(carrying *tdh* gene [*tdh*⁺] and/or *trh* gene [*trh*⁺]) *V. parahaemolyticus* counts in bloody clams were followed from harvest to the retail stage *(33)*. Nevertheless, pathogenic *V. parahaemolyticus* was detected in only a small portion of the bloody clam samples, with low MPN values close to the lower detection limit (3 MPN/10 g), raising a need for a more sensitive procedure.

Compared with conventional PCR, real-time PCR and loop-mediated isothermal amplification (LAMP) are more sensitive, specific, time- and labor-saving alternatives for DNA detection, and they have been applied in an MPN format *(3, 10, 19, 22)*. LAMP, in particular, has great advantages in field settings; it can be performed in a simple isothermal chamber without trained personnel and yields robust results that allow visual judgment *(18, 35)*. A lyophilized reagent that no longer requires a cold chain system is another advantage *(20)*. To find a method to measure the total and pathogenic *V. parahaemolyticus* levels in shellfish products that can be used by any health authority in the world, we have focused on LAMP (we previously developed a LAMP assay for sensitive and rapid detection of *tdh*⁺ and *trh*⁺ *V. parahaemolyticus* *(35)*). When applied in an MPN format in conjunction with the three-step enrichment procedure, the *tdh*-LAMP assay can be a powerful tool to enumerate *tdh*⁺ *V. parahaemolyticus* in shellfish samples. In contrast, our subsequent studies suggested that the *trh*-LAMP assay can be improved by using new primer sets, currently being tested, that can tolerate the *trh* gene sequence variations widely observed in *trh*⁺ environmental strains *(20)*. Thus, whereas this study does not deal with *trh*⁺ *V. parahaemolyticus*, this MPN-LAMP format can potentially be expanded to enumerate *trh*⁺ environmental strains.

Our previous isolation of O3:K6 pandemic strains from bloody clams using an immunomagnetic separation (IMS) technique targeting the K antigen, an outermost structure of *V. parahaemolyticus* cells *(29)*, prompted interest in screening clinically important *V. parahaemolyticus* populations with all 69 K serotypes from shellfish samples. This approach is based on the facts that (i) *V. parahaemolyticus* O:K serotypes have been established based on clinical isolates submitted to the Committee on the Serological Typing of *Vibrio parahaemolyticus* (Laboratory of Genomic Research on Pathogenic Bacteria, International Research Center for Infectious Diseases, Research Institute for Microbial Diseases, Osaka University, Osaka, Japan) and (ii) studies from various parts of the world have generally reported that approximately 90% of clinical isolates possessed established K antigens *(4, 8, 24, 25)*, whereas a study from the United States reported that the proportion was only 56% *(11)*. Moreover, whereas the inhibitory effect of shellfish homogenate is a concern in any DNA amplification–based assay *(1, 12, 27)*, IMS can alleviate this problem.

In this study, we prepared immunomagnetic beads specific for as many as 69 established K antigens and, additionally, incorporated IMS into the MPN format by introducing PickPen, an eight-channel intrasolution magnetic particle separation device (designated as PickPen-IMS)

(23). 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 contain relatively low and high levels of total *V. parahaemolyticus*, respectively.

MATERIALS AND METHODS

Preparation of immunomagnetic beads. Commercially available *V. parahaemolyticus* polyvalent K antisera I to IX (Denka Seiken Co. Ltd., Tokyo, Japan), which were raised against a mixture of K antigens, as polyvalent I (K1 and K3 to K8), II (K9 to K13, K15, and K17), III (K18 to K24), IV (K25, K26, and K28 to K32), V (K33, K34, and K36 to K40), VI (K41 to K47), VII (K48 to K54), VIII (K55 to K61), and IX (K63 to K69), were physically absorbed to ~1-μm-diameter magnetic beads separately. Since polyvalent K antiserum specific for the rest of the K antigens, K70 to K75, is not available, the corresponding *V. parahaemolyticus* monovalent K antisera (Denka Seiken Co. Ltd.) were preliminarily mixed together in equal proportions in terms of total protein and were physically absorbed to the same magnetic beads. All antisera were purified by ammonium sulfate fractionation before absorption. The resulting 10 immunomagnetic bead sets were mixed together in equal proportions and were used for PickPen-IMS. The specificity of the immunomagnetic beads was confirmed as follows: two K serotypes were selected from each group of serotypes, and *V. parahaemolyticus* laboratory strains possessing the corresponding K antigens were subjected to agglutination tests with the 10 immunomagnetic bead sets (data not shown).

Processing of Japanese shellfish samples. Twenty-one Japanese shellfish samples consisting of 13 short-necked clams (*Tapes japonica*), six Japanese hard clams (*Meretrix lusoria*), and two freshwater clams (*Cyrenidae*) were purchased at a supermarket in Mie, Japan, at 2- to 4-week intervals from May 2012 to October 2012, were transported at temperatures below 10°C to the Mie Prefecture Health and Environment Research Institute, and were processed within 1 hour of purchase. The Japanese shellfish sample was shucked and homogenized in a plastic bag. A three-tube MPN dilution series was prepared as described in the U.S. Food and Drug Administration's *Bacteriological Analytical Manual (6)*, with slight modifications. Briefly, a 25-g portion of the homogenate was weighed into 225 ml of alkaline peptone water (APW; Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) and was used for qualitative and quantitative analyses. For the quantitative analysis, 10-ml aliquots of the shellfish homogenate diluted 1:10 in APW were transferred to three empty tubes, and subsequent 10-fold dilutions were prepared by transferring 1-ml aliquots of each 1-log-higher dilution to three tubes containing 9 ml of APW. The rest of the shellfish homogenate diluted 1:10 in APW (~220 ml) was stored for the qualitative analysis. Although the culture tubes for the qualitative and quantitative analyses were incubated in parallel, only those derived from the shellfish samples shown to be positive for the *tdh* gene in the qualitative analysis as explained below were further examined in the quantitative analysis, resulting in 201 culture tubes examined.

Enrichment procedures for qualitative analysis of Japanese shellfish samples. The procedures are shown schematically in Figure 1.

The A-S¹-S² procedure was performed as previously described *(9)*, with the following two modifications. (i) Throughout this study, the incubation time of the APW preenrichment was
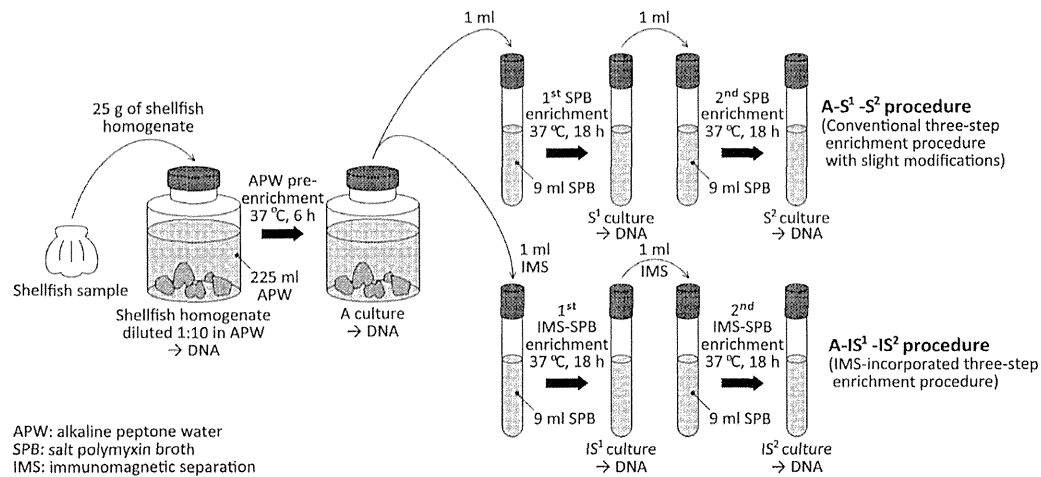
FIGURE 1. *Enrichment procedures for qualitative analysis of Japanese shellfish samples.*

shortened to 6 h because fast-growing species such as *V. parahaemolyticus* still remain dominant in such a short incubation time *(29, 30)*. (ii) The incubation time of the second salt polymyxin broth (SPB) enrichment was changed only in the examination of these Japanese shellfish samples, with the aim of precisely examining the effectiveness of PickPen-IMS, for the following reason. We performed the A-IS^1-IS^2 procedure in parallel and found that the number of bacterial cells captured by the immunomagnetic beads was so small that the incubation time of the second SPB enrichment needed to be extended from 6 to 18 h to ensure sufficient bacterial growth in the IS^2 culture. Accordingly, we changed the incubation time of the corresponding SPB enrichment in the parallel A-S^1-S^2 procedure.

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 of SPB (Nissui Pharmaceutical Co., Ltd.) and was 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 of SPB and was incubated at 37°C for 18 h (IS^2 culture). One-milliliter aliquots from each culture (the shellfish homogenate diluted 1:10 in APW prior to incubation and the A, S^1, S^2, IS^1, and 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 1-ml aliquots from each A, S^1, S^2, IS^1, and IS^2 culture 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 undulata*) were purchased at a local morning market in Hat Yai, Thailand, in March 2012. The Thai shellfish samples were transported at ambient temperatures to the Prince of Songkla University, Hat Yai, Thailand, and were processed within 1 hour of purchase. A three-tube MPN dilution series was prepared as described above.

Enrichment procedures for Thai shellfish samples. Due to the high levels of bacteria, including *V. parahaemolyticus,* in the Thai shellfish samples purchased at the local morning market (explained below), unusual bacterial growth such as aggregation and surface biofilm formation interfered with proper PickPen-IMS. Two changes were thus made, although the procedures were essentially the same as described for the Japanese shellfish samples. (i) The A-S^1-S^2 procedure (Fig. 1) was performed in the MPN format, except that the incubation time of the second SPB enrichment was shortened to 6 h, as described for the original procedure *(9)*. (ii) The A-IS^1 procedure was performed using a small volume (5 ml) of SPB with shaking. Briefly, as shown in Figure 2, the MPN dilutions were incubated at 37°C for 6 h (A culture). One-milliliter aliquots from each A culture were used for PickPen-IMS as described below. The resulting 1 ml of each bead suspension was inoculated into 4 ml of SPB and was shaken at 37°C at 160 rpm for 18 h (IS^1 culture). One-milliliter aliquots from each S^2 and IS^1 culture were removed and used for DNA template preparation.

PickPen-IMS. PickPen-IMS was performed as previously described *(23)*, with slight modifications. Briefly, 1-ml aliquots of each culture were transferred to individual wells in a 96-well (2-ml capacity) microtiter plate. The cultures were incubated with 25 µl of the immunomagnetic beads at room temperature for 30 min, with gentle pipetting every 10 min. The subsequent bead washing and bead suspension steps were performed with new tips and wells. The beads were captured with an eight-channel magnetic particle separation device (PickPen, Bio-Nobile, Turku, Finland) by gently stirring the cultures with an up-and-down motion for 1 min. The captured beads were then washed twice by release into and recapture from 1 ml of peptone water (1% polypeptone, 0.3% yeast extract, 2% NaCl) and were suspended in 1 ml of SPB.

DNA template preparation. A 1-ml aliquot of a test culture was centrifuged at 10,000 × *g* for 5 min, and the supernatant was discarded. The pellet was washed with and suspended in 1 ml of saline (0.9% [wt/vol] NaCl), heated at 100°C for 10 min, and immediately cooled on ice for 10 min. After centrifugation at 10,000 × *g* for 5 min, the supernatant was transferred to a new tube and was stored at −20°C until it was used.

Conventional PCR assay. Detection of *tdh*$^+$ *V. parahaemolyticus* was conducted as previously described *(28)* using D3 and D5 primers and 2 µl of DNA template solution in each reaction,
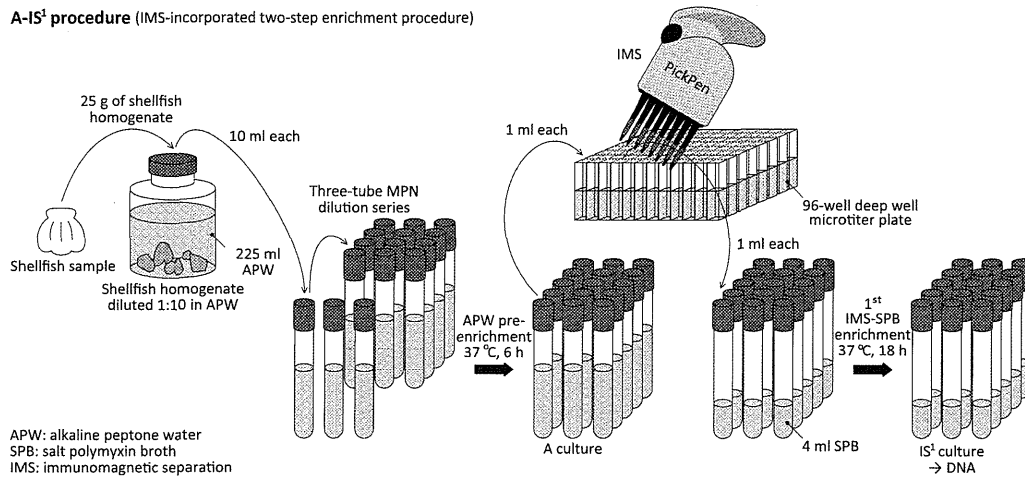
FIGURE 2. Recommended enrichment procedure.

with the exception that 1 µl of DNA template solution was used in the examination of the Thai shellfish samples to follow the bloody clam risk assessment procedure (33).

LAMP assay. Detection of tdh⁺ V. parahaemolyticus was conducted as previously described (35), except that 2 µl of DNA template solution was used in each reaction according to the manufacturer's instructions for the Loopamp DNA amplification kit (Eiken Chemical Co. Ltd., Tokyo, Japan), and the enzyme inactivation step was performed at 80°C for 10 min. Loopamp real-time turbidimeters from Eiken Chemical Co., LA-320A and LoopampEXIA, were used to examine the Japanese and Thai shellfish samples, respectively.

Real-time PCR assay. Detection of tdh⁺ V. parahaemolyticus was conducted as previously described (32) using the LightCycler 480 System (Roche Diagnostics K. K., Tokyo, Japan), with these exceptions: a probe labeled with FAM at the 5′ end and TAMRA at the 3′ end was used, and the initial denaturation step was performed at 95°C for 300 s according to the manufacturer's instructions for the LightCycler 480 Probe Master (Roche Diagnostics K. K.).

RESULTS AND DISCUSSION

Evaluation of the A-IS¹-IS²-LAMP procedure for the enumeration of tdh⁺ V. parahaemolyticus in Japanese shellfish samples. Based on the conventional three-step enrichment procedure (A-S¹-S²), consisting of the APW preenrichment step followed by the first and second SPB enrichment steps (9), PickPen-IMS (23) was incorporated in between the APW preenrichment and the first SPB enrichment step as well as between the first SPB and second SPB enrichment step (A-IS¹-IS²) (Fig. 1). The introduction of PickPen was essential to accommodate the IMS technique in the MPN format: the design of the eight-channel intrasolution magnetic device enables (i) a straightforward microtiter plate–based IMS procedure that dramatically improves sample throughput; (ii) a reduced carry-over of background microflora, which can highly enhance the effectiveness of the IMS treatment; and (iii) more consistent results by skipping aspiration steps, which often lead to inconsistent bead recovery in conventional IMS procedures (23).

To evaluate effectiveness in testing shellfish samples containing low levels of total V. parahaemolyticus, we applied the A-IS¹-IS²-LAMP procedure to Japanese shellfish products. Twenty-one Japanese shellfish samples were used with the A-S¹-S² and A-IS¹-IS² procedures and were tested for the presence of the tdh gene in each of the various cultures (Fig. 1) using conventional PCR (28) and LAMP (35). Four samples (19%) had detectable levels (>1 CFU/22 g) of tdh⁺ V. parahaemolyticus (qualitative analysis). To quantify tdh⁺ V. parahaemolyticus in the tdh-positive shellfish samples, the MPN dilutions were examined for the tdh gene in each of the various cultures (not shown in Fig. 1) using conventional PCR, LAMP, and real-time PCR (32) (quantitative analysis). The qualitative and quantitative analyses resulted in the examination of 201 culture tubes. Table 1 shows that some shellfish samples had low but detectable levels (<3 to 11 MPN/10 g) of tdh⁺ V. parahaemolyticus. According to Jones et al. (10), variation in calculated MPN values with a single tube difference is approximately 0.5 log MPN/g. In this study, we defined differences in MPN values less than or equal to 0.5 log MPN/10 g as insignificant, and we will express the relationship as similar; otherwise, the relationship will be expressed as lower or higher. Although the number of the tdh-positive shellfish samples was small, Table 1 seems more to highlight the difference in the three DNA detection methods (LAMP, conventional PCR, and real-time PCR) rather than the two enrichment procedures (A-S¹-S² and A-IS¹-IS²). LAMP and real-time PCR yielded similar MPN values compared with conventional PCR; and, more importantly, LAMP and real-time PCR successfully determined MPNs in more cultures for which conventional PCR gave negative results. The cultures with (IS¹ and IS² cultures) and without (S¹ and S² cultures) PickPen-IMS yielded similar MPN values.

Examination of the effectiveness of PickPen-IMS for the concentration of tdh⁺ V. parahaemolyticus from Japanese shellfish samples. Because the effect of PickPen-IMS was not shown in the MPN determination (Table 1), we examined whether the effect can be detected at an

TABLE 1. *Levels of* tdh $^+$ *V. parahemolyticus in Japanese shellfish samples*[a]

	IS^1 culture			S^1 culture			IS^2 culture			S^2 culture		
Shellfish sample designation	LAMP	Conventional PCR	Real-time PCR	LAMP	Conventional PCR	Real-time PCR	LAMP	Conventional PCR	Real-time PCR	LAMP	Conventional PCR	Real-time PCR
Short-necked clam 1	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	0.8	1.0
Short-necked clam 2	<0.5[b]	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5
Short-necked clam 3	0.6	<0.5	0.6	1.0	<0.5	0.6	0.6	<0.5	0.6	0.9	<0.5	0.6

[a] Only shellfish samples shown to be positive for the *tdh* gene in the qualitative analysis are included. Another *tdh*-positive shellfish sample is not included because its MPN dilutions were lost due to a laboratory accident. Values are expressed as log MPN/10 g. IS^1 culture, taken after the first IMS-SPB enrichment step of the IMS-incorporated three-step enrichment (A-IS^1-IS^2) procedure; S^1 culture, taken after the first SPB enrichment step of the conventional three-step enrichment (A-S^1-S^2) procedure; IS^2 culture, taken after the second IMS-SPB enrichment step of the IMS-incorporated three-step enrichment (A-IS^1-IS^2) procedure; S^2 culture, taken after the second SPB enrichment step of the conventional three-step enrichment (A-S^1-S^2) procedure.

[b] Values <0.5 were below the detection limit in this quantitative analysis but were detected in the qualitative analysis (>1 CFU/22 g).

individual tube level. The 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*. The analysis of the four *tdh*-positive Japanese shellfish samples yielded eight pairs of IS^1-S^1 culture tubes, for which one or both tubes were positive for the *tdh* gene using real-time PCR. The numbers of total and *tdh* $^+$ *V. parahaemolyticus* cells in each culture tube quantified using real-time PCR as explained above are shown in Table 2. The proportions of *tdh* $^+$ *V. parahaemolyticus* cells to the total *V. parahaemolyticus* cells (*tdh* $^+$/total) were determined for the IS^1 and S^1 cultures. The ratios of *tdh* $^+$/total of the IS^1 cultures to the S^1 cultures were calculated, and their average value was 32 (Table 2), indicating that the first PickPen-IMS treatment was effective in concentrating *tdh* $^+$ *V. parahaemolyticus* from the Japanese shellfish samples.

Next, to examine whether *tdh* $^+$ *V. parahaemolyticus* can further be enriched by repeating the IMS-SPB enrichment step, eight pairs of the IS^2-S^2 culture tubes derived from the above eight pairs of the IS^1-S^1 culture tubes were examined for the ratio of *tdh* $^+$/total of IS^2 to S^2. Their average value decreased to 19 (range, 0.031 to 87)

(data available upon request), suggesting a negative effect of the further IMS-SPB enrichment. Therefore, the second IMS-SPB enrichment step will hereafter be omitted, and the resulting IMS-incorporated two-step enrichment procedure (A-IS^1) will be further validated.

Comparison of conventional PCR and LAMP for the detection of the *tdh* gene from Japanese shellfish samples.

To show that LAMP improves the detection rate for *tdh* $^+$ *V. parahaemolyticus*, a comparison between conventional PCR and LAMP was made for the culture tubes ($n = 201$) examined in the quantitative and qualitative analyses of the 21 Japanese shellfish samples. Conventional PCR detected the *tdh* gene in 28 (14%) of 201 cultures, whereas LAMP detected the *tdh* gene in an additional 20 cultures, totaling 48 cultures (24%). To investigate features common to the cultures yielding the conventional PCR–negative and LAMP-positive result, the numbers of total and *tdh* $^+$ *V. parahaemolyticus* cells in each culture tube quantified using real-time PCR as explained above were examined. The cultures for which only LAMP gave positive results had a tendency to contain

TABLE 2. *Numbers of total and* tdh $^+$ *V. parahaemolyticus cells in cultures derived from* tdh-*positive Japanese shellfish samples*[a]

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

[a] IS^1 culture, taken after the first IMS-SPB enrichment step of the IMS-incorporated three-step enrichment (A-IS^1-IS^2) procedure; S^1 culture, taken after the first SPB enrichment step of the conventional three-step enrichment (A-S^1-S^2) procedure; *tdh* $^+$ Vp, number of *tdh* $^+$ *V. parahaemolyticus* cells; total Vp, number of total *V. parahaemolyticus* cells; *tdh* $^+$/total, proportion of *tdh* $^+$ *V. parahaemolyticus* cells to the total *V. parahaemolyticus* cells.

TABLE 3. *Levels of* tdh⁺ V. *parahaemolyticus in Thai shellfish samples*[a]

| Shellfish sample designation | IS[1] culture | | S[2] culture | |
	LAMP	Conventional PCR	LAMP	Conventional PCR
Bloody clam 1	2.4	2.6	3.0	3.0
Bloody clam 2	3.4	3.4	3.4	3.4
Bloody clam 3	3.0	1.6	3.0	3.0
Bloody clam 4	3.0	1.6	3.9	3.0
Hard clam 1	2.5	2.5	3.4	3.4
Hard clam 2	5.0	5.0	5.0	5.0
Hard clam 3	3.4	3.4	3.6	3.6
Green mussel	5.0	3.9	3.4	3.9
Undulated surf clam	4.2	4.2	4.2	4.2

[a] Values expressed as log MPN/10 g; IS[1] culture, taken after the first IMS-SPB enrichment step of the IMS-incorporated two-step enrichment (A-IS[1]) procedure; S[2] culture, taken after the second SPB enrichment step of the conventional three-step enrichment (A-S[1]-S[2]) procedure.

(i) a small amount of *tdh⁺ V. parahaemolyticus* cells, which is consistent with previous studies showing the high sensitivity of LAMP *(34, 36, 37)*, 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 nonspecific DNA and too much DNA template can inhibit PCR *(32)*. Compared with conventional PCR, LAMP may be less susceptible to potential DNA amplification inhibition.

Evaluation of the A-IS¹-LAMP procedure for the enumeration of *tdh⁺ V. parahaemolyticus* in Thai shellfish samples. In contrast to the results from the bloody clam risk assessment *(33)*, our previous study indicated a high incidence of patients with *tdh⁺ V. parahaemolyticus* infection in Hat Yai, Southern Thailand *(14)*, stimulating the search for a possible source of the infection. It is known that some retailers store shellfish products overnight and sell them the following morning. Our preliminary studies indicated that shellfish sold at the morning markets contained relatively high levels of pathogenic *V. parahaemolyticus* (data not shown), prompting us to enumerate the level using the A-IS¹-LAMP procedure.

Nine Thai shellfish samples were used with the A-S¹-S² procedure (modification of the conventional three-step enrichment procedure *(9)*) and the A-IS¹ procedure (Fig. 2) in the MPN format, 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 (930 to 110,000 MPN/10 g) of *tdh⁺ V. parahaemolyticus* (Table 3). This suggests that shellfish microflora, including *tdh⁺ V. parahaemolyticus*, had proliferated during overnight storage at the retailers. This hypothesis is supported by previous studies in which preincubation of shellfish samples prior to examination allowed total and pathogenic *V. parahaemolyticus* to grow substantially *(9, 10)*. Although the data shown here are based on only nine shellfish samples collected within 1 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 that LAMP yielded similar or higher MPN values than conventional PCR, with significant differences ranging from 0.9 to 1.4 log MPN/10 g, showing the high performance of LAMP (Table 3).

When the two enrichment procedures (A-S¹-S² and A-IS¹) are compared, the cultures with PickPen-IMS (IS¹) yielded similar or lower MPN values than the cultures without PickPen-IMS (S²) (Table 3). Although the reasons for this are unclear, an environmental study conducted in Hat Yai showed that more than half of the *tdh*-negative *V. parahaemolyticus* isolates from seafood possessed established K antigens *(29)*. Such *tdh*-negative strains can compete with *tdh*-positive strains for specific binding to the immunomagnetic beads. This may be of particular concern with shellfish samples containing total *V. parahaemolyticus* at high levels in tropical coastal environments.

In conclusion, we recommend the A-IS¹-LAMP procedure (the enrichment procedure shown in Fig. 2 in conjunction with LAMP). LAMP showed similar or higher performance than conventional PCR in the detection and quantification of a wide range of *tdh⁺ V. parahaemolyticus* levels in shellfish products. In addition, as indicated in the examination of Japanese shellfish samples at the individual tube level, PickPen-IMS can further facilitate target detection for the shellfish samples containing relatively low levels of total *V. parahaemolyticus*. The result suggests that PickPen-IMS could be used as an optional tool for shellfish samples that require a further improvement in sensitivity of the MPN-LAMP procedure. Although further validation with a large number of shellfish samples harvested in various parts of the world and cost-effectiveness studies are required, the A-IS¹-LAMP procedure could offer a method to measure *tdh⁺ V. parahaemolyticus* levels in shellfish products that can be used by any health authority in the world, contributing to domestic shellfish safety as well as fair world trade.

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Food Control

Detection and quantification of pathogenic *Vibrio parahaemolyticus* in shellfish by using multiplex PCR and loop-mediated isothermal amplification assay

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ARTICLE INFO

ABSTRACT

Vibrio parahaemolyticus is a halophilic bacterium that commonly inhabits the marine and estuarine environments. This organism is also one of the leading causative pathogen of gastroenteritis often related to consumption of raw or undercooked seafood. In this study, molluscan shellfish (bloody clams and surf clams) and crustaceans (shrimps) were monitored in wet markets and hypermarkets. Two molecular methods were employed and compared to detect total and pathogenic *V. parahaemolyticus* in MPN enrichments: multiplex PCR and LAMP assay. The multiplex PCR was optimized to detect the total (*toxR*+), *tdh*+ and *trh*+ *V. parahaemolyticus*. On the other hand, the LAMP assay was employed to target the pathogenic strains only, the *tdh*+ and *trh*+, respectively. Out of 232 samples examined, 229 (98.7%) were positive for *V. parahaemolyticus* with counts ranging from 30 to >110, 000 MPN/g. Positive samples for *tdh*+ *V. parahaemolyticus* were obtained in 77 out of 232 (33.1%) samples ranging from 30 to >110, 000 MPN/g. Meanwhile, positive samples for *trh*+ were identified in 16 out of 232 (6.9%) samples examined ranging from 30 to 9600 MPN/g. Detection of samples with presence of *tdh*+ genes did not vary between methods, but a significant difference was observed when the LAMP assay was compared to PCR to detect *trh*+ *V. parahaemolyticus*. Therefore, on occasions where the density of the targeted genes is low, the LAMP assay serves as a better alternative. Nonetheless, this study constitutes an assessment of presence of total and potentially pathogenic *V. parahaemolyticus* in shellfishes for domestic consumption revealing the potential risk of contracting vibriosis if precautions and safety measures are not properly managed.

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1. Introduction

Vibrio parahaemolyticus was first discovered by Fujino et al. (1953) during the investigation of a shirasu food poisoning outbreak in the 1950s (Shinoda, 2011). Like other members of the genus *Vibrio*, *V. parahaemolyticus* is a Gram-negative and halophilic bacterium commonly found in the estuarine and coastal marine waters. As an opportunistic foodborne pathogen, *V. parahaemolyticus* is often harbored on marine reservoirs, mainly shellfish, serving as a vehicle of infection to human. Consequently, consumption of raw or undercooked seafood with presence of *V. parahaemolyticus* may lead to the development of disease characterized by severe diarrhea, abdominal cramps, nausea, vomiting, headaches, fever and chills (Shimohata &

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Takahashi, 2010). Occasional exposure of skin excisions to *V. parahaemolyticus* may initiate wound infection that may even progress to septicemia in severe case (Daniels & Shafaie, 2000).

Even though *V. parahaemolyticus* is one of the leading causes of foodborne illness related to seafood consumption, not all strains are pathogenic. The comprehensive understanding of pathogenicity of *V. parahaemolyticus* remains unresolved; however, two virulent factors have been clearly identified in association with clinical cases: the thermostable direct hemolysin (TDH) and TDH-related hemolysin (TRH) (Nishibuchi & Kaper, 1995). The TDH protein is encoded by virulent strains bearing the *tdh* gene and shows a characteristic hemolytic activity on the Wagatsuma agar, known as Kanagawa phenomenon. Meanwhile, *trh*+ *V. parahaemolyticus* strains are able to form a weak hemolytic zone in normal blood agar (Shinoda, 2011), although these strains were found to be Kanagawa negative. The close similarity between the TDH and TRH (67% homology) proteins is associated with the mechanism of *V. parahaemolyticus* to cause disease, where both proteins induce the Ca^{2+}-inactivated Cl^- channel of the human colonic epithelial cells leading to alteration of ion flux causing gastroenteritis in infected people (Shimohata & Takahashi, 2010). Although other genetic signatures of virulence has been described for *V. parahaemolyticus*, *tdh*+ and *trh*+ genes are still considered as the main virulent factors contributing to the pathogenicity of this organism.

Conventional methods of detection using biochemical and microbiological tests are often limiting and not determinative (Bej et al., 1999; No, Okada, Kogure, & Park, 2011). These techniques are mainly applicable for obtaining an estimation of the total load of *V. parahaemolyticus* in a sample as an estimate of the potential risk of presence of pathogenic populations. However, rising concerns about the use of total *V. parahaemolyticus* as indicative of risk has arose over the recent years, suggesting the direct detection of virulent markers (*tdh*+ and *trh*+) as a more reliable approach from a food safety perspective, since these pathogenic strains are the ones associated with illness. Alternatively, molecular techniques such as gene-specific probe and polymerase chain reaction (PCR) employed to identify the presence of pathogenic *V. parahaemolyticus* strains were relatively specific and sensitive. However, the former was found to be more laborious compared to the latter, thus PCR have been widely adopted to detect the presence of pathogenic *V. parahaemolyticus* strains in food and in the environment (Bej et al., 1999; Panicker, Call, Krug, & Bej, 2004; Yamamoto et al., 2008). The subsequent introduction of real-time PCR which conferred an additional advantage to the conventional PCR since visualization of PCR products by gel electrophoresis is not required and results are obtained in real-time, facilitating a rapid detection of *V. parahaemolyticus* (Nordstorm, Vickery, Blackstone, Murray, & DePaolo, 2007). However, this assay is quite sophisticated and needs expensive equipment.

A novel molecular method known as the Loop-mediated Isothermal Amplification (LAMP) was subsequently introduced by Notomi et al. (2000). This technique amplifies DNA rapidly with high specificity and sensitivity under isothermal condition. A specific LAMP procedure for the detection of total *V. parahaemolyticus* was further developed (Nemoto et al., 2011; Yamazaki, Ishibashi, Kawahara, & Inoue, 2008) followed by the progressive introduction of a set of primers to detect the *tdh* and *trh* genes in the same assay in order to screen for pathogenic *V. parahaemolyticus* strains (Yamazaki, Kumeda, Misawa, Nakaguchi, & Nishibuchi, 2010). Prominently, LAMP offers several advantages with respect to previous approaches: firstly, only a simple isothermal chamber is required to perform the assay; secondly, results are easily determined through visual judgment or in real-time with the aid of a turbidimeter platform; and thirdly, the procedure is completed within one hour.

The increasing concern of foodborne pathogens to the public health enhanced the need of rapid detection and identification methods. In this study, we adopted two molecular detection methods: a multiplex PCR and a LAMP assay to investigate the incidence of the total and pathogenic *V. parahaemolyticus* in the commonly eaten shellfish, namely the mollusks (bloody clams and surf clams) and the crustacean (shrimps). Besides being rapid and sensitive, both assays are easily applicable in the laboratory settings without the needs of sophisticated equipment or trained personnel, facilitating the surveillance control. Therefore, as an effort of food surveillance, monitoring the *V. parahaemolyticus* in the Malaysian shellfish may provide helpful information to the locals regarding safe consumption. Moreover, the successive information may further facilitate the development of the seafood industry to strife for greater food hygiene with improved stringency on the microbial control.

2. Materials and methods

2.1. Sample collection

A total of 232 shellfish samples were randomly purchased from various wet markets and hypermarkets located in Selangor, Malaysia within a period of nine months (March 2013 to November 2013). One hundred nineteen samples were collected from wet markets comprising of 44 blood clams (*Anadara granosa*), 38 surf clams (*Paphia undulata*) and 37 shrimps (*Penaeus* spp.). The remaining 113 samples were collected from the hypermarkets comprising of 40 blood clams, 38 surf clams and 35 shrimps. During collection, all the samples were transferred to sterile plastic bags for transportation and were analyzed immediately on their arrival at the laboratory.

2.2. Sample processing

A 10-g portion of the shellfish was mixed with 90 ml of Alkaline Peptone Water (APW; Merck, Germany) in a sterile stomacher bag and was homogenized using a stomacher (Interscience, France) for a minute. Subsequently, the homogenate was subjected to qualitative and quantitative analysis as described in the schematic diagram shown in Fig. 1.

2.2.1. Enrichment protocol for qualitative analysis of shellfish

Ten milliliters of homogenate was transferred into a sterile tube and incubated for 18 h at 35 ± 2 °C. Subsequently, one ml of the enriched sample was removed and subjected to DNA extraction for PCR and LAMP assay. The qualitative analysis served as a preliminary study; consequently, shellfish samples showing negative results for presence of *V. parahaemolyticus* were not subjected to quantitative analysis.

2.2.2. Enrichment protocol for quantitative analysis of shellfish

The three serial dilutions of three-tube most-probable number (MPN) method was employed for quantification as described in the U.S. Food and Drug Administration's *Bacteriological Analytical Manual* with slight modifications. Briefly, a series of 10-fold dilution was performed by transferring 1 ml of the homogenate into 9 ml of APW; 1 ml of the dilutions were then transferred into sterile tubes, with each tube containing a final amount of sample of 10^{-2} to 10^{-5} g in triplicates. Tubes were incubated at 35 ± 2 °C for 18 h. Total *V. parahaemolyticus* was determined using the PCR. Simultaneously, shellfish samples positive for pathogenic *V. parahaemolyticus* (*tdh*+ or *trh*+) were quantified by MPN with PCR and LAMP assay. However, due to the high loads of *V. parahaemolyticus* in samples, all the tubes from the first dilution yielded positive results and consequently were
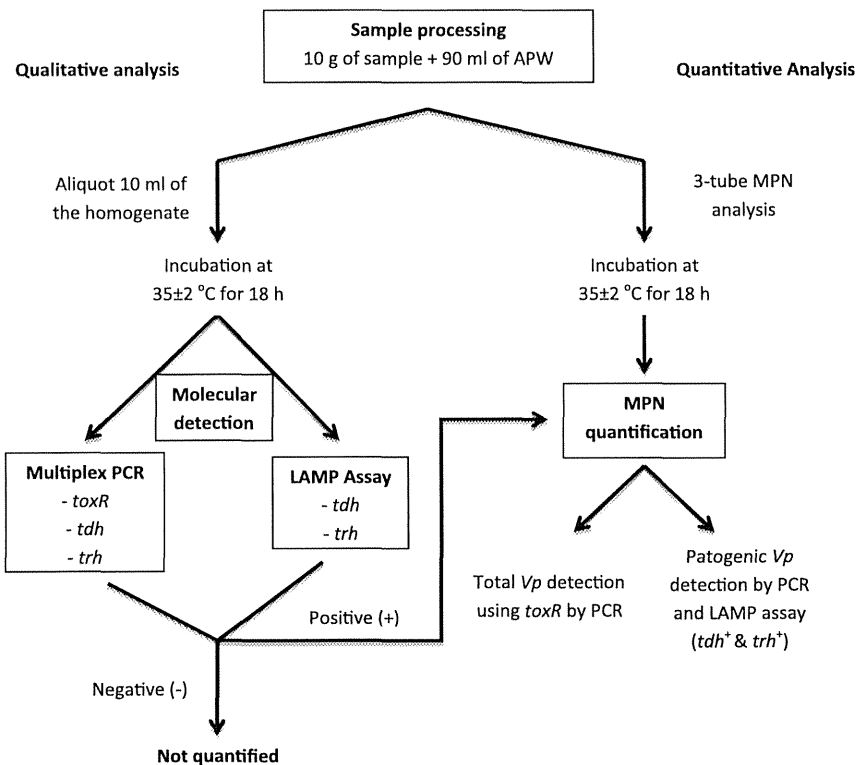
Fig. 1. Schematic representative of qualitative and quantitative analyses of total and pathogenic (tdh+ and trh+) V. parahaemolyticus in shellfishes.

non-informative to estimate the total count (data not shown). Hence, the first dilution was not considered afterward.

2.3. DNA template preparation

DNA extraction was carried out using the boiling cell method. A 1-ml aliquot of the inoculated APW after incubation was centrifuged at 10,000 RPM for 5 min. The supernatant was removed and the pellet was re-suspended in 200 µl of sterile distilled water and boiled for 15 min. Suspensions were stored at −20 °C until further use. Before being used, suspensions were centrifuged at 10,000 RPM for 1 min to sediment the cell debris and the supernatant was used as the DNA template.

2.4. Multiplex PCR assay

Three sets of primers (SIGMA; USA) were used, respectively, the species-specific gene toxR (F: 5′-GTCTTCTGACGCAATCGTTG-3′ & R: 5′-ATACGAGTGGTTGCTGTCATG-3′; Kim et al., 1999) and the virulent markers genes tdh (F: 5′-CCACTACCACTCTCATATGC-3′ & R: 5′-G GTACTAAATGGCTGACATC-3′; Tada et al., 1992) and trh (F: 5′-TTG GCTTCGATATTTTCAGTATCT-3′ & R: 5′-CATAACAAACATATGCCCATTT CCG -3′; Bej et al., 1999). The primer sets used in this study have been reported to be effective in detecting V. parahaemolyticus and the respective pathogenic strains; however, an improvement was made to facilitate the primers to perform in a multiplex format. The optimized mPCR was performed in 25 µL reaction mixture (PROMEGA; USA) using 1.4× PCR Buffer, 2.5 mM of MgCl₂, 0.2 mM of dNTP, 0.2 µM of each primers, 2.5U of Taq Polymerase and 2 µL of DNA, topped up until 25 µL with dH₂O. The following cycling condition were applied; 3 min of pre-denaturation at 95 °C followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 60 °C for 45 s and extension at 68 °C for 1 min. A final extension of 72 °C for 3 min was used and the product was left indefinitely at 10 °C.

### 2.4.1. Specificity and sensitivity test of multiplex PCR

Under the optimum condition the specificity of the mixed oligonucleotide primers was tested on the strains: VP81 (trh−/tdh+), AT4 (trh+/tdh−) and a mixture of both strains at ratio of 1:1. Other Vibrio strains (Vibrio cholerae, Vibrio alginolyticus and Vibrio vulnificus) and non-Vibrio strains (E. coli, K. pneumonia, Salmonella spp., Campylobacter spp., Listeria spp.) that were previously isolated in our laboratory were also included in the evaluation. The sensitivity of the multiplex PCR was evaluated on VP81 and AT4 determined by purified DNA template and pure culture. The detection limits were defined as the highest dilutions that resulted in positive amplification, with templates used ranged from 1 to 5 µL. Briefly, the extracted DNA (100 ng each) from the respective strains was serially diluted in sterile distilled water in 10-fold steps, and the assay was performed individually and in combination of both strains. Sensitivity on pure culture was performed by serially diluting an overnight culture of each strain in 10-fold steps, and 100 µL of each diluent was boiled followed by the multiplex PCR assay as described. Simultaneously, an aliquot of 100 µL from each dilution was plated on Tryptic Soy Agar plate (3% NaCl) and incubated overnight at 35 °C to determine the cell density.

2.5. LAMP assay

The LAMP assay for the respective virulent factors (tdh or trh) was performed using the Loopamp DNA amplification kit (EIKEN; Japan) as described below:

1) tdh-LAMP Assay (Tanaka et al., 2014; Yamazaki et al., 2010)

    The tdh-LAMP assay was performed using 25-µl of a 1X reaction mixture containing: 2 µl of DNA template, 1 µl of BST DNA polymerase, 1.6 µM of each inner primers FIP and BIP, 0.2 µM of each outer primers F3 and B3 and 0.8 µM of each loop primers LF and LB.
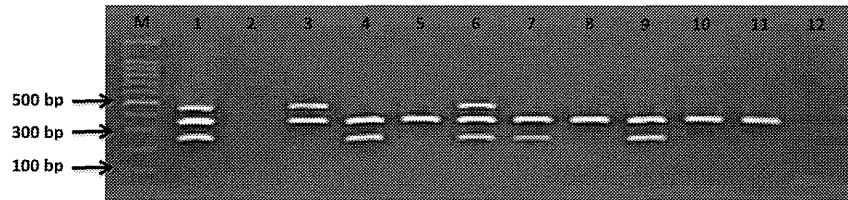
Fig. 2. Agarose gel electrophoresis of PCR products corresponding to amplification of *trh* gene (484 bp), *toxR* gene (368 bp) & *tdh* gene (251 bp). Lane 1 = mixture of *tdh* (VP81) and *trh*-positive (AT4) *V. parahaemolyticus;* Lane 2 = negative control; Lane 3 & Lane 4 = *trh*-positive (AT4) and *tdh*-positive (VP81) *V. parahaemolyticus* strains, respectively; Lane 5 to Lane 12 = Shellfish samples examined; M = 100 bp DNA marker.

2) *trh*-LAMP Assay (Nishibuchi M., Unpublished data)

The *trh*-LAMP assay was performed using 25-μl of a 1X reaction mixture containing: 2 μl of DNA template, 1 μl of BST DNA polymerase, 1.6 μM of each sets of inner primers FIP-BIP and FIP2-BIP2, 0.2 μM of each outer primers F3 and B3 and 0.8 μM of each loop primers LF, LB and LB2.

The reaction mixtures were incubated in a Loopamp real-time turbidimeter (EXIA, Teramees, Co. Ltd., Kyoto Japan) at 63 °C for 60 min and 80 °C for 10 min to complete the reaction. The real time detection was determined based on the turbidity at 650 nm using the EXIA turbidimeter. A positive reaction was identified when the turbidity reached 0.1 within 60 min.

2.6. Data analysis

Difference between the LAMP and PCR were assessed by sample-by-sample comparison, individual MPN tubes scoring (positive versus negative) and the MPN estimates of abundance. The analysis and significant differences for sample-by-sample and individual MPN tubes were determined using the chi-square (χ^2) test, where the level of significance was set at $p < 0.05$. The estimates of abundance on the other hand were determined using the standard MPN calculation of each target for each assay. Difference in $\log_{10}$ was determined between the assays.

3. Results and discussion

3.1. Optimization, specificity and sensitivity of the multiplex PCR assay

Conventional PCR uses a set of primers to amplify a single gene of interest and is rather time consuming and cost ineffective. The fact is that a more sophisticated approach such as the multiplex PCR has considerably overcome such limitations. For instance, additional information may be gained from a single test run that otherwise would require several times the reagents and time to perform. However, an underoptimized multiplex PCR often lead to low sensitivity or random amplification. In this study, the multiplex PCR for *V. parahaemolyticus* detection was successfully optimized as described in materials and methods (Fig. 2). The presence of *V. parahaemolyticus* is indicated by the amplification of the *toxR* gene (368 bp), a well-conserved sequence among the *V. parahaemolyticus* strains. Meanwhile, the *tdh*+ and *trh*+ *V. parahaemolyticus* are distinguished by the respective amplicons: *tdh* gene (251 bp) and *trh* gene (484 bp). For instance, the *tdh*+ strain used as control was the VP81 (lane 4 in Fig. 2) and the *trh*+ was represented by the AT4 strain (lane 3 in Fig. 2), where the *toxR* gene amplicon was seen in both strains but the pathogenicity marker was different for the respective strains. Specificity of the assay was achieved, as no bands were amplified from non-

V. parahaemolyticus isolates (data not shown). The detection limit for the *toxR* and *tdh* gene was 20 pg (3.7 CFU per reaction), whereas the *trh* gene was 1-fold less sensitive (40 pg; 8.3 CFU per reaction) when the assay was evaluated with VP81 and AT4 strains, respectively. Even in combination of the purified DNA templates, the multiplex PCR sensitivity was not compromised. Although the *trh* gene was less sensitive, the difference was rather negligible as the assay provided a sensitivity of <10 CFU cells per reaction well within the range of 10^3 to 10^4 cells/ml.

Three parameters were manipulated for successive amplification; the PCR buffer concentration, $MgCl_2$ concentration and the annealing temperature. The PCR buffer and $MgCl_2$ were found to be influential in the amplification process, whereby the change in concentration for either component may lead to random or lack of amplification. Our study found that by increasing the PCR buffer concentration up to 1.4× random amplification was significantly reduced or eliminated, whereas further increment resulted in lack of product amplification. Worth to note that by manipulating the PCR buffer concentration, we significantly manipulated the KCl concentration. According to Henegariu, Heerema, Dlouhy, Vance, and Vogt (1997), increasing the concentration of KCl reduces the unwinding of DNA during denaturation, hence, reducing the likeliness of random binding during primer annealing. Significantly, above the threshold concentration, reduced amplification was observed reasonably due to the tight bonding between the DNA strands. Noteworthy that $MgCl_2$ serves as a co-factor for the Taq polymerase and dNTP, concurrently promotes the binding between the primers and the target genes. In our study, the $MgCl_2$ concentration of 2 mM and 2.5 mM was found to be significantly effective. The presence of free Mg^{2+} is necessary for successive PCR amplification; however, the abundance of free Mg^{2+} significantly promotes random binding. It was also found that increased or decreased concentration of $MgCl_2$ resulted in random amplification and weak/no product amplification, respectively. Hence, an equilibrate concentration of $MgCl_2$ is essential.

The annealing temperature of 60 °C was found to be optimum in pairing of the primers. Notably, the change of annealing temperature resulted in significant reduction in the intensity of *trh* amplification, but exhibited a minor effect on the *toxR* and *tdh* markers (data not shown). The reason for such observation may be unclear, but it is hypothesized that the close homology (approximately 68% homology) between the *tdh* and *trh* marker may have influenced the PCR amplification, considering the fact that the *trh* and *tdh* primers may overlap each other resulting in weak amplification of *trh* marker which is the larger product (484 bp) (Yamazaki et al., 2010).

3.2. Prevalence and enumeration of total V. parahaemolyticus

The incidence and level of *V. parahaemolyticus* in shellfish samples obtained from wet markets and hypermarkets are summarized in Table 1. Out of 232 samples, 229 (98.7%) were

Table 1
The incidence and level of total *V. parahaemolyticus* in shellfish samples examined from hypermarkets and wet markets.

Sampling Sites	Samples	Number of samples tested	Number of positive samples (%)	Level of total *V. parahaemolyticus* (MPN/g)				
				<100	10^2–10^3	10^3–10^4	10^4–10^5	>10^5
Wet market	Bloody clam (CW)	44	44 (100%)	—	1	1	9	33
	Surf Clam (LW)	38	38 (100%)	—	1	2	1	34
	Shrimp (SW)	37	36 (97.3%)	3	2	6	11	14
Hypermarket	Bloody Clam (CH)	40	40 (100%)	—	—	4	11	25
	Surf Clam (LH)	38	37 (97.4%)	10	7	10	5	5
	Shrimp (SH)	35	34 (97.1%)	—	1	12	9	12
Total		232	229 (98.7%)	13	12	35	46	123

V. parahaemolyticus positive, prevalently from the wet markets as seen in all (100%) the bloody clams and surf clams, and 97.3% of the shrimps examined ranging from 62 to >110, 000 MPN/g of *V. parahaemolyticus*. On the other hand, *V. parahaemolyticus* was present in all bloody clams, 97.4% surf clams and 97.1% shrimps screened from the hypermarkets ranging from 30 to >110,000 MPN/g. In the absence of a standard guideline in Malaysia, the standard safety level of <10^4 MPN/g of *V. parahaemolyticus* in seafood recommended by the Food and Drug Administration (FDA) was adopted as a reference. A total of 102 of 119 (85.7%) samples from the wet markets and 67 of 113 (59.3%) samples from the hypermarkets were found to be positive with *V. parahaemolyticus* above the legislative level. Noteworthy, *V. parahaemolyticus* is often found in the coastal waters of the tropical and sub-tropical countries. Such environments often facilitate the survival or growth of this pathogen, hence, the presence of *V. parahaemolyticus* in Malaysian shellfishes could be considered natural and expected (Nakaguchi, 2013). However, the question remains unclear whether the high microbial counts were mainly contributed by the pre-harvesting or the post-harvesting phase. We postulate that the latter would serve as the primary factor considering the fact that proper practices from the post-harvesting and by the intermediaries (retailers) are often neglected (Bilung et al., 2005; Noorlis et al., 2011).

Limited to the accessibility from farm to fork, we scrutinized the preservation managements by the intermediaries instead, where we found that proper handling of the shellfishes are considerably lacking, primarily by the wet market retailers. For instance, the bloody clams and surf clams are displayed at ambient temperature by the wet markets handlers and similar approach was adopted by the hypermarkets where the bloody clams are also displayed at room temperature. Such practice by the retailers is highly discouraged because it would allow the growth of *V. parahaemolyticus* inside these filter feeders, thus may have significantly contributed to the high counts of *V. parahaemolyticus* as seen in our study. Comparatively, the surf clams collected from the hypermarkets were found to be lower in *V. parahaemolyticus* density as these mollusks are maintained on ice during retailing. Similar approach was adopted to preserve the shrimps, hence the *V. parahaemolyticus* counts in the crustacean samples were comparable for both retails and considerably lower than in the molluscan shellfishes. Worth to note that *V. parahaemolyticus* are cold-sensitive, hence the cell viability may have been limited or reduced by maintaining the shellfishes on ice (Su & Liu, 2007). Therefore, the knowledge of maintaining the shellfishes at refrigerated temperatures (<10 °C) should be disseminated to the retailers to reduce the risk of *V. parahaemolyticus* food poisoning. Storing of shellfishes at refrigerating temperature may be worthwhile, however, on certain occasions the *V. parahaemolyticus* counts may still remain high in samples examined suggesting the possibility of the presence of cold-tolerant strains capable of adapting to low temperatures (Burnham et al., 2009). Thus, other approaches to reduce the microbial loads in shellfishes during post-harvesting, for

instance, the relaying and depuration process, mild heat treatment, high-pressure processing and irradiation are encouraged (Yeung & Boor, 2004).

3.3. Prevalence and enumeration of pathogenic *V. parahaemolyticus*

Not all strains of *V. parahaemolyticus* cause illness; on the contrary, only a small percentage of the total *V. parahaemolyticus* is pathogenic. Pathogenic *V. parahaemolyticus* strains are more likely to produce symptomatic infections and have one or more distinctive traits that are generally absent in non-pathogenic strains. The incidence and level of the pathogenic *V. parahaemolyticus* screened from wet markets and hypermarkets are summarized in Table 2 (*tdh*+) and Table 3 (*trh*+). Out of 232 samples, 77 (33.1%) were *tdh*+ *V. parahaemolyticus* positive. Thirty-three of 44 (75%) samples of bloody clams and 19 of 38 (50%) of surf clams collected in wet markets were found to be positive for *tdh*+ *V. parahaemolyticus* with values ranging from 30 to >110,000 MPN/g. On the other hand, 16 of 232 (6.9%) samples examined showed positive values for *trh*+ *V. parahaemolyticus*. Eleven shellfish samples from the wet markets were positive for *trh*+ *V. parahaemolyticus*, with levels ranging from 36 to 4300 MPN/g, whereas the remaining 5 samples were screened from hypermarkets ranging from 30 to 9300 MPN/g. However, pathogenic *V. parahaemolyticus* was only detected in mollusks but not in shrimps. The ability for the former to bioconcentrate may have facilitated the accumulation of the pathogenic strains in their guts, thus contributing to the high prevalence (Lee, Liu, & Huang, 2003). Besides, a possible explanation to such observation may be due to the difference in total *V. parahaemolyticus* concentration. As reported previously by Hara-Kudo et al. (2003), the incidence of pathogenic *V. parahaemolyticus* was relatively higher in seafood with high density of *V. parahaemolyticus*. Hence, the absence of these pathogens in shrimps is not intriguing since the presence of total *V. parahaemolyticus* counts in shrimps was relatively lower compared to the mollusks examined.

Studies carried out in other regions of the world, for instance in the United States (Parveen et al., 2008), Europe (Martinez-Urtaza et al., 2008; Rosec, Simon, Causse, & Boudjemaa, 2009), North (Zimmerman et al., 2007) and South America (Garcia et al., 2009) reported a higher incidence of *trh*+ strains over the *tdh*+ ones. However, in this study, the presence of *tdh*+ *V. parahaemolyticus* was relatively higher compared to the *trh*+ strains, regardless of qualitative or quantitative count which is in agreement with the findings of DePaola, Nordstrom, Bowers, Wells, and Cook (2003), where *trh*+ *V. parahaemolyticus* are uncommon contaminants in the Asian environment and most strains isolated from diarrheal patients were TDH producers (Shinoda, 2011). Besides, a possible explanation for such observation may due to the difference in temperature affiant among strains, where the *tdh*+ strain has a higher tendency towards warmer coastal water, whereas the *trh*+ strains are more dominant in colder water (Rodriguez-Castro et al.,

Table 2

The incidence and level of pathogenic (tdh+) V. parahaemolyticus in shellfish samples examined from hypermarkets and wet markets.

Sampling sites	Samples	Number of samples tested	Number of positive samples (%)	Level of (tdh+) V. parahaemolyticus (MPN/g)				
				<100	10^2–10^3	10^3–10^4	10^4–10^5	> 10^5
Wet market	Bloody Clam (CW)	44	33 (75%)	15	15	3	–	–
	Surf Clam (LW)	38	19 (50%)	1	5	6	–	7
	Shrimp (SW)	37	ND	–	–	–	–	–
Hypermarket	Bloody Clam (CH)	40	18 (45%)	9	8	1	–	–
	Surf Clam (LH)	38	7 (18.4%)	6	1	–	–	–
	Shrimp (SH)	35	ND	–	–	–	–	–
Total		232	77 (33.1%)	31	29	10	–	7

Table 3

The incidence and level of pathogenic (trh+) V. parahaemolyticus in shellfish samples examined from hypermarkets and wet markets.

Sampling Sites	Samples	Number of samples tested	Number of positive samples (%)	Level of (trh+) V. parahaemolyticus (MPN/g)				
				<100	10^2–10^3	10^3–10^4	10^4–10^5	> 10^5
Wet market	Bloody clam (CW)	44	7 (15.9%)	3	3	2	–	–
	Surf Clam (LW)	38	4 (10.5%)	3	1	–	–	–
	Shrimp (SW)	37	ND	–	–	–	–	–
Hypermarket	Bloody Clam (CH)	40	5 (12.5%)	2	1	2	–	–
	Surf Clam (LH)	38	ND	–	–	–	–	–
	Shrimp (SH)	35	ND	–	–	–	–	–
Total		232	16 (6.9%)	8	6	2	–	–

2009). As seen in this study, the presence of trh+ strains were often accompanied with by tdh+ strains as seen in 13 (81.3%) of the 16 positive samples. The co-occurrence of both pathogenic strains suggest a clear dominance of tdh+ strains over the trh+ strains, where the latter often co-populate when the former is highly enumerated in our samples. Therefore, tdh+ strains are expected to prevail over the trh+ strains in tropical and sub-tropical countries, for instance in Malaysia (Mohammad, Hashim, Gunasalam, & Radu, 2005; Sujeewa, Norrakiah, & Laina, 2009) and several countries in Southeast Asia (Nakaguchi, 2013).

The legislative standard by the FDA may serve as a preventive measurement, nonetheless may not represent the real scenario. For instance, 6 mollusk samples with total V. parahaemolyticus counts of <10^4 MPN/g were found to be positive with tdh+ strains ranging from 30 to 420 MPN/g. Vice versa, high total counts of V. parahaemolyticus sample may not necessarily contain strains bearing pathogenic genes. Therefore, the quantification of pathogenic V. parahaemolyticus is often critical in food safety management, which is preferred over the total V. parahaemolyticus count, where several referrals V. parahaemolyticus outbreak reported by the FDA were subjected by <10^4 V. parahaemolyticus/g of contaminants (Kaysner & DePaola, 2000; Yeung & Boor, 2004). Contributively, the insights from our study demonstrated that these pathogenic V. parahaemolyticus are frequently found in the commonly eaten mollusks in Malaysia raising considerable public health concerns concerning the domestic consumptions of these filter feeders. The bloody clams are consumed raw or undercooked whereas the surf clams are well-cooked in Malaysian culture. Thus, potentially the former may serve as the vehicle for V. parahaemolyticus infection over the surf clams. Alarmingly, seven surfs clams purchased from the wet markets were found to be positive with >10^5 MPN/g of the tdh+ V. parahaemolyticus. The reason for the latter is not known, hence, further investigation is required as surf clams may be a significant vehicle for V. parahaemolyticus infection if they are improperly cooked.

3.4. Comparative study between the multiplex PCR and the LAMP assay

Two methods of detection were compared; the multiplex PCR and LAMP assay. In this study, multiplex PCR was found to be cost

and workload effective when quantitative studies were conducted, where simultaneous detection of toxR, tdh and trh gene were applicable. In the contrary, the LAMP assay does not facilitate such detection; hence individual assays were conducted for the detection of tdh+ and trh+ V. parahaemolyticus, respectively. Comparatively, the LAMP assay was rather promising in qualitative studies, where the presence or absence of the pathogenic (tdh+ or trh+) was determined within an hour. Therefore, as a diagnostic tool, LAMP offers a rapid and robust detection of virulent V. parahaemolyticus strains over the multiplex PCR. For instance, the LAMP assay may come in handy when an outbreak occurs where imminent confirmation is required. The multiplex PCR on the other hand serves well as a food safety surveillance tool to monitor the microbial counts within a time frame.

Noteworthy, both assays offer advantages over the conventional detection using the biochemical methods: less laborious and time effective, overgrowth of other naturally occurring contaminants may mask the detection of V. parahaemolyticus, tentatively the pathogenic strains (tdh+ or trh+) and false negative due to the inhibitory effects of the biochemical compounds (Copin, Robert-Pillot, Malle, Quilici, & Gay, 2012). LAMP has been demonstrated to be at least 10–100 folds more sensitive compared to PCR in

Table 4

Discrepancies between the LAMP and PCR assay in detecting pathogenic V. parahaemolyticus, tdh+ and trh+, respectively.

Pathogenicity	Sample/Designation	Conc. (log_{10} MPN/g)		Log_{10} difference
		LAMP	PCR	
tdh+	Bloody Clam (CW38)	2.36	1.96	0.4
V. parahaemolyticus	Bloody Clam (CH24)	3.63	3.18	0.45
	Surf Clam (LW14)	3.63	2.56	1.07
	Surf Clam (LW17)	1.96	1.48	0.48
trh+	Bloody Clam (CW37)	2.36	1.48	0.88
V. parahaemolyticus	Bloody Clam (CW41)	2.36	1.48	0.88
	Bloody Clam (CW42)	2.36	ND[a]	2.36
	Bloody Clam (CW44)	1.56	ND	1.56
	Bloody Clam (CH38)	3.363	2.363	1.00
	Bloody Clam (CH39)	2.18	ND	2.18
	Bloody Clam (CH40)	1.96	ND	1.96
	Surf Clam (LW22)	1.96	ND	1.96
	Surf clam (LW23)	2.18	ND	2.18

[a] ND; Not detected by the assay quantitatively.

Table 5
Chi-square (χ^2) test for the calculation of significant differences.

Pathogenicity	Sample-by-sample			Tubes-by-tubes (MPN)		
	LAMP	PCR	p-Value	LAMP	PCR	p-Value
tdh+ V. parahaemolyticus	77/232 (33.2%)	77/232 (33.2%)	1.00	305/308 (99.0%)	302/308 (98.1%)	0.3137
trh+ V. parahaemolyticus	16/232 (6.9%)	12/232 (5.1%)	0.4354	52/64 (81.3%)	30/64 (46.9%)	5.06E-5

detecting *V. parahaemolyticus* (Chen and Ge, 2010; Yamazaki et al., 2008), the *tdh* (Yamazaki et al., 2010) and *trh* strains (Nishibuchi M., Unpublished data). Since the samples were enriched, both methods offered similar detection sensitivity, however, the LAMP assay was rather superior over the multiplex PCR on occasions (Table 4). The *tdh*-LAMP assay was found to be 0.48 to 1.07 log MPN/g more sensitive than PCR. The disagreements between the two methods were seen in 4 of 77 (0.05%) samples detected with *tdh*+ *V. parahaemolyticus*. However, there were no significant differences between the two assays based on the statistical analysis based on sample-by-sample and individual MPN tubes scoring (Table 5). The *trh*-LAMP on the other hand detected 0.88 to 2.362 log MPN/g more than the multiplex PCR where discrepancies were seen in 9 of 16 (56.3%) of the *trh*+ samples. Although there were no significant differences when the two assays were compared sample-by-sample, a substantial difference was observed when the individual MPN tubes scorings were determined (Table 5).

We postulate that the sensitivity of LAMP is superior over the multiplex PCR on occasions where the contaminant microbes appeared low in density. Although MPN enrichment may facilitate microbial cell recovery and growth, but the presence of competing microbes (high ratio) or injuries sustained during sampling may have limited the cell growth. For instance, a significant difference ($p < 0.05$) between assays was seen for the detection of *trh*+ harboring *V. parahaemolyticus* when individual MPN tubes were compared, further supports our hypothesis. In such condition, PCR may not facilitate the detection, rather in some cases, subsequent enrichment or double PCR may be required which is time consuming (Alam, Tomochika, Miyoshi, & Shinoda, 2001; Hara-Kudo et al., 2003). Such observation was also reported by Tanaka et al. (2014), where they were able to detect better in MPN enrichment format with the LAMP assay when the target cells were present at a low concentration, however, the difference was minute when the samples were highly contaminated. Hence, the *trh*+ *V. parahaemolyticus* detection by the LAMP assay was rather convincing where the *trh*+ strains were only detected when the samples tended to be highly contaminated with *V. parahaemolyticus* (>10^4 MPN/g) suggesting that ratio of *trh*+ over the total *V. parahaemolyticus* is relatively small in the environment. Comparatively, the detection for the *tdh*+ *V. parahaemolyticus* did not vary much between both methods.

4. Conclusion

The sensitivity of LAMP over the multiplex PCR may come in handy on occasions where determination of low counts of pathogenic (*tdh*+ or *trh*+) *V. parahaemolyticus* cells in a diverse background of competing microflora is desired, for instance the *trh*+ strains as seen in our study. Nonetheless, routine screening of *V. parahaemolyticus* is necessary to monitor changes in levels of this foodborne pathogen to reduce the incidence of foodborne infections. The presence of *V. parahaemolyticus* in shellfishes is inevitable, though not all the *V. parahaemolyticus* detected in our shellfish samples were pathogenic, rather only the molluscan shellfishes (bloody clams and surf clams) containing pathogenic strains of *V. parahaemolyticus*. To our utmost knowledge, no

outbreaks or sporadic cases due to *V. parahaemolyticus* have been recently reported in Malaysia. However, some mollusks were found to contain high levels of virulent strains of *V. parahaemolyticus* addressing the concern towards public health. Therefore, proper handling of these mollusks should be channeled not only to the retailers, but also to the consumers. For instance, the consumers should be educated on the risk involved and preventive measures, such as proper storage and cooking practices that should be prioritized. If the filter feeders are not properly handled, they may serve as a vehicle of food poisoning by *V. parahaemolyticus* through consumption or as cross-contaminants.

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Effect of detergents as antibacterial agents on biofilm of antibiotics-resistant *Vibrio parahaemolyticus* isolates

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ABSTRACT

Vibrio parahaemolyticus (V. parahaemolyticus) is a halophilic, Gram-negative human pathogen known as a leading cause of seafood-derived food poisoning. Due to high contamination rate of seafood in Asian countries, V. parahaemolyticus is considered as a food safety concern. V. parahaemolyticus is able to produce biofilm which is more resistant toward disinfectants and antibodies than its planktonic form. Thirty six V. parahaemolyticus isolates from seafood were tested for their susceptibility using 18 different antibiotics. Two V. parahaemolyticus isolates were resistant to bacitracin, chloramphenicol, rifampin, ampicillin, vancomycin, nalidixic acid, penicillin and spectinomycin. Fourteen V. parahaemolyticus isolates were found to be resistant to bacitracin, tetracycline, rifampin, ampicillin, vancomycin, penicillin and spectinomycin. The remaining two isolates were resistant to more than 2 antibiotics. Majority of the V. parahaemolyticus isolates (97.2%) showed MAR index > 0.2, indicating that these isolates were originated from high risk sources. To investigate effect of three common detergents on antibacterial-resistant V. parahaemolyticus, 16 V. parahaemolyticus isolates resistant to more than 7 antibiotics were selected. V. parahaemolyticus (ATCC 17802) was used as reference strain. Detergents were tested for their minimum inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) and time–kill curves were constructed to assess the concentration between MIC and bactericidal activity of detergents. Detergents D1 (Linear alkyl benzene based) was found to be the most effective with MIC and MBC ranged between 97.656 and 1562.5 μg/ml and 781.25–3125 μg/ml, respectively. The time–kill curves demonstrated that the bactericidal endpoint for resistant V. parahaemolyticus isolates reached after 30 min incubation with D1 at concentration 8 × MIC. The isolate VP003 was killed at 8 × MIC within 0.5 h and the reduction in CFU/ml was 3 log units (99.9%). V. parahaemolyticus biofilms were formed in 96 wells microtiter plates at 37 °C and 24 h-old biofilm were used to test antibacterial activity of detergents. Results showed that biofilm-producing ability of antibacterial-resistant V. parahaemolyticus isolates were inhibited at 1562.5–6250 μg/ml of D1 and eradicated at 3125 – ≥50,000 μg/ml of D1. Detergents showed potential antimicrobial activity against V. parahaemolyticus

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1. Introduction

Vibrio parahaemolyticus (V. parahaemolyticus) is a halophilic and Gram-negative human pathogen (Broberg, Calder, & Orth, 2011), known as a leading cause of seafood-derived food poisoning (Su & Liu, 2007; Yeung & Boor, 2004). *V. parahaemolyticus* is widely distributed within estuarine and marine environments in warm and tropical areas (Vora et al., 2005; Xu, Wang, Sun, Liu, & Li, 2013). *V. parahaemolyticus* usually attach to underwater surfaces (McCarter, 1999) and is generally isolated from a variety of raw seafood. It is mainly associated with outbreaks related to consumption of shellfish (Kawatsu, Ishibashi, & Tsukamoto, 2006; Xu et al., 2013). Virulent strains of *V. parahaemolyticus* cause acute gastroenteritis, septicaemia and wound infections. Consumption of raw/undercooked seafood results in acute gastroenteritis with the following symptoms: headache, abdominal cramps, vomiting, diarrhea and nausea (Broberg

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et al., 2011; Liston, 1990). *V. parahaemolyticus* is a food safety concern in many Asian countries as the rate of seafood consumption is high (Su & Liu, 2007). In eastern China, 40.1% form total of 802 outbreaks of food-borne disease was related to *V. parahaemolyticus* (Chao et al., 2010). *V. parahaemolyticus* can infect individuals through open wounds and almost 66% of wound infections result in death (CDC, 2005). Due to the harmful effects and high prevalence of *V. parahaemolyticus* in Asian countries, efficient diagnostic and treatment methods are highly needed (Wang et al., 2011). Seafood products are the main parts of daily food in the South and South-eastern parts of Asia. Bloody clam, lala, shrimp and squid are among the favorite fishery products. Shrimp is the most susceptible one which can be contaminated with variety of bacteria especially *Vibrio* family (Krupesha Sharma et al., 2010). Even water treatment processes (such as chlorination, addition of antibiotics and using sand filters in shrimp farms) could not reduce *Vibrio* contamination of shrimps. It is assumed that *Vibrio* form biofilms on surfaces of shrimp farms (Karunasagar, Otta, & Karunasagar, 1996). Bacterial biofilms are defined as complex aggregation of bacteria that are encased in a self secreted exopolymeric matrix consisting of exopolysaccharides (EPS) (Blascher et al., 2007; Sybiya Vasantha Packiavathy, Agilandeswari, Syed Musthafa, Pandian, & Ravi, 2012). Bacterial biofilms are reported to be more resistant to action of disinfectants, antibodies and antibiotics than their planktonic forms (Caraher, Reynolds, Murphy, McClean, & Callaghan, 2007; Krupesha Sharma et al., 2010). According to Centres for Disease Control and Prevention, almost 65% of all reported infections are caused by bacterial biofilms (Lewis, 2007). Like other biofilm producer microorganisms, *V. parahaemolyticus* is capable of producing some distinct type of adherence factors that enables the bacterium to stick onto the surface and initiate biofilm formation (Donlan, 2002). Antibiotics are the only effective therapy for the food-borne infections (Mao, Yu, You, Wei, & Liu, 2007). One of the most important food safety concerns is the increasing antibiotic resistance of food-borne pathogens. Recently, many aerobic and anaerobic bacteria were reported to show antibiotic resistance (Yong et al., 2004). Emergence of antibiotic resistance strains of *V. parahaemolyticus* has become a serious threat in the seafood industries (Tendencia & Pena, 2001). After couple of generations, antibiotic resistant bacteria can become more frequent in the environment. In many parts of the world such as US, Europe and Asian countries, eating raw or partially cooked seafood is a part of the daily diet. As mentioned before, seafood can easily be contaminated with *V. parahaemolyticus* and consumption of raw or partially cooked seafood can increase the chance of seafood-derived food poisoning. Cross contamination with *V. parahaemolyticus* usually occur during transportation, handling and processing. Since *V. parahaemolyticus* is able to survive at refrigeration temperatures, the bacteria can grow rapidly in contaminated products at storage condition (Burnham et al., 2009). Fresh food coming in direct contact with a dirty and contaminated surface can easily be contaminated. Use of proper cleaning and disinfecting methods is essential to avoid the risk of contamination. The goal of disinfecting is to totally eradicate food poisoning microorganisms. Detergents are surface-active chemicals widely used in cleaning purposes in different industries. Most detergents posses' antibacterial activity but little studies were reported on the effect of detergents on microbial biofilm. In this study, effect of 3 common detergents on biofilm formation by antibacterial-resistant *V. parahaemolyticus* isolates from seafood was investigated.

2. Materials and methods

2.1. Antibiotic susceptibility test of V. parahaemolyticus

Seafood cockles (*Anadara granosa*), clams (*Mya arenaria*, shrimps (*Penaeus* spp.) and squids (*Loligo opalescens*) were screened for

V. parahaemolyticus. The isolates were confirmed by PCR targeting the species-specific *toxR* region in *V. parahaemolyticus* (Kim et al., 1999; Lesley et al., 2005). A total of 36 *V. parahaemolyticus* isolates were used for antibiotics susceptibility test. Antibiotic susceptibility of the isolates was determined through disc diffusion tests according to the guidelines of The National Committee for Clinical Laboratory Standards (NCCCLS, 2001) (Bauer, Kirby, Sheris, & Turck, 1966; Tunung et al., 2007; Zulkifli, Alitheen, Raha, et al., 2009; Zulkifli, Alitheen, Son, et al., 2009). Using sterile non-toxic cotton swab, *V. parahaemolyticus* cultures were uniformly swabbed on Mueller-Hinton (MH) agar (Merck, Darmstadt, Germany) plates and left to dry for 3–5 min. *Escherichia coli* (ATCC 25922) used as control. Eighteen antibacterial agents were used in this study. Antibiotic discs (8 mm diameter) of Gentamicin (CN, 10 μg), Ampicillin (AMP, 10 μg), Penicillin (P, 10 μg), Erythromycin (E, 15 μg), Streptomycin (S, 25 μg), Imipenem (IPM, 10 μg), Tetracycline (TE, 30 μg), Nalidixic acid (NA, 30 μg), Vancomycin (VA, 30 μg), Norfloxacin (NOR, 10 μg), Ciproflaxin (CIP, 5 μg), Amikacin (AM, 30 μg), Bacitracin (B, 10 μg), Kanamycin (K, 30 μg), Spectinomycin (SH, 100 μg), Ceptazimine (CAZ, 30 μg), Rifampin (RD, 2 μg), and Chloramphenicol (C, 30 g μg), were supplied by Oxoid (Hampshire, United Kingdom). Antibiotic discs were placed on the inoculated plates and incubated at 37 °C overnight. The results were reported as resistant, intermediate or susceptible.

2.2. Multiple antibiotic resistance (MAR) index

Multiple antibiotic resistances (MAR) index of *V. parahaemolyticus* isolates was determined according to Krumperman (1983). Based on the definition MAR index is defined as a/b, where 'b' represents the number of multiple antibiotics to which *V. parahaemolyticus* isolates are exposed and 'a' the number of multiple antibiotics to which *V. parahaemolyticus* isolates are resistant (Gwendellynne et al., 2005).

2.3. Bacterial strains and inoculums preparation

A control of *V. parahaemolyticus* (ATCC 17802) from the American Type Culture Collection (Rockville, MD, USA) and 16 resistant *V. parahaemolyticus* isolates (Pattern I, II and III as referring to Table 2) toward antibiotics were used for inoculums preparation. *V. parahaemolyticus* strains were cultured on Tryptic Soy Broth (TSB) with 3% NaCl (Difco, Spark, MD, USA) or in Mueller Hinton Broth (MHB) for 24 h at 37 °C. *V. parahaemolyticus* was picked from broth and diluted in 1 ml of fresh TSB with 3% NaCl to make serial dilutions and get approximately 10^6 CFU/ml.

2.4. Preparation of detergent assay as antibacterial agent

Antibacterial resistant isolates of *V. parahaemolyticus* strains and *V. parahaemolyticus* (ATCC 17802) were tested for effect of detergents. The following abbreviations were used for 3 detergents commonly used in cleaning process in Malaysia: D1 (Linear alkyl benzene based) approved for food industry in Malaysia, D2 (Ammonium based) used for household and D3 (Sodium Hydroxide based) generally used in dishwashing and cleaning purpose. The stock solution was prepared according to Rukayadi, Lee, Han, Yong, and Hwang (2009) with some modification. Detergents were diluted in the DMSO (100%) to get the final 10% stock solution as it was the minimum concentration can inhibit the growth of *V. parahaemolyticus*. Standard control, Chlorhexidine (CHX)(1,1-hexa-methylenebis (5-p-chlorphenyl biguanide)) was purchased from Sigma Chemical (St Louis, MO, USA), and dissolved in sterile-distilled water for 10,000 μg/ml (1% stock solution).

2.5. In vitro susceptibility test for detergents

The standard paper blank disc-diffusion assay (CLSI, 2003), was used to test the susceptibility of V. parahaemolyticus isolates to detergents. One ml of V. parahaemolyticus from TSB was transferred to new plate and added with 15 ml of TSA. Sterile filter paper discs (6 mm diameter) (Schleicher and Schuell, Dassel, Germany), were placed on TSA plates and 20 μl of 10% stock solution of each detergent was loaded on the discs. A negative control (10% of DMSO) and standard control were included in the assay. The plates were observed for clear zones after 24 h incubation at 37 °C. All experiments were conducted in duplicate. The method proposed by Rukayadi et al. (2009) modified as follows: antibacterial was diluted in 10% DMSO followed by 2-fold dilutions in the test wells; thus, the final concentration of DMSO was serially decreased. The effect of DMSO has been examined on the growth and viability of resistant strains tested. DMSO at <10% was found not to affect growth or viability of the strain tested. These results suggested that DMSO had no effect on activity and that all measured antimicrobial activity was due to local detergents.

2.6. Minimum inhibitory concentrations (MICs) and minimal bactericidal concentrations (MBCs) determination

MICs and MBCs tests were performed in 96-well microtiter plates according to the method described in the CLSI M7-A6 guidelines. MICs for V. parahaemolyticus isolates were determined using McFarland standard (5×10^6 CFU ml^{-1}) by diluting 1: 1000 using TSB. Each antibacterial agent were diluted 1: 10 in TSB containing 5×10^3 CFU ml^{-1} inoculums. Dilutions started from wells in column 12 of the microtiter plates. Therefore, column 12 of microtiter plates contained the highest concentration of antibacterial and column 3 contained the lowest concentration of detergent. Column 2 served as the positive control (antimicrobial agent-free wells, only medium and inoculum), and column 1 was the negative control (only medium, no inoculum, no antibacterial agent). After 24 h incubation at 37 °C, the MIC was measured as the lowest concentration of antimicrobial agent resulting in complete inhibition of visible growth. The MIC was reported as the lowest concentration at which no visible was observed (Sybiya Vasantha Packiavathy et al., 2012). To determine MBCs, wells with no visible growth were used. The medium (approximately of 100 μl) of each well was removed and was spread onto agar plates supplemented with 3%NaCl and incubated at 37 °C for 24 h (or until visible growth in the positive control). The positive controls in column 2, (antimicrobial agent-free wells), and growth-negative controls in column 1, were included in the MBC test. MBC was defined as the lowest concentration of antimicrobial agent at which V. parahaemolyticus in the culture were killed or the lowest concentration with no visible growth on TSA plates.

2.7. Times–kill assay

Time–kill assays were performed in TSB with 3% NaCl medium, according to the method proposed by Lorian (2005) and Pankey and Ashcraft (2009) with some modification. following concentrations were used to kill the resistant V. parahaemolyticus isolates and control strain (ATCC 17802): 0 MIC, 1 × MIC, 2 × MIC, and 4 × MIC and 8 × MIC. The cultures were enriched in TSB with 3% NaCl (10 ml tubes) in a shaking incubator (150 rpm) at 37 °C for 24 h. A volume of 10 μl of the culture was transferred into new 10 ml TSB with 3% NaCl tube. At the given time (0 h, 1/2 h, 1 h, 2 h, 4 h, 6 h and 8 h) 100 μl of each concentration was spread in the TSA with 3% NaCl plates and incubated at 37 °C for 24 h. The number of colonies was recorded for calculation of CFU/ml and log (cfu/ml) to get viable cell results.

2.8. Assessment of in vitro biofilm formation

V. parahaemolyticus were allowed to form biofilm in the wells of presterilized, polystyrene flat-bottomed 96-well microtiter plates, according to Sandoe, Wysome, West, Heritage and Wilcox (2006). Briefly, the wells of microtiter plates were filled with 100 μl of TSB with 3% NaCl. To generate biofilms, 100 μl of the standard inoculum was transferred into each well. The plates were covered and sealed with parafilm and incubated at 37 °C for 24 h.The medium was then discarded and non adherent cells were removed through washing the biofilm with sterile phosphate buffered saline (PBS). The washing step was repeated 3 times and plates were inverted to remove residual medium.

2.9. Quantification V parahaemolyticus biofilm

Biofilm staining was performed using crystal violet solution described by Djordjevic, Wiedmann, and McLandsborough (2002). Briefly, the biofilm-coated wells of microtitre plates were vigorously shaken to remove all nonadherent bacteria. The remaining attached bacteria were washed twice with 200 μl of 50 mmol PBS (pH 7) and air-dried in room temperature for 45 min ach well was stained using 110 μl of 0.4% aqueous crystal violet solution for 45 min wells were washed twice with 350 μl of sterile distilled water and immediately de-stained with 200 μl of 95% ethanol. After 45 min of de-staining, 100 μl of de-staining solution was transferred to a new well and the amount of the crystal violet stain in the de-staining solution was measured with atunable microplate reader (VERSAMAX, Sunnyvale, CA, USA) at OD$_{650}$ nm.

2.10. Sessile minimum inhibitory concentration (SMICs) and minimum eradication bactericidal concentration (MBECs)

To measure SMICs and MBECs of detergents washed adherent cells in the 96-well microtiter plates were filled with 200 ml of the stock solution in TSB with 3% NaCl, ranging from the 97.656–$\geq$ 50000 μg/ml. Dilutions started from the wells in column 12 of the microtiter plate, meaning that column 12 of the microtiter plate contained 100 mg/ml of stock solution and column 3 contained 97.656 μg/ml of stock solution. Column 2 served as the positive control (medium and inoculum) and column 1 was the negative control (only medium). The plates were incubated at 37 °C for 24 h and biofilms where then washed and stained, as described above. The optical density (OD$_{650}$) after incubation for 24 h. The SMIC was defined as the lowest concentration where no growth occurred in the supernatant fluid, confirmed by no increase in OD$_{650}$ compared to the initial reading. SMIC$_{50}$ was defined as the lowest concentration of antibacterial agent at which 50% of resistant V. parahaemolyticus biofilms OD$_{650}$ were inhibited. SMIC$_{90}$ was reported as the lowest concentration of antibacterial agent at which 90% of resistant V. parahaemolyticus biofilms OD$_{650}$ were inhibited.

To determine MBECs, the biofilms at the bottom of treated wells were rinsed and then scarred with a metal loop and spread over the surface of TSA with 3% NaCl plates. Plates were incubated at 37 °C for 24 h and the MBEC was determined as the lowest concentration at which no bacterial growth was observed on the TSA with 3% NaCl plates. MBEC$_{50}$ was defined as the lowest concentration of antibacterial agent at which 50% of V. parahaemolyticus biofilms showed no growth on the TSA with 3% NaCl plates. MBEC$_{90}$ was reported as the lowest concentration of antibacterial agent at which 90% of V. parahaemolyticus biofilms had no growth on the TSA with 3% NaCl plates. All experiments were performed in triplicate.

Table 1

Distribution of antimicrobial resistance of *V. parahaemolyticus* for 36 isolates obtained from cultured seafood.

Antibiotics	No. of resistant (n)	Percentage of V. parahaemolyticus resistant to selected antibiotics (%)
Aminoglycosides		
Gentamicin (CN10)	0	0
Streptomycin (S10)	23	63.89
Amikacin AM30)	0	0
Kanamycin (K30)	0	0
β-lactams		
Ampicillin (AMP10)	36	100
Penicillin (P10)	36	100
Imipenen (IPM10)	0	0
Glycopeptides		
Vancomycin(VA30)	34	96.44
Macrolides		
Erythromycin (E15)	0	0
Quinolones		
Ciprofloxacin (CIP5)	0	0
Nalidixic Acid (NA30)	2	5.56
Norfloxacin (NOR10)	0	0
Tetracyclines		
Tetracyclines (TE30)	0	0
Others		
Chloramphenicol (C30)	0	0
Rifampicin (RD5)	8	22.22
Ceptazimine (CAZ30)	0	0
Bacitracin (B10)	36	100
Spectinomycin(SH25)	32	88.89

2.11. Statistical analysis

To perform the statistical analysis, Microsoft word 2007 was used to analyze the statistic while Fisher's exact test was used for the comparison. *P* value < 0.05 was considered statistically significant.

3. Results and discussions

3.1. Antibiotics susceptibility tests against V. parahaemolyticus

Antibiotic susceptibility test were performed on *V. parahaemolyticus* isolates. A total of 36 *V. parahaemolyticus* isolates were tested against 18 antibiotics from different groups. As referred in Table 1, two *V. parahaemolyticus* isolates were resistant (100%) to bacitracin,

ampicillin, penicillin followed by spectinomycin (88.89%), vancomycin (96.44%), streptomycin (63.89%), rifampin (22.24%) and nalixidic acid (5.56%). The findings in this research were in agreement with the findings of Abraham, Manly, Palaniappan, & Dhevendaran, 1997; Bhattacharya, Choudhury, & Kumar, 2000; and Molina-Aja et al., 2002, who reported that *V. parahaemolyticus* were resistance to erythromycin, streptomycin, penicillin and ampicillin. Resistancy level to beta-lactams group (ampicillin and penicillin) was observed in 100% of the analyzed isolates. The penicillin family is one of the most valuable groups of antibiotics in primary care. Penicillins are bactericidal, well distributed and highly efficacious against susceptible organisms. However, emergence of resistant bacterial strains has limited the usefulness of penicillins in recent years (Miller, O'Neill, & Chopra, 2002).

V. parahaemolyticus isolates demonstrated individual and multiple resistant toward tested antibiotics (Table 2) and most of the *V. parahaemolyticus* isolates from seafood in this study were found to be resistance to at least 7 antibiotics. According to Queensland government (2013), multi-resistant organisms (MROs) are defined as microorganisms that are predominantly bacteria and are resistant to one or more classes of antimicrobial agents. This is not surprising since other reports on multi-drug resistance pattern in *V. parahaemolyticus* isolates from raw seafood were reported in Indonesia and Nigeria (Adeleye, Vivian, Rita, Stella, & Emmanuel, 2008; Zulkifli, Alitheen, Raha, et al., 2009; Zulkifli, Alitheen, Son, et al., 2009). The high susceptibility (94.4%) to nalidixic acid is in contradict with the findings of Manjusha et al. (2005) and Zulkifli, Alitheen, Raha, et al. (2009) and Zulkifli, Alitheen, Son, et al. (2009). *V. parahaemolyticus* isolates high resistance (100%) to bacitracin. Furthermore, bacitracin is the most effective antibiotic in minor wounds treatment (Han, Walker, Janes, & Prinyawiwatkul, 2007; Smack et al., 1996). High resistance of *V. parahaemolyticus* towards Bacitracin was reported by other researchers (Adeleye et al., 2008; Norlis et al., 2011; Zulkifli, Alitheen, Raha, et al., 2009; Zulkifli, Alitheen, Son, et al., 2009).

3.2. Multiple antibiotic resistance (MAR)

V. parahaemolyticus isolates with MAR index values >0.2 are considered to be originated from high risk sources of contamination such high risk sources include human and farm animals (e.g. poultry, swine and dairy cattle) that are frequently exposed to antibiotics. *V. parahaemolyticus* isolates with MAR index values <0.2 are considered to be obtained from low risk sources (seldom or

Table 2

The antibiotic resistance profile patterns and Multiple Antibiotic Resistance (MAR) index of *V. parahaemolyticus* obtained from seafood.

Pattern	Strain no.	Antibiotic resistant profiles[a]	MAR index	No. of isolate/total isolates (% of occurrence)
I	VP003, VP027	B10S10RD2Am10VA20 NA30PG10SH25	0.44	2/36 (8.33)
II	VP002	B10Te30RD2Am10VA30 PG10SH25	0.38	1/36 (2.98)
III	VP005, VP007, VP008, VP010, VP012, VP015, VP016, VP017, VP021,VP022, VP030, VP032, VP033	B10S10RD2Am10VA30 PG10SH25	0.38	13/36 (36.11)
III	VP004, VP006, VP009, VP014, VP020, VP023, VP026	B10RD2Am10VA30PG10 SH25	0.33	7/36 (19.44)
IV	VP011, VP018, VP019	B10S10Am10VA30PG10 SH25	0.33	3/36 (8.33)
VI	VP034, VP035, VP036	B10S10RD2Am10VA30PG10	0.33	3/36 (8.33)
VII	VP001, VP013,	B10Am10VA30PG10 SH25	0.28	2/36 (11.11)
VIII	VP026,VP029,	B10RD2VA30PG10SH25	0.28	2/36 (11.11)
IX	VP031	B10S10Am10PG10SH25	0.28	1/36 (2.98)
X	VP024	B10RD2Am10PG10	0.22	1/36 (2.98)
XI	VP028	B10VA30	0.11	1/36 (2.98)

[a] Tested for B10, Bacitracin; Am10,Ampicilin; S10,Streptomycin; RD2,Rifampin; VA30, Vancomycin; NA30, Nalixidic Acid; PG10, Penicillin; S H25,Spectinomycin.

Table 3
Results of antibacterial susceptibility testing formed by resistant V. parahaemolyticus isolates including ATCC 17802 against detergents as antibacterial.

V. parahaemolyticus resistant isolates	Detergent 1 (D1) (µg/ml)		Detergent 2 (D2) (µg/ml)		Detergent 3 (D3) (µg/ml)		CHX (µg/ml)	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
ATCC17802	781.25	1562.5	3125	6250	1562.5	3125	9.656	39.062
VP003	1562.5	3125	3125	6250	1562.5	3125	19.531	39.062
VP027	781.25	1562.5	1562.5	6250	1562.5	3125	19.351	39.062
VP002	97.656	1562.5	390.25	3125	781.25	3125	9.656	19.351
VP005	1562.5	3125	3125	6250	1562.5	3125	9.656	19.531
VP007	3125	6250	3125	6250	3125	6250	19.531	39.062
VP008	781.25	1562.5	3125	6250	1562.5	3125	19.531	39.062
VP010	781.25	1562.5	1562.5	3125	1562.5	3125	19.531	39.062
VP012	1562.5	3125	1562.5	3125	1562.5	3125	19.531	39.062
VP015	781.25	1562.5	1562.5	6250	1562.5	3125	19.531	39.062
VP016	781.25	3125	1562.5	3125	1562.5	3125	9.656	39.062
VP017	1562.5	3125	3125	6250	1562.5	6250	9.656	19.351
VP021	1562.5	3125	3125	6250	3125	6250	9.656	19.351
VP022	781.25	1562.5	1562.5	6250	1562.5	6250	19.531	19.351
VP030	781.25	3125	3125	6250	1562.5	3125	9.656	39.062
VP032	195.312	781.25	3125	6250	3125	3125	19.351	39.062
VP033	1562.5	3125	1562.5	3125	3125	6250	19.351	39.062

Table 4
Comparative in vitro activities of detergents against resistant V. parahaemolyticus isolates.

V. parahaemolyticus resistant isolates or antibacterial agent	MIC (µg/ml)			MBC (µg/ml)		
All isolates (n = 16)	Range	50%	90%	Range	50%	90%
Detergent 1 (D1)	97.656–1562.5	781.25	1562.5	781.25–3125	1562.5	3125
Detergent 2 (D2)	390.25–3125	1562.5	3125	3125–6250	6250	6250
Detergent 3 (D3)	781.25–3125	1562.5	3125	3125–6250	3125	6250
CHX (Standard control)	9.656–19.351	19.351	19.351	19.351–39.062	39.062	39.062

never been exposed to antibiotics). Wide use of antibiotics in human therapy resulted in emergence of MAR pathogenic microorganisms in human feces and subsequently contamination of aquatic systems and environments. The genetics exchange between MAR pathogenic microorganisms and other bacteria facilitate emergence of MAR forms with high frequency (Krumperman et al., 1983). Since the 1950s, antibiotics have been used to control bacterial diseases in vegetables and fruits and streptomycin is the most commonly used antibiotics for plants (Kummerer, 2009). In Table 2, MAR index of V. parahaemolyticus isolates ranged from 0.11 to 0.44. Majority of the V. parahaemolyticus isolates (97.2%) showed MAR index > 0.2, indicating that these isolates were originated from high risk source

samples (Gwendelynne et al., 2005). Table 2 states, more than 8.33% (2/36) of the isolates had a MAR index value of 0.44 and 2.98% of the V. parahaemolyticus isolates showed MAR index value of <0.2.

3.3. Minimum inhibitory concentrations (MICs) and minimal bactericidal concentrations (MBCs) determination

V. parahaemolyticus isolates, which were resistant to antibiotics, were selected to investigate the threshold concentration of detergents that can inhibit bacterial growth. According to MICs and MBCs results of V. parahaemolyticus isolates (Table 3) detergents in 96 wells microtitre plates can inhibited the growth of resistant

Table 5
Results of antibacterial susceptibility testing to biofilm formed by resistant V. parahaemolyticus strains including ATCC 17802.

V. parahaemolyticus resistant isolates	Detergent 1 (D1) (µg/ml)		Detergent 2 (D2) (µg/ml)		Detergent 3 (D3) (µg/ml)		CHX (µg/ml)	
	SMIC	MBEC	SMIC	MBEC	SMIC	MBEC	SMIC	MBEC
ATCC17802	3125	6250	6250	12,500	3125	12,500	78.125	625
VP003	3125	6250	25,000	≥50,000	3125	25,000	78.125	1250
VP027	3125	6250	12,500	≥50,000	3125	25,000	78.125	1250
VP002	3125	6250	12,500	25,000	3125	25,000	19.531	312.5
VP005	6250	12,500	12,500	≥50,000	6250	25,000	19.531	625
VP007	6250	12,500	12,500	≥50,000	6250	25,000	78.125	625
VP008	3125	6250	12,500	25,000	6250	25,000	78.125	312.5
VP010	3125	12,500	12,500	≥50,000	6250	25,000	78.125	625
VP012	3125	6250	12,500	25,000	6250	25,000	78.125	625
VP015	3125	6250	6250	25,000	6250	25,000	78.125	312.5
VP016	3125	6250	6250	≥50,000	3125	12,500	78.125	312.5
VP017	6250	12,500	6250	≥50,000	3125	25,000	78.125	312.5
VP021	3125	6250	6250	≥50,000	3125	12,500	78.125	312.5
VP022	1562.5	3125	6250	≥50,000	3125	12,500	78.125	312.5
VP030	1562.5	6250	25,000	≥50,000	6250	25,000	78.125	312.5
VP032	1562.5	3125	25,000	≥50,000	6250	12,500	312.5	625
VP033	3125	12,500	25,000	≥50,000	6250	25,000	312.5	625