Investigation of *Vibrio parahaemolyticus* and its bacteriophages in shellfish for using as a tool to predict *Vibrio parahaemolyticus* outbreaks

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Abstract

Previous study demonstrated that DNA profiles of *V. parahaemolyticus* isolates from bloody clam and patient were identical. Therefore, in this study, correlation between numbers of *V. parahaemolyticus* and vibriophages in shellfish was investigated for 1 year (June 2009 to May 2010). Monthly sampling of bloody clams was performed in a local market in Hat Yai, Thailand, to determine numbers of *V. parahaemolyticus* using MPN technique. Vibriophages in bloody clam was examined by soft agar overlay technique using 10 strains of pandemic and non-pandemic *V. parahaemolyticus*. Clinical isolates of *V. parahaemolyticus* were obtained from Hat Yai hospital. A total of 139 bloody clam samples were determined. All of them were positive for *V. parahaemolyticus*. It was found that over the full testing period, number of *V. parahaemolyticus* correlated to the number of clinical isolates of *V. parahaemolyticus*. Seventy five samples were positive for vibriophages. Correlation between dynamic of vibriophages specific to pandemic and non-pandemic strains of *V. parahaemolyticus* was not significantly observed. Most of the increased numbers of clinical isolates of *V. parahaemolyticus* seems to coincide with the numbers of this organism in bloody clam. Thus, it is suggests that numbers of *V. parahaemolyticus* in shellfish may be a useful tool for prediction incidence of infections due to *V. parahaemolyticus* in this area.

Introduction

Vibrio parahaemolyticus is a halophilic bacterium found worldwide in marine ecosystems and is an important causative agent of gastroenteritis after consumption of improperly cooked seafood. It requires 0.5- 8% sodium chloride for growth with the optimal temperature ranging from 30°C to 35°C. When grown with favorable conditions, the generation time is around 12 min [28]. Not all strains of *V. parahaemolyticus* are pathogenic. Most clinical strains of *V. parahaemolyticus* possess a major virulence factors, a thermostable direct hemolysin (TDH). Another virulence factor, the TDH- related hemolysin (TRH) has also been involved in some food-poisoning outbreaks [12]. Both TDH and TRH increased intracellular Ca^{2+} and Cl^- secretion from colonic cell [25-26]. TDH and TRH are encoded by the *tdh* and *trh* genes, respectively [13, 29]. ToxR, a transmembrane regulatory protein encoded by the *toxR* gene, plays an important role in stimulation *tdh* [17]. Around 90% of clinical isolates possess either the *tdh*, the *trh* gene or both whereas those genes are rarely detected in the environmental isolates [30, 32].

Marine bacterial viruses (bacteriophages) are ubiquitous in the environment and their high host specificity for bacterial species makes them effective agents for the control of microbial communities [11]. In vitro study of bacteriophages and population dynamics of marine bacteria (*Pseudoaltermonas* sp, *Photobacteria* sp. and *Vibrio* sp.) indicated that bacteriophages influenced population dynamics, the species composition, and the clonal composition of the bacterial community in marine system [16]. In Dhaka, Bangladesh, a decrease in the bacteriophages that preyed on *Vibrio cholerae* in local waters was correlated with an increase in cholera patient admissions as well as an increase in the rate of isolation of pathogenic *V. cholerae* from the same water samples [6]. In addition, an increase in the environmental vibriophages was

correlated with a decrease in *V. cholerae* and tended to coincide with the collapse of a concurrent cholera epidemic [7]. Data corroborating these conclusions have been reported by Nelson and colleagues [19]. In the case of *V. cholerae*, lytic phages adversely affect the infectivity of this organism [20, 34] and thus transmission of *V. cholerae* in endemics settings where phages can amplify on *V. cholerae* in either the environment or within cholera victims [7, 13, 21]

The first isolation of *V. parahaemolyticus* bacteriophages was reported in 1966 [17]. The three bacteriophages described were isolated from seawater, human feces, and a lysogenic strain of *V. parahaemolyticus,* and were found to be distinctly different from each other based on plaque morphology, host range, and serological specificity. In 2000, a filamentous bacteriophage f237 was isolated from a culture supernatant of *tdh*$^+$ *V. parahaemolyticus* O3:K6 pandemic strain [18]. It was speculative that epidemic potency of the pandemic *V. parahaemolyticus* was due to acquisition of the open reading frame 8 of f237. However, relationship between vibriophages and *V. parahaemolyticus* in environment has not been extensively investigated. In general, *Vibrio* spp. are more abundant in shellfish (~10^4 cfu/g) than in seawater (~10^2cfu/ml) and vibriophages are more prevalent in mollusk extracts than in seawater [1, 2]. After studying the seasonal variations in *V. parahaemolyticus*, bacteriophages and *Vibrio* spp. in oysters and the water column, Comeau and colleagues [4] concluded that phages from *Vibrio* species in oysters were the major sources of viruses that infect *V. parahaemolyticus*. In Thailand, consumption of molluscan shellfish, especially the cockle, *Andara granosa*, contaminated with *V. parahaemolyticus*, is a major cause of acute gastroenteritis because semi-cooking is the popular way for consumption of this kind of mollusk. In a previous study we reported that the DNA profiles of *V. parahaemolyticus* isolates from mollusks and patients were identical [30]. In this study, we focused on the possibility that cockle was a potential reservoir for both *V.*

parahaemolyticus and their infecting phages and performed a year-long study on the numbers of bacteriophage and *V. parahaemolyticus* isolated from cockles and their relationship to the incidence of *V. parahaemolyticus* infections in Hat Yai city, southern Thailand.

Materials and Methods

Collection of clinical samples of *V. parahaemolyticus*

Clinical isolates of *V. parahaemolyticus* were obtained from patients in a Hat Yai hospital, Songkhla, Thailand. A rectal swab from each patient was cultured on Thiosulfate citrate bile salt sucrose agar [5]. The sucrose non-fermenting colonies were selected after overnight incubation at 37^{o}C and confirmed as *V. parahaemolyticus* by a polymerase chain reaction (PCR) targeted to the *V. parahaemolyticus tox*R gene (Vp-*toxR*).

Collection of environmental *V. parahaemolyticus*

After they were harvested from nearby cultivation farms, cockles were obtained from a local market in Hat Yai City. From each animal, 25 g of flesh was homogenized in 225 ml of alkaline peptone water (APW) containing 1% NaCl, pH 8.6. Serial dilutions of these extracts were inoculated into a series of three APW tubes, and incubated overnight at 37^{o}C. The highest dilution tubes that exhibited turbidity were plated on CHROMagar^{TM} Vibrio (CHROMagar, France) and after incubation; 1-5 mauve colored colonies were selected and used as a source of genomic DNA template to detect the Vp-*tox*R gene by PCR. If at least one colony was PCR positive, the corresponding tube and sample was considered positive for *V. parahaemolyticus* and used to calculate the most probable number (MPN) for the concentration of viable *V. parahaemolyticus*

per gram in the corresponding cockle extract [Mike Curiale, http://i2workout.com/mcuriale/mpn/index.html]. In addition, both the *tdh* and *trh* virulence genes of *V. parahaemolyticus* were examined by PCR as described below.

V. parahaemolyticus confirmation and detection of virulence genes

The test strains of *V. parahaemolyticus* were inoculated into one ml of Luria Bertani broth supplemented with 1% NaCl and grown overnight with shaking at 150 rpm at 37^{o}C. Template DNA was obtained by boiling for 10 min; the supernatant was diluted 1:10 in sterile deionized water and used for the PCR assay. The *toxR* gene was amplified using primers T4 and T7 as previously described [14]. Testing for the *tdh* and *trh* genes by PCR was carried out using the previously reported primers D3–D5 and R2–R6, respectively [24]. The PCR conditions were as follows: the reaction mixture consisted of 1.5 µl template DNA, 2 µl 10x buffer, 1.6 µl 25 mM $MgCl_2$, 0.1 µl *Taq* DNA polymerase, 1.6 µl 2.5mM dNTPs, 5 µl 2 µM primer-mix and 8.2 µl sterile MilliQ water. The amplification conditions were 35 cycles of amplification including a denaturation step at 94^{o}C for 1 min, annealing at 63^{o}C (Vp-*tox*R) for 1.5 min or 55^{o}C (*tdh* and *trh*) for 1 min, and extension at 72^{o}C for 1.5 min for Vp-*tox*R, or for 1 min for *tdh* and *trh*.

Isolation and enumeration of *V. parahaemolyticus* bacteriophages

All other bacteria used in this study were from the culture collection of the Department of Microbiology, Prince of Songkla University, Thailand. Different O and K serotype strains of *V. parahaemolyticus* including 5 clinical pandemic strain isolates (PSU2598-O4:K68, PSU3916-O1:KUT, PSU4099-O1:KUT, PSU4118-O1:K25 and PSU4211-O3:K6) and 5 non-pandemic clinical isolates

(PSU3622-O3:K29, PSU3862-O5:K17, PSU3909-O3:K5, PSU3922-O3:K45, PSU4117-O2:K56) were used as host strains for screening for *V. parahaemolyticus* bacteriophages in the cockle filtrates.

In each experiment, 25 g of cockle was squeezed out and the liquid extract obtained was centrifuged three times at 1,100× *g* for 3 min. The supernatant was collected and subjected to Millipore filtration (0.45 μm, Corning, Germany) to produce the starting filtrates. Enumeration of vibriophages was performed by plaque assay using the agar overlay method [3, 6]. Briefly, 200 μl of *V. parahaemolyticus* culture (~10^8 cells per ml, optical density 0.2–0.3 at 600 nm) was mixed with 200 μl of cockle filtrate and 3 ml of soft agar, which was then overlain on nutrient agar containing 17 mM NaCl. The plate was incubated overnight at 37°C. Plaques observed on the bacterial lawn indicated a positive *V. parahaemolyticus* bacteriophages and were counted to estimate the concentration of phage particles in the cockle filtrates. Single plaque isolates of the detected phages were amplified on a susceptible strain of *V. parahaemolyticus* by harvesting the plate lysates and stored at 4°C over chloroform.

Susceptibility of clinical and environmental *V. parahaemolyticus* isolates to bacteriophages

Each of eight bacteriophages that were propagated on the most three common pandemic serotypes of *V. parahaemolyticus* strains isolated in this area (PSU2598-O4:K68, PSU4118-PSUO1:K25 and PSU 4211-O3:K6) (32) and the one non-pandemic *V. parahaemolyticus* strain (PSU 3622-O3:K29) were randomly selected from different plaque morphologies and amplified by a plate-lysate method. The high-titer bacteriophages (10^{10} PFU/ml) were subsequently examined for their host range specificity against 18 clinical and 18

environmental isolates of *V. parahaemolyticus*. Bacterial strains were first grown to an optical density ~0.2-0.3 at 600 nm and were then added to nutrient soft agar; the mixture was overlain onto nutrient agar supplemented with 17 mM NaCl to make the bacterial lawns. Ten µl of high-titer phage stocks were spotted into agar-overlay lawns of bacteria. The plates were incubated overnight at 37°C and inspected on the next day for the single plaques or bacterial growth inhibition zones. Serotypes and virulence genes of those clinical and environmental *V. parahaemolyticus* isolates were determined for their correlation with bacteriophage susceptibility. In addition, the host range of bacteriophages that could be propagated on these three pandemic indicator strains was also investigated with 10 clinical isolates of pandemic *V. parahaemolyticus* that possessed the same serotype. Moreover, bacteriophages were tested against four pathogenic *Vibrio* spp. and four other enteric bacteria including *Shigella flexneri*, *Salmonella* Typhi, *Aeromonas hydrophila* and *Escherichia coli* O157:H7 using the spotting assay as described above.

Serotyping

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

Statistical analysis

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

Results and Discussion

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

Table 1 Numbers of bacteriophage positive cockle samples and susceptible hosts

Time	No. of samples collected	No. of samples positive for phage	Number of susceptible *V. parahaemolyticus* hosts[a]
Jun 09	14	12	9
Jul 09	14	7	10
Aug 09	22	8	9
Sept 09	14	2	4
Oct 09	7	2	3
Nov 09	9	4	6
Dec 09	10	4	7
Jan 10	10	9	9
Feb 10	10	7	10
Mar 10	10	8	9
Apr 10	10	9	10
May 10	9	4	8
Total	139	76	-[b]

[a] The same ten host strains of *V. parahaemolyticus* were determined per month

[b] In each month, three to ten of *V. parahaemolyiticus* host strains were susceptible to phages

Table 2 *V. parahaemolyticus* strains susceptible to bacteriophages isolated from cockles between June 2009 and May 2010

Time	Positive sample(s) for bacteriophage									
	Vp pandemic strains					Vp non-pandemic strains				
	PSU 2598 O4:K68	PSU 3916 O1:KUT	PSU 4099 O1:KUT	PSU 4118 O1:K25	PSU 4211 O3:K6	PSU 3622 O3:K29	PSU 3862 O5:K17	PSU 3909 O3:K5	PSU 3922 O3:K45	PSU 4117 O1:K56
Jun 09	3	2	3	4	10	8	0	4	4	4
Jul 09	4	3	1	3	1	2	3	2	2	3
Aug 09	1	1	0	1	3	2	2	4	1	1
Sept 09	1	0	0	2	0	1	0	0	0	2
Oct 09	0	0	1	0	0	0	0	0	1	1
Nov 09	1	1	0	1	2	3	0	2	0	0
Dec 09	1	2	0	0	2	0	1	1	1	3
Jan 10	1	0	1	2	5	4	1	3	5	2
Feb 10	2	1	3	3	3	3	4	2	1	2
Mar 10	1	0	3	4	4	4	6	2	1	2
Apr 10	3	9	1	3	7	5	3	3	5	7
May 10	1	4	1	0	1	0	1	1	1	1
Total	19	23	14	23	38	32	21	24	22	28

In the southern part of Thailand, cockle is a very popular raw seafood item and it is frequently contaminated with *V. parahaemolyticus* [33]. We sought to determine if there was any correlation between the frequency of patients infected with *V. parahaemolyticus* and the presence of this bacterium and its bacteriophages in cockles from the same local area. In this study, 97 isolates of *V. parahaemolyticus* were obtained from patients in the Hat Yai hospital during December 2009 and February 2010 (Fig. 1). The following four different categories of clinical isolates based on PCR analysis were: 1) tdh^+ trh^- 2) tdh^+ trh^+ 3) tdh^- trh^- and 4) tdh^- trh^+. PCR types 1-4 were identified in 62, 3, 13 and 2 of the isolates, respectively. Seventeen isolates were not examined for virulence genes because of their death during transport to the laboratory. The number of patients infected locally significantly coincided at the 95% confidence level (p value = 0.02, R^2= 0.40) with the increase in the number of *V. parahaemolyticus* in the cockle extracts (Figs. 2 and 3a). Correlation between the decrease in the numbers of *V. parahaemolyticus* bacteriophage in the cockle filtrates and an increase in the level of *V. parahaemolyticus* in the cockle extracts was detected in some months but there was no significant correlation obtained over the whole period of the study time (p value = 0.07, R^2= 0.27) (Fig. 3b). The low numbers of bacteriophage in the cockle filtrates seemed to correlate with an increase in the number of *V. parahaemolyticus* in some months (Jun-Aug 09, Oct-Dec 09) (Fig. 2). An increase in the numbers of bacteriophage appeared to follow the high numbers of *V. parahaemolyticus* in the former period of time (Dec 09-Feb 10). However this inverse correlation was not consistent perhaps because some *V. parahaemolyticus* might develop resistance to the local phages as has been seen with *Listeria* spp. co-cultured with listeriophages [29]. In addition, Middelboe and colleagues [16] demonstrated the temporary effects on dynamics and diversity of

the individual bacterial host species after interaction with their specific phages because phage-resistant bacteria were present after the first lysis of hosts. Thus, we conclude that the levels of *V. parahaemolyticus* present in cockle are useful to assess the relative risk of cockles as a source of *V. parahaemolyticus* infection in Southern Thailand.

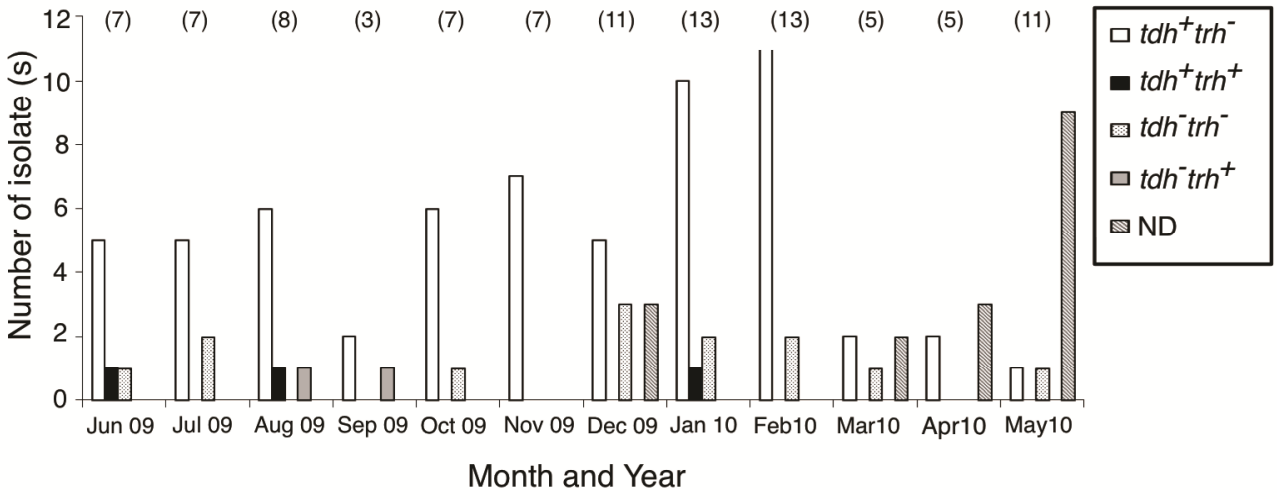

Fig.1 Clinical isolation of *V. parahaemolyticus* detected in patients between June 2009 and May 2010.

NB. Number in parenthesis is total number of isolates/month; ND = no PCR result

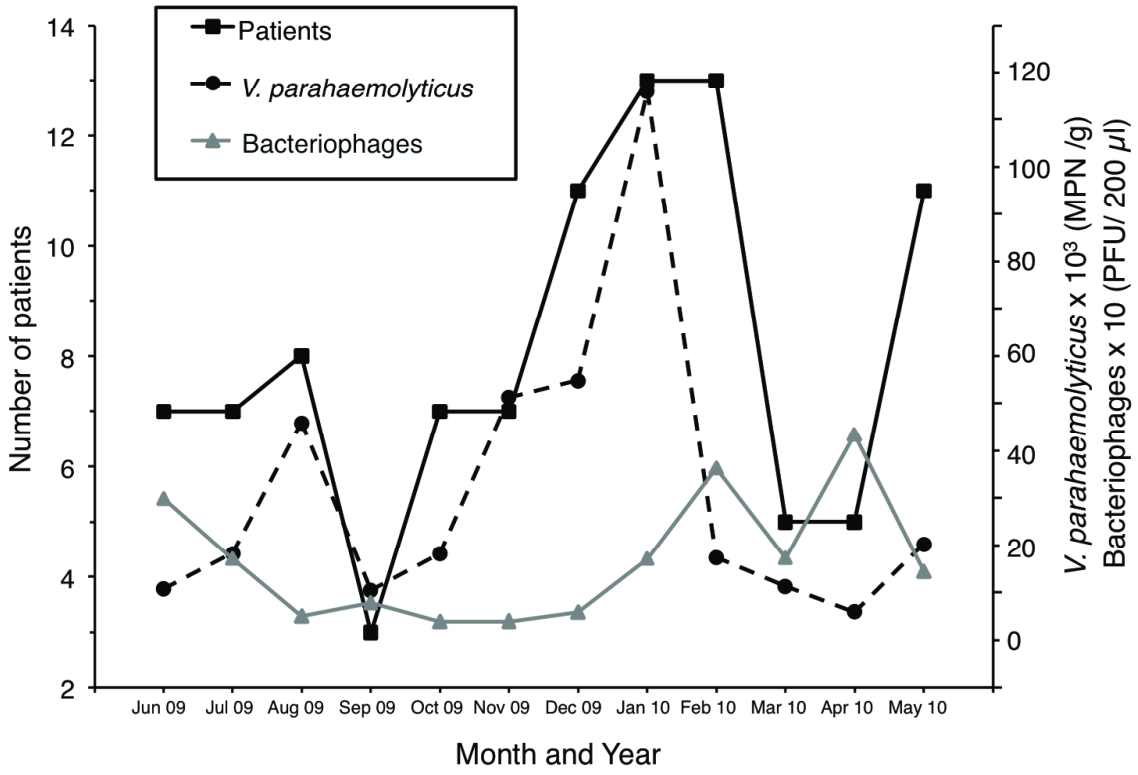

Fig. 2 Correlation between the number of patients, *V. parahaemolyticus* and bacteriophages obtained in one year (June 2009 – May 2010)

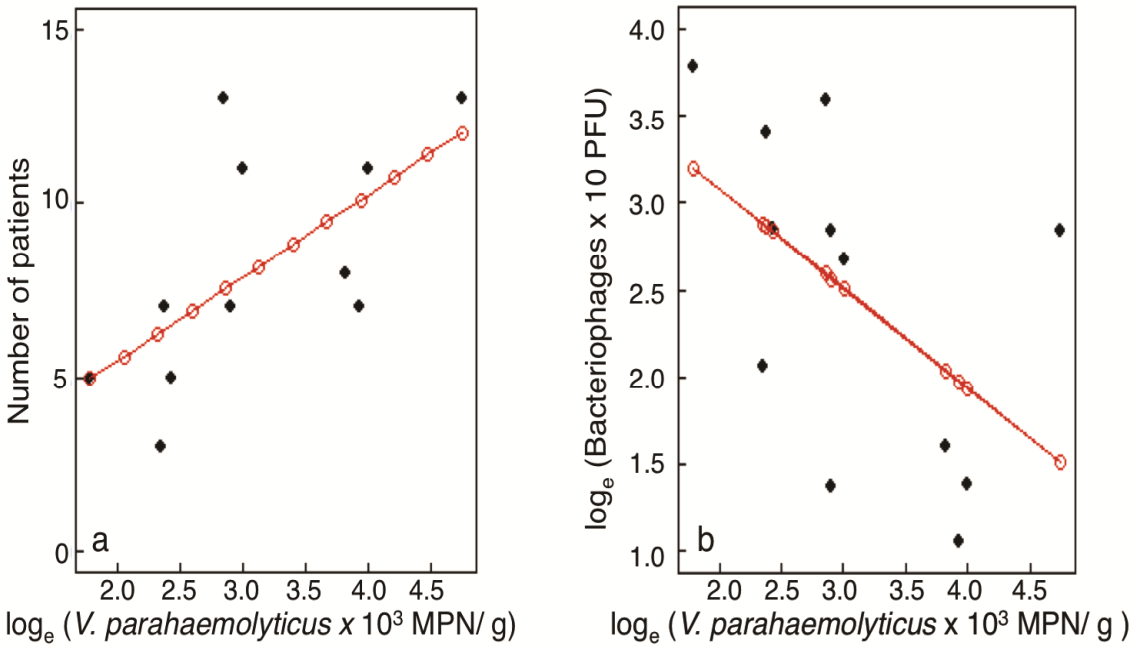

Fig. 3 (a) Pearson's product-moment correlation showing correlations between *V. parahaemolyticus* level in cockle extracts and the number of patients; p=0.02 (95% confidence) (b) No correlation between *V. parahaemolyticus* level and bacteriophages level in cockle extracts and filtrates, respectively; p=0.07 (95% confidence)

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

Each set of eight bacteriophages isolated from the O4:K68, O1:K25 and O3:K6 pandemic strains (PSU2598, PSU4118 and PSU 4211 respectively) were capable of forming plaque on the same serotypes of each set of 10 pandemic strains of *V. parahaemolyticus* (data not shown). In addition, seven bacteriophages that were isolated on the O3:K6 pandemic strain PSU 4211 were able to infect different serotypes of pandemic *V. parahaemolyticus* (O4:K68-PSU2598 and O1:K25-PSU4118) (data not shown). In Hat Yai City, around 60% of patients in one study were infected with these pandemic strains [32]. Thus, these may be useful both as indicators of the presence of the pandemic strain as well as for use as a possible biological control to suppress *V. parahaemolyticus* in food items that are at high risk for contamination by this organism.

In this study, 25 and 26 of the *V. parahaemolyticus* bacteriophages obtained from the 3 pandemic (PSU 2598, PSU 4118 and PSU4211) and 1 non-pandemic (PSU3622) *V. parahaemolyticus* hosts were active against *V. alginolyticus* and *V. mimicus* respectively (Table 4). This would seem to indicate that these three *Vibrio* species migh occupy the same ecological niche. None of the bacteriophages active on *V. parahaemolyticus* grew on other enteric pathogens including *V. cholerae*. Therefore, it is possible that they may participate in the processes of genetic exchange between the *V. parahaemolyticus* and those *V. alginolyticus* and *V. mimicus* [8, 10, 23, 31]. Recently, two bacterial isolates obtained from Alaskan oysters were identified as *V. alginolyticus* and expressed a *trh* gene with 98% homology to the *trh*2 of *V. parahaemolyticus* [9]. In addition, it has been demonstrated that one clinical isolate of *V. mimicus* harbored a *tdh* with a 97% homology to *tdh*2 of *V. parahaemolyticus* [22].

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

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

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

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

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

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

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