2, IS^1, IS^2 cultures) were removed and used for DNA template preparation.

Enrichment procedures for quantitative analysis of Japanese shellfish samples. Enrichment procedures for the quantitative analysis were performed in the same manner as described above for the qualitative analysis but in the MPN format. The MPN dilutions were subjected to the A-S^1-S^2 and A-IS^1-IS^2 procedures; and one ml aliquots from each A, S^1, S^2, IS^1 and IS^2 cultures were removed and used for DNA template preparation.

Processing of Thai shellfish samples. Nine Thai shellfish samples consisting of four bloody clams (*Anadara granosa*), three hard clams (*Meretrix lusoria*), one green mussel (*Perna viridis*) and one undulated surf clam (*Paphia undulate*) were purchased at a local morning market in Hat Yai, Thailand in Mar 2012. The Thai shellfish samples were transported at ambient temperatures to the Prince of Songkla University, Hat Yai, Thailand, and processed within an hour of purchase. A three-tube MPN dilution series was prepared as described above.

Enrichment procedures for Thai shellfish samples. The A-S^1-S^2 procedure was performed as previously described *(7)* except the incubation time of the APW pre-enrichment was shortened to 6 h. The A-IS^1 procedure

1 was performed as schematically shown in Fig. 2: The MPN dilutions were incubated at 37 °C for 6 h (A culture).
2 One ml aliquots from each A culture were used for PickPen-IMS as described below. The resulting 1 ml of each
3 bead suspension was inoculated into 4 ml SPB, and shaken at 37 °C at 160 rpm for 18 h (IS^1 culture). One ml
4 aliquots from each S^2 and IS^1 culture were removed and used for DNA template preparation.
5
6 **PickPen-IMS.** PickPen-IMS was performed as previously described *(18)* with slight modifications.
7 Briefly, one ml aliquots of each culture were transferred to individual wells in a 96-well (2 ml capacity)
8 microtiter plate. The cultures were incubated with 25 μl of the immunomagnetic beads at room temperature for
9 30 min with gentle pipetting every 10 min. The subsequent bead washing and bead suspension steps were
10 performed with new tips and wells. The beads were captured with an eight-channel magnetic particle separation
11 device (PickPen, BioNobile, Finland) by gently stirring the cultures in an up-and-down motion for 1 min. The
12 captured beads were then washed twice by releasing into and re-capturing from 1 ml peptone water (1%
13 polypeptone, 0.3% yeast extract, 2% NaCl), and suspended in 1 ml SPB.
14
15 **DNA template preparation.** A 1 ml aliquot of a test culture was centrifuged at 10,000 × g for 5 min, and
16 the supernatant was discarded. The pellet was washed with and suspended in 1 ml saline (0.9 w/v% NaCl),
17 heated at 100 °C for 10 min, and immediately cooled on ice for 10 min. After centrifugation at 10,000 × g for 5
18 min, the supernatant was transferred to a new tube and stored at -20 °C until used.
19
20 **Conventional PCR assay.** Detection of tdh^+ *V. parahaemolyticus* was conducted as previously described
21 *(22)* using D3/D5 primers and 2 μl DNA template solution in each reaction, except that 1 μl DNA template
22 solution was used in the examination of the Thai shellfish samples to follow the bloody clam risk assessment
23 procedure *(26)*.
24
25 **LAMP assay.** Detection of tdh^+ *V. parahaemolyticus* was conducted as previously described *(28)*, except
26 that 2 μl DNA template solution was used in each reaction according to the manufacturer's instructions for the
27 Loopamp DNA amplification Kit (Eiken Chemical Co. Ltd., Tokyo, Japan), and the enzyme inactivation step
28 was performed at 80 °C for 10 min. The reactions were in the LA-320A (Eiken Chemical Co., Ltd., Tokyo,
29 Japan) or the LoopampEXIA (Teramecs Co. Ltd., Kyoto, Japan) for the examination of the Japanese and Thai
30 shellfish samples, respectively.
31

1 **Real-time PCR assay.** Detection of *tdh*$^+$ *V. parahaemolyticus* was conducted as previously described *(25)*
2 using the LightCycler 480 System (Roche Diagnostics K. K., Tokyo, Japan), except a probe labeled with FAM at
3 the 5'- end and TAMRA at the 3'- end was used, and the initial denaturation step was performed at 95 °C for 300
4 s according to the manufacturer's instructions for the LightCycler 480 Probe Master (Roche Diagnostics K. K.,
5 Tokyo, Japan).
6
7 **RESULTS AND DISCUSSION**
8
9 **Evaluation of the A-IS^1-IS^2-LAMP procedure for the enumeration of *tdh*$^+$ *V. parahaemolyticus* in**
10 **Japanese shellfish samples.** To evaluate the effectiveness in testing shellfish samples at low total *V.*
11 *parahaemolyticus* levels, we applied the A-IS^1-IS^2-LAMP procedure to Japanese shellfish products. Based on the
12 conventional three-step enrichment procedure (A-S^1-S^2 procedure), consisting of the APW pre-enrichment step
13 followed by the first and second SPB enrichment steps *(7)*, PickPen-IMS *(18)* was incorporated in between the
14 APW pre-enrichment and the first SPB enrichment step as well as between the first SPB and second SPB
15 enrichment step (A-IS^1-IS^2 procedure) (Fig. 1). The introduction of PickPen was essential to accommodate the
16 IMS technique in the MPN format: The design of the eight-channel intra-solution magnetic device enables (i) a
17 straightforward microtiter plate-based IMS procedure that dramatically improves sample throughput, (ii) reduced
18 carry-over of background microflora, which can highly enhance the effectiveness of the IMS treatment, and (iii)
19 more consistent results by skipping aspiration steps which often lead to inconsistent bead recovery in
20 conventional IMS procedures *(18)*.
21 Twenty-one Japanese shellfish samples were used with the A-S^1-S^2 and A-IS^1-IS^2 procedures, and tested
22 for the presence of the *tdh* gene using conventional PCR *(22)* and LAMP *(28)*. Four samples (19%) had
23 detectable levels (>1 CFU/22 g) of *tdh*$^+$ *V. parahaemolyticus* (qualitative analysis). To quantify the *tdh*-positive
24 shellfish samples, the MPN dilutions were examined for the *tdh* gene using conventional PCR and LAMP
25 (quantitative analysis). Table 1 shows some shellfish samples had low but detectable levels (<0.3 ~ 11 MPN/g)
26 of *tdh*$^+$ *V. parahaemolyticus*. LAMP yielded similar MPN values compared to PCR; and more importantly,
27 LAMP successfully determined MPNs in more cultures for which PCR gave negative results. The cultures with
28 (IS^1 and IS^2 cultures) and without (S^1 and S^2 cultures) PickPen-IMS yielded comparable MPN values with the
29 differences of <0.5 log MPN/g. The difference of ~0.5 log MPN/g in calculated MPN values corresponds to a
30 single tube difference *(8)*.
31

Examination of the effectiveness of PickPen-IMS for the concentration of *tdh*$^+$ *V. parahaemolyticus* from Japanese shellfish samples. To examine the effectiveness of PickPen-IMS, cultures with (IS^1 and IS^2 cultures) or without (S^1 and S^2 cultures) PickPen-IMS were compared for the abundance of *tdh*$^+$ *V. parahaemolyticus*. Among all of the cultures (n = 201) derived from the four Japanese shellfish samples, those shown to be positive for the *tdh* gene in the qualitative and/or quantitative analyses were examined for the numbers of total and *tdh*$^+$ *V. parahaemolyticus* cells using a real-time PCR assay *(25)* (Table 2). When the proportions of *tdh*$^+$ *V. parahaemolyticus* cells to the total *V. parahaemolyticus* cells (*tdh*$^+$/total) are compared, the IS^1 cultures have *tdh*$^+$/total thirty-two times greater, on average, than the S^1 cultures, indicating that the first PickPen-IMS was effective in concentrating *tdh*$^+$ *V. parahaemolyticus* from the Japanese shellfish samples. PickPen-IMS can considerably save time and effort for quantitative purposes and also for qualitative purposes, e.g., isolating *tdh*$^+$ *V. parahaemolyticus* strains from environmental samples.

Although an attempt was made to examine whether *tdh*$^+$ *V. parahaemolyticus* can further be enriched by repeating the IMS-SPB enrichment step, such an effect was not observed: The average ratio of *tdh*$^+$/total of the IS^2 cultures to the S^2 cultures decreased to nineteen-fold (data available upon request). Therefore and hereafter, the second IMS-SPB enrichment step will be omitted, and the resulting IMS-incorporated two-step enrichment procedure (A-IS^1 procedure) will be further validated.

Comparison of PCR and LAMP for the detection of the *tdh* gene from Japanese shellfish samples. To show LAMP improves the detection rate for *tdh*$^+$ *V. parahaemolyticus*, a comparison between conventional PCR and LAMP was examined for all of the cultures (n = 201) derived from the four *tdh*-positive Japanese shellfish samples. PCR detected the *tdh* gene in 28 (14%) out of 201 cultures; whereas LAMP detected the *tdh* gene in an additional 20 cultures, totaling 48 cultures (24%). To show features common to the cultures yielding the PCR-negative and LAMP-positive result, as for all of the *tdh*-positive cultures (n = 48), the numbers of total and *tdh*$^+$ *V. parahaemolyticus* cells quantified using the real-time PCR assay were plotted. Fig. 3 shows the cultures where only LAMP gave positive results (PCR$^-$/LAMP$^+$; indicated by a filled circle) had a tendency to contain (i) a small amount of *tdh*$^+$ *V. parahaemolyticus* cells, which is consistent with previous studies showing the high sensitivity of LAMP *(27, 29, 30)* or (ii) large amounts of both total and *tdh*$^+$ *V. parahaemolyticus* cells. Although the reasons for the latter are unclear, it has been noted that a large amount of non-specific DNA and too much DNA template can inhibit PCR *(25)*. Compared to conventional PCR, LAMP may be less susceptible to potential DNA amplification inhibition.

Evaluation of the A-IS^1-LAMP procedure for the enumeration of *tdh*$^+$ *V. parahaemolyticus* in Thai shellfish samples.

In contrast to the results from the bloody clam risk assessment *(26)*, our previous study indicated high incidence of patients with *tdh*$^+$ *V. parahaemolyticus* infection in Hat Yai, Southern Thailand *(10)*, stimulating the search for a possible source of the infection. It is known that some retailers store shellfish products overnight and sell them in the following morning. Our preliminary studies indicated shellfish sold at the morning markets were naturally-contaminated with relatively high levels of pathogenic *V. parahaemolyticus* (data not shown), prompting us to enumerate the level using the A-IS^1-LAMP procedure.

Nine Thai shellfish samples were used with the A-S^1-S^2 and A-IS^1 procedures in the MPN format (Fig. 2); and the MPN dilutions were tested for the presence of the *tdh* gene using conventional PCR and LAMP. All of the Thai shellfish samples showed considerably higher levels (93 ~ 11,000 MPN/g) of *tdh*$^+$ *V. parahaemolyticus* (Table 3). This suggests shellfish microflora including *tdh*$^+$ *V. parahaemolyticus* had proliferated during the overnight storage at the retailers. This hypothesis is supported by previous studies in which pre-incubation of shellfish samples prior to examination allowed total and pathogenic *V. parahaemolyticus* to grow substantially *(7, 8)*. Although the data shown here is based only on nine shellfish samples collected within one week and may not represent the general hygienic situation of the shellfish products sold at the local morning markets, the finding raises considerable public health concern about shellfish safety for domestic consumers.

A comparison between the two DNA detection methods shows LAMP yielded the same or higher MPN values with the difference of 0.5 log MPN/g or greater than PCR, showing the high performance of LAMP (Table 3). Even in the rare cases where LAMP yielded lower MPN values than PCR, the differences were less than 0.5 log MPN/g.

Considering factors influencing enrichment and gene analysis, such as background microflora and DNA amplification inhibitors that can substantially vary from shellfish to shellfish, depending on the sample of interest, the effectiveness of PickPen-IMS can be either adversely or positively affected. Table 3 shows the A-IS^1 procedure (IS^1 culture) yielded the same or lower MPN values than the A-S^1-S^2 procedure (S^2 culture). The reasons for this are unclear, but it is important to note a potential disadvantage in applying the IMS technique to environmental samples. An environmental study conducted in Hat Yai, southern Thailand showed more than half of the *V. parahaemolyticus* isolates from seafood possessed K antigens despite lack of the pathogenic genes *(23)*. This finding suggests the possibility that during an IMS process, immunomagnetic beads can be saturated by a majority of such non-pathogenic strains, resulting in screening out the target strains. This may be of particular concern in applying IMS to shellfish samples with high total *V. parahaemolyticus* levels, such as the Thai

shellfish samples. However, depending on the purpose and/or shellfish sample of interest, our strategy of screening clinically-important *V. parahaemolyticus* populations with all of the sixty-nine K serotypes, to concentrate targets present in low numbers as well as to eliminate a great abundance of background microflora, can overweigh this potential disadvantage. Moreover, while the inhibitory effect of shellfish homogenate is a concern in any DNA amplification-based assay *(1, 9, 21)*, IMS can alleviate this problem.

In conclusion, LAMP consistently showed higher performance than conventional PCR in the detection and quantification of a wide range of *tdh*$^+$ *V. parahaemolyticus* levels in shellfish products. Also, depending on the shellfish sample of interest, such as the Japanese shellfish samples which have relatively low levels of total *V. parahaemolyticus*, PickPen-IMS can further facilitate the target detection. Although further validation with a large number of shellfish samples are required, the A-IS^1-LAMP procedure offers a practical means for the measurement and management of the *tdh*$^+$ *V. parahaemolyticus* levels in shellfish products and can be used by any health authority in the world.

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REFERENCES

1. Blackstone, G. M., J. L. Nordstrom, M. C. L. Vickery, M. D. Bowen, R. F. Meyer, and A. DePaola. 2003. Detection of pathogenic *Vibrio parahaemolyticus* in oyster enrichments by real time PCR. *J. Microbiol. Methods* 53:149-155.
2. Blanco-Abad, V., J. Ansede-Bermejo, A. Rodriguez-Castro, and J. Martinez-Urtaza. 2009. Evaluation of different procedures for the optimized detection of *Vibrio parahaemolyticus* in mussels and environmental samples. *Int. J. Food Microbiol.* 129:229-236.
3. Cantet, F., D. Hervio-Heath, A. Caro, C. L. Mennec, C. Monteil, C. Quéméré, A. Jolivet-Gougeon, R. R. Colwell, and P. Monfort. 2013. Quantification of *Vibrio parahaemolyticus*, *Vibrio vulnificus* and *Vibrio cholerae* in French Mediterranean coastal lagoons. *Res. Microbiol.* 164:867-874.
4. Cook, D. W., P. O'Leary, J. C. Hunsucker, E. M. Sloan, J. C. Bowers, R. J. Blodgett, and A. DePaola. 2002. *Vibrio vulnificus* and *Vibrio parahaemolyticus* in U.S. retail shell oysters: a national survey June 1998 to July 1999. *J. Food Prot.* 65:79-87.
5. DePaola, A., and C. A. Kaysner. 2004. *Vibrio*. In: Bacteriological Analytical Manual online, 9th ed. Available at: http://www.fda.gov/Food/FoodScienceResearch/LaboratoryMethods/ucm070830.htm
6. FAO/WHO. 2011. Risk Assessment of *Vibrio parahaemolyticus* in Seafood: Interpretative Summary and Technical Report. Microbiological Risk Assessment Series No. 16. Food and Agriculture Organization of the United Nations/World Health Organization, Rome.
7. Hara-Kudo, Y., K. Sugiyama, M. Nishibuchi, A. Chowdhury, J. Yatsuyanagi, Y. Ohtomo, A. Saito, H. Nagano, T. Nishina, H. Nakagawa, H. Konuma, M. Miyahara, and S. Kumagai. 2003. Prevalence of pandemic thermostable direct hemolysin-producing *Vibrio parahaemolyticus* O3:K6 in seafood and the coastal environment in Japan. *Appl. Environ. Microbiol.* 69:3883–3891.
8. Jones J. L., Y. Hara-Kudo, J. A. Krantz, R. A. Jr. Benner, A. B. Smith, T. R. Dambaugh, J. C. Bowers, and A. DePaola. 2012. Comparison of molecular detection methods for *Vibrio parahaemolyticus* and *Vibrio vulnificus*. *Food Microbiol.* 30:105–111.
9. Kaufman, G. E., G. M. Blackstone, M. C. L. Vickery, A. K. Bej, J. Bowers, M. D. Bowen, R. F. Meyer, and A. DePaola. 2004. Real-time PCR quantification of *Vibrio parahaemolyticus* in oysters using an alternative matrix. *J. Food Prot.* 67:2424–2429.
10. Laohaprertthisan, V., A. Chowdhury, U. Kongmuang, S. Kalnauwakul, M. Ishibashi, C. Matsumoto, and M. Nishibuchi. 2003. Prevalence and serodiversity of the pandemic clone among the clinical strains of *Vibrio*

1 *parahaemolyticus* isolated in southern Thailand. *Epidemiol. Infect.* 130:395-406.

2 11. Luan, X., J. Chen, Y. Liu, Y. Li, J. Jia, R. Liu, and X. H. Zhang. 2008. Rapid quantitative detection of *Vibrio*
3 *parahaemolyticus* in seafood by MPN-PCR. *Curr. Microbiol.* 57:218-221.

4 12. Matsumoto, C., J. Okuda, M. Ishibashi, M. Iwanaga, P. Garg, T. Rammamurthy, H. Wong, A. DePaola, Y. B.
5 Kim, M. J. Albert, and M. Nishibuchi. 2000. Pandemic spread of an O3:K6 clone of *Vibrio*
6 *parahaemolyticus* and emergence of related strains evidenced by arbitrarily primed PCR and *toxRS*
7 sequence analysis. *J. Clin. Microbiol.* 38:578-585.

8 13. Miwa, N., M. Kashiwagi, F. Kawamori, T. Masuda, Y. Sano, M. Hiroi, and H. Kurashige. 2006. Levels of
9 *Vibrio parahaemolyticus* and thermostable direct hemolysin gene-positive organisms in retail seafood
10 determined by the most probable number-polymerase chain reaction (MPN-PCR) method. *Shokuhin*
11 *Eiseigaku Zasshi* 47:41-45.

12 14. Mori, Y., K. Nagamine, N. Tomita, and T. Notomi. 2001. Detection of loop-mediated isothermal
13 amplification reaction by turbidity derived from magnesium pyrophosphate formation. *Biochem. Biophys.*
14 *Res. Commun.* 289:150-154.

15 15. Nemoto, J., M. Ikedo, T. Kojima, T. Momoda, H. Konuma, and Y. Hara-Kudo. 2011. Development and
16 evaluation of a loop-mediated isothermal amplification assay for rapid and sensitive detection of *Vibrio*
17 *parahaemolyticus*. *J. Food Prot.* 74:1462-1467.

18 16. Nishibuchi, M., and J. B. Kaper. 1995. Thermostable direct hemolysin gene of *Vibrio parahaemolyticus*: a
19 virulence gene acquired by a marine bacterium. *Infect. Immun.* 63:2093-2099.

20 17. Nordstrom, J. L., M. C. L. Vickery, G. M. Blackstone, S. L. Murray, and A. DePaola. 2007. Development of
21 a multiplex real-time PCR assay with an internal amplification control for the detection of total and
22 pathogenic *Vibrio parahaemolyticus* bacteria in oysters. *Appl. Environ. Microbiol.* 73:5840-5847.

23 18. Nou, X., T. M. Arthur, J. M. Bosilevac, D. M. Brichta-Harhay, M. N. Guerini, N. Kalchayanand, and M.
24 Koohmaraie. 2006. Improvement of immunomagnetic separation for *Escherichia coli* O157:H7 detection by
25 the PickPen magnetic particle separation device. *J. Food Prot.* 69:2870-2874.

26 19. Okuda, J., M. Ishibashi, E. Hayakawa, T. Nishino, Y. Takeda, A. K. Mukhopadhyay, S. Garg, S. K.
27 Bhattacharya, G. B. Nair, and M. Nishibuchi. 1997. Emergence of a unique O3:K6 clone of *Vibrio*
28 *parahaemolyticus* in Calcutta, India, and isolation of strains from the same clonal group from Southeast
29 Asian travelers arriving in Japan. *J. Clin. Microbiol.* 35:3150-3155.

30 20. Ripabelli, G., M. L. Sammarco, G. M. Grasso, I. Fanelli, A. Caprioli, and I. Luzzi. 1999. Occurrence of
31 *Vibrio* and other pathogenic bacteria in *Mytilus galloprovincialis* (mussels) harvested from Adriatic Sea,

1 Italy. *Int. J. Food Microbiol.* 49:43-48.

2 21. Rizvi, A. V., and A. K. Bej. 2010. Multiplexed real-time PCR amplification of *tlh*, *tdh* and *trh* genes in
3 *Vibrio parahaemolyticus* and its rapid detection in shellfish and Gulf of Mexico water. *Antonie Van*
4 *Leeuwenhoek.* 98:279-290.

5 22. Tada, J., T. Ohashi, N. Nishimura, Y. Shirasaki, H. Ozaki, S. Fukushima, J. Takano, M. Nishibuchi, and Y.
6 Takeda. 1992. Detection of the thermostable direct hemolysin gene (*tdh*) and the thermostable direct
7 hemolysin-related hemolysin gene (*trh*) of *Vibrio parahaemolyticus* by polymerase chain reaction. *Mol. Cell.*
8 *Probes* 6:477-487.

9 23. Vuddhakul, V., A. Chowdhury, V. Laohaprertthisan, P. Pungrasamee, N. Patararungrong, P. Thianmontri, M.
10 Ishibashi, C. Matsumoto, and M. Nishibuchi. 2000. Isolation of *Vibrio parahaemolyticus* strains belonging
11 to a pandemic O3:K6 clone from environmental and clinical sources in Thailand. *Appl. Environ. Microbiol.*
12 66:2685-2689.

13 24. Wagatsuma, S. 1974. Ecological studies on Kanagawa phenomenon positive strains of *Vibrio*
14 *parahaemolyticus*. International symposium on *Vibrio parahaemolyticus*. Saikon Publishing Co., Tokyo.
15 91-96.

16 25. Ward, L. N., and A. K. Bej. 2006. Detection of *Vibrio parahaemolyticus* in shellfish by use of multiplexed
17 real-time PCR with TaqMan fluorescent probes. *Appl. Environ. Microbiol.* 72:2031-2042.

18 26. Yamamoto, A., J. Iwahori, V. Vuddhakul, W. Charernjiratragulc, D. Vose, K. Osaka, M. Shigematsu, H.
19 Toyofuku, S. Yamamoto, M. Nishibuchi, and F. Kasuga. 2008. Quantitative modeling for risk assessment of
20 *Vibrio parahaemolyticus* in bloody clams in southern Thailand. *Int. J. Food Microbiol.* 124:70-78.

21 27. Yamazaki, W., M. Ishibashi, R. Kawahara, and K. Inoue. 2008. Development of a loop-mediated isothermal
22 amplification assay for sensitive and rapid detection of *Vibrio parahaemolyticus*. *BMC Microbiol.* 8:163.

23 28. Yamazaki, W., Y. Kumeda, N. Misawa, Y. Nakaguchi, and M. Nishibuchi, 2010. Development of a
24 loop-mediated isothermal amplification assay for sensitive and rapid detection of the *tdh* and *trh* genes of
25 *Vibrio parahaemolyticus* and related *Vibrio* species. *Appl. Environ. Microbiol.* 76:820-828.

26 29. Yamazaki, W., K. Seto, M. Taguchi, M. Ishibashi, and K. Inoue. 2008. Sensitive and rapid detection of
27 cholera toxin-producing *Vibrio cholerae* using a loop-mediated isothermal amplification. *BMC Microbiol.*
28 8:94.

29 30. Zhao, X., Y. Li, L. Wang, L. You, Z. Xu, L. Li, X. He, Y. Liu, J. Wang, and L. Yang. 2010. Development and
30 application of a loopmediated isothermal amplification method on rapid detection *Escherichia coli* O157
31 strains from food samples. *Mol. Biol. Rep.* 37:2183-2188.

1 **FIGURE LEGEND**

2

3 FIGURE 1. Enrichment procedures for qualitative analysis of Japanese shellfish samples.

4

5 FIGURE 2. Recommended enrichment procedure.

6

7 FIGURE 3. Relationship between numbers of total and *tdh*$^{+}$ *V. parahaemolyticus* cells in cultures derived from
8 Japanese shellfish samples. ND; not detected using real-time PCR, but detected using conventional PCR and/or
9 LAMP.

Table 1. *Levels of* tdh$^+$ V. parahaemolyticus *in Japanese shellfish samples (log MPN/g).*

	IS^1 culture[b]		S^1 culture[c]		IS^2 culture[d]		S^2 culture[e]	
Shellfish sample[a]	LAMP	PCR	LAMP	PCR	LAMP	PCR	LAMP	PCR
Short-necked clam	1.0	1.0	1.0	1.0	1.0	1.0	1.0	0.8
Short-necked clam	ND	ND	ND	ND	ND	ND	ND	ND
Short-necked clam	0.6	ND	1.0	ND	0.6	ND	0.9	ND

[a] Only shellfish samples shown to be positive for the *tdh* gene in the qualitative analysis are included.

[b] Culture taken after the first IMS-SPB enrichment step of the IMS-incorporated three-step enrichment (A-IS^1-IS^2) procedure.

[c] Culture taken after the first SPB enrichment step of the conventional three-step enrichment (A-S^1-S^2) procedure.

[d] Culture taken after the second IMS-SPB enrichment step of the IMS-incorporated three-step enrichment (A-IS^1-IS^2) procedure.

[e] Culture taken after the second SPB enrichment step of the conventional three-step enrichment (A-S^1-S^2) procedure.

ND; not detected (<0.3 MPN/g).

9 Table 2. *Numbers of total and* tdh$^+$ V. parahaemolyticus *cells in cultures derived from* tdh-*positive Japanese shellfish samples.*

IS^1 culture[a]			S^1 culture[b]			
tdh$^+$ Vp[c] (log CFU/ml)	Total Vp[d] (log CFU/ml)	*tdh*$^+$/total[e]	*tdh*$^+$ Vp[c] (log CFU/ml)	Total Vp[d] (log CFU/ml)	*tdh*$^+$/total[e]	Ratio of *tdh*$^+$/total of IS^1 to S^1
4.5	7.6	0.00086	4.0	7.3	0.00055	1.6
4.1	4.6	0.29	3.5	6.2	0.0021	139
5.5	7.3	0.014	3.3	6.6	0.00056	25
4.7	8.0	0.00050	3.8	7.4	0.00021	2.4
4.0	5.9	0.014	3.0	6.7	0.00018	77
5.7	7.8	0.0085	5.4	6.8	0.038	0.23
5.5	6.7	0.068	5.2	6.9	0.018	3.7
6.5	7.9	0.037	5.8	8.2	0.0045	8.1
						32 (average)

10 [a] Culture taken after the first IMS-SPB enrichment step of the IMS-incorporated three-step enrichment (A-IS^1-IS^2) procedure.

11 [b] Culture taken after the first SPB enrichment step of the conventional three-step enrichment (A-S^1-S^2) procedure.

12 [c] Number of *tdh*$^+$ *V. parahaemolyticus* cells.

13 [d] Number of total *V. parahaemolyticus* cells.

14 [e] Proportion of *tdh*$^+$ *V. parahaemolyticus* cells to the total *V. parahaemolyticus* cells.

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