

## Total number, virulence genes, and heterogeneity of *Vibrio parahaemolyticus* in a single shellfish

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**ABSTRACT:** Infection by *Vibrio parahaemolyticus* is established if seafood contaminated with pathogenic strains of this bacterium at a sufficiently high dose is consumed. We reported recently that *V. parahaemolyticus* isolates from 67% of patients constituted a homogeneous population of a pathogenic strain and a heterogeneous population of *V. parahaemolyticus* varying in the serotype and/or the virulence gene profile found in 33% of the patients. To assess the possibility that this result was due to the dominant presence of a homogeneous strain of *V. parahaemolyticus* in shellfish, we enumerated and characterized *V. parahaemolyticus* in shellfish samples including three kinds of molluscan bivalves. Using CHROMagar *Vibrio* isolation medium, presumed total *Vibrio* strains and presumed *V. parahaemolyticus* in each of 52 shellfish samples were enumerated. The highest numbers of presumed total *Vibrio* strains and presumed *V. parahaemolyticus* were detected in bloody clam and mussel, respectively. There was no correlation between the number of presumed total *Vibrio* strains and that of presumed *V. parahaemolyticus*. Five to ten strains of *V. parahaemolyticus* isolated from each shellfish sample were characterized. Virulence genes (*tdh* and *trh*) could be detected in none of the isolated strains. Diversities in the serotype and DNA fingerprints were confirmed among the isolated strains from a single shellfish sample representing six different shellfish groups. We therefore conclude that most of the single shellfish harbour a pathogenic strain-bearing heterogeneous population of *V. parahaemolyticus* and the concentrations of pathogenic strains are low.

**KEYWORDS:** enumeration, serotype, genotype, CHROMagar

### INTRODUCTION

*Vibrio parahaemolyticus* is a halophilic bacterium that causes seafood-borne gastroenteritis<sup>1</sup>. The clinical symptoms develop within 4–96 h after consumption of seafood contaminated with pathogenic strain of this bacterium at or higher than an infectious dose ( $2 \times 10^5$  to  $10^9$  organisms of pathogenic strains)<sup>2</sup>. The symptoms include abdominal cramps, nausea, vomiting, headache, diarrhoea, mild fever, and chills<sup>3</sup>. Only *V. parahaemolyticus* strains that produce one or both of two important virulence factors, a thermostable direct haemolysin (TDH) and a TDH-related haemolysin, have been considered to be pathogenic strains<sup>4,5</sup>. These haemolysins are encoded by the *tdh* and *trh* genes, respectively; either *tdh*, *trh* or both genes are present in most clinical isolates of *V. parahaemolyticus*<sup>6</sup>. However, only 3–6% of environmental isolates harbour these genes<sup>7,8</sup>. A clas-

sification based on combination of the serotypes of two antigens, somatic O and capsular-like K, have been established using clinical strains and serves as a useful tool in epidemiological investigations<sup>9</sup>. At present time 13 O and 74 K serotypes have been reported. However, there are many environmental strains of *V. parahaemolyticus* that are untypeable to these existing serotypes because environmental strains have not been included in establishment of the O:K serotype. Since 1996, the increase in incidence of gastroenteritis due to *V. parahaemolyticus* O3:K6 has been reported from many countries in Asia, America, Africa, and Europe<sup>10</sup>. These strains are considered to belong to a pandemic clone and the strains belonging to some other serotypes (O1:K25, O4:K68, O1:KUT, etc.) are considered to have diverged from the O3:K6 pandemic clone<sup>11,12</sup>.

Results of the attempts to isolate the O3:K6 pandemic strains from shellfish in Songkla Province

in southern Thailand demonstrated that the O3:K6 pandemic strains are accumulated in molluscan bivalves but not in other shellfish<sup>8,13</sup>. Investigations of clinical isolates of *V. parahaemolyticus* from Hat Yai Hospital, Songkla Province have demonstrated that 67% of patients were infected with a homogeneous population of pathogenic strains of *V. parahaemolyticus* belonging to a single O:K serotype and most of these patients were infected by the strains belonging to the pandemic O3:K6 clone (hereinafter abbreviated as O3:K6 pandemic strains)<sup>14</sup>. However, a heterogeneous population varying in serotype and virulence gene profile were found from the rest (33%) of the patients. To investigate whether this result was due to consumption of molluscan bivalves containing a homogeneous population of *V. parahaemolyticus* bearing a pathogenic strain at or higher than the infectious dose with an ability to subsequently predominate in human digestive tracts, we examined total numbers, virulence genes, and homogeneity/heterogeneity represented by the O:K serotype (and DNA fingerprints when needed) of *V. parahaemolyticus* strains isolated from single shellfish samples.

## MATERIALS AND METHODS

### Enumeration of *V. parahaemolyticus* in shellfish

Shellfish samples were obtained from morning markets in Hat Yai City, Songkhla Province, Thailand between June and October 2010. They were examined within 1 h of collection. Their meat was removed and crushed in alkaline peptone water pH 8.6 supplemented with 1% NaCl at the ratio of 1:10. Then 0.1 ml of each sample was spread on CHROMagar Vibrio (CHROMagar Microbiology, Paris, France) in duplicate and inoculated plates were incubated at 37 °C for 24 h. The numbers of the total colonies and that of mauve colonies, presumed to be *V. parahaemolyticus*, were counted and their concentrations in the original shellfish in cfu/g were calculated. Five to ten mauve colonies were selected for confirmation of *V. parahaemolyticus* and detection of virulence genes.

### Confirmation of *V. parahaemolyticus* and detection of virulence genes

The mauve colonies on CHROMagar Vibrio medium were presumed to be *V. parahaemolyticus*. To confirm this presumptive identification, the isolated mauve colonies were examined for *V. parahaemolyticus*-specific *toxR* gene sequence by a PCR method<sup>15</sup>. The test isolate was grown in Luria-Bertani (LB) broth containing 1% NaCl with shaking (160 rpm) at 37 °C overnight. One ml of the broth culture was

centrifuged, and the bacterial cells were washed and resuspended in sterile saline (0.85% NaCl). The cell suspension was boiled for 10 min, and the supernatant was obtained by centrifugation, diluted 10-fold in distilled water, and used as the template for PCR amplification. Amplification of the *toxR* gene was performed using primers T4 and T7 as described previously<sup>15</sup>. Since the urease-positive phenotype of *V. parahaemolyticus* has been reported to be associated with the presence of the *trh* gene<sup>16</sup>, all *toxR* positive isolates were screened for urease activity. Presence or absence of the *tdh* and *trh* genes was examined by PCR using the previously reported primers D3-D5 and R2-R6, respectively<sup>17</sup>. The isolates that exhibited urease activity but were negative for the *trh* gene by the PCR assay were examined by Southern blot hybridization using *trh* probes as described previously<sup>18</sup>.

### Serotyping

Five isolates of *V. parahaemolyticus* from each of six selected shellfish were subjected to O:K serotyping. The O:K serotype of each isolate was determined by the slide-agglutination test using anti-O and anti-K antibodies (Denka Seiken, Tokyo). Briefly, the test isolate was grown in tryptic soy broth containing 3% NaCl at 37 °C for 18 h, and the bacterial cells were suspended in saline (3% NaCl). The bacterial cell suspension was subjected to agglutination with specific anti-K antibodies to determine the K serotype. For determination of the O serotype, the bacterial cell suspension was autoclaved at 121 °C for 30 min. Autoclaved bacterial cells were subjected to agglutination with specific anti-O antibodies.

### Arbitrarily primed polymerase chain reaction (AP-PCR)

In order to determine DNA profiles of *V. parahaemolyticus*, DNA of each test strain was extracted by the phenol-chloroform extraction method<sup>19</sup>. AP-PCR was performed using primer 2 (5'-GTTTCGCTCC-3') as described previously<sup>14</sup>. Briefly, amplification was performed in a 30- $\mu$ l mixture composed of 0.33 mM dNTPs (TaKaRa Biochemicals, Tokyo), 25 ng template DNA, 2.5 U *Ex Taq* (TaKaRa), 0.83 pmol primer, and 1 $\times$  *Ex Taq* Buffer (TaKaRa). The PCR was performed in a thermal cycler (Program Temp Control System PC-808, Astec Co., Tokyo). The thermocycle was started with a cycle at 95 °C for 4 min. This was followed by 45 cycles of denaturation at 95 °C for 1 min, annealing at 36 °C for 1 min, and extension at 72 °C for 2 min, in which a transition time of 5 min was set between the denaturation and annealing, annealing and extension, and extension and denaturation

**Table 1** Meat weight, total number of bacteria and *V. parahaemolyticus* (VP) detected on CHROMagar Vibrio from single shellfish samples.<sup>a</sup>

Seafood samples (no. of tested samples)	Average meat weight (g/single seafood)	No. of total bacteria (cfu/g)		No. of VP (cfu/g)		Proportion of VP (%) <sup>b</sup>
		Average	Min–Max	Average	Min–Max	
Shrimp (10)	8.5 ± 0.2 <sup>c</sup>	1.0 × 10 <sup>5</sup>	4.0 × 10 <sup>2</sup> –4.9 × 10 <sup>5</sup>	5.8 × 10 <sup>3</sup>	1.0 × 10 <sup>2</sup> –4.6 × 10 <sup>4</sup>	5.8
Crab (2)	34.3 ± 1.4	1.1 × 10 <sup>5</sup>	8.0 × 10 <sup>4</sup> –1.4 × 10 <sup>5</sup>	1.0 × 10 <sup>2</sup>	0–2.0 × 10 <sup>2</sup>	0.1
Mussel (10)	7.6 ± 0.7	4.3 × 10 <sup>5</sup>	8.0 × 10 <sup>3</sup> –1.5 × 10 <sup>6</sup>	4.7 × 10 <sup>4</sup>	1.0 × 10 <sup>3</sup> –2.1 × 10 <sup>5</sup>	10.9
Hard clam (10)	3.8 ± 0.2	3.0 × 10 <sup>4</sup>	4.0 × 10 <sup>2</sup> –1.2 × 10 <sup>5</sup>	3.2 × 10 <sup>3</sup>	4.0 × 10 <sup>2</sup> –1.6 × 10 <sup>4</sup>	10.6
Bloody clam (10)	4.1 ± 0.1	7.7 × 10 <sup>6</sup>	3.0 × 10 <sup>3</sup> –7.5 × 10 <sup>7</sup>	5.1 × 10 <sup>3</sup>	4.4 × 10 <sup>2</sup> –3.6 × 10 <sup>4</sup>	0.07
Squid (10)	8.6 ± 0.2	1.6 × 10 <sup>4</sup>	1.2 × 10 <sup>3</sup> –4.0 × 10 <sup>4</sup>	6.6 × 10 <sup>2</sup>	1.0 × 10 <sup>2</sup> –2.0 × 10 <sup>3</sup>	4.1

<sup>a</sup> Mauve colonies on CHROMagar Vibrio was presumed as that of *V. parahaemolyticus* and this assumption was confirmed as described in the text.

<sup>b</sup> Average no. of *V. parahaemolyticus*/average no. of total bacteria.

<sup>c</sup> Mean ± SD.

steps. The thermocycle was finished with one cycle at 72 °C for 7 min. The amplification products were analysed by agarose (1.5%) gel electrophoresis.

### Pulsed field gel electrophoresis (PFGE)

*V. parahaemolyticus* was grown in LB broth supplemented with 1% NaCl at 37 °C overnight. An agarose plug was prepared by mixing equal volumes of bacterial suspension with melted agarose. Bacterial lysis in an agarose plug was achieved with 950 µl lysis solution (containing 50 mM Tris-HCl, pH 8.0; 50 mM EDTA; 1% *N*-lauryl sarcosine; and 1 mg/ml proteinase K), and the DNA was cleaved for 48 h and digested with 50 U of *NotI* restriction enzyme (TOYOBO Co. Ltd., Osaka, Japan). The digested DNA fragments and DNA markers were separated in 1% Pulsed-Field Certified agarose (Bio-Rad Laboratories, Hercules, CA, USA) using 0.5 × TBE buffer on a CHEF-DRIII system (Bio-Rad). The running condition was set 6 V/cm at 14 °C for 24 h at a field angle of 120° and switch times were 1–18 s for 12 h and 3–80 s for 12 h. After completion of the electrophoresis, the gel was stained with ethidium bromide for 30 min, destained in distilled water for 1 h, and photographed using a UV transilluminator.

## RESULTS

### Enumeration of *V. parahaemolyticus* in shellfish

A total of 52 shellfish samples including shrimp, crab, mussel, squid, hard clam and bloody clam were investigated. The average total number of bacteria was the highest for bloody clam at 7.7 × 10<sup>6</sup> cfu/g (ranged from 3.0 × 10<sup>3</sup> to 7.5 × 10<sup>7</sup> cfu/g) (Table 1). The average lowest number of bacteria was observed for squid at 1.6 × 10<sup>4</sup> cfu/g (ranged from 1.2 × 10<sup>3</sup> to 4.0 × 10<sup>4</sup> cfu/g). Five to ten mauve colonies selected from CHROMagar were confirmed to be *V. parahaemolyticus*.

**Table 2** Serotype of *V. parahaemolyticus* detected in single shellfish samples.

Shellfish	O:K serotypes					
Shrimp	O1:KUT	O4:K34	O10:K24	O8:KUT	O8:KUT	O8:KUT
Crab	O3:K6	O4:K4	O4:K4	O4:K4	O4:KUT	O5:K17
Mussel	O1:KUT	O1:KUT	O1:KUT	O2:KUT	O11:KUT	O8:KUT
Hard clam	O3:KUT	O4:KUT	O8:KUT	O8:KUT	O8:KUT	O8:KUT
Bloody clam	O3:K48	O3:KUT	O4:K63	O4:KUT	ND <sup>a</sup>	ND <sup>a</sup>
Squid	O3:KUT	O4:K8	O4:KUT	O10:K52	O10:KUT	O10:KUT

Five isolates of *V. parahaemolyticus* were determined for each shellfish sample.

The O:K serotype of each isolate was determined by agglutination with anti-O and anti-K antibodies.

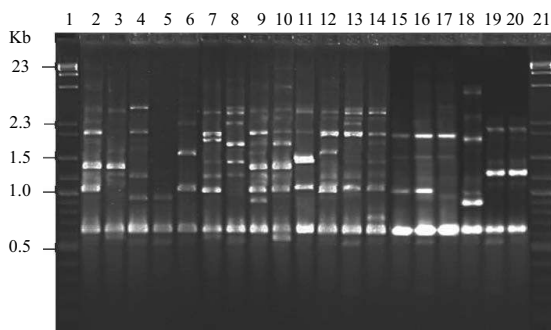
<sup>a</sup> Not determined.

*molyticus* by PCR for each shellfish sample. All the isolates were *toxR* positive but they harboured neither the *tdh* nor *trh* virulence genes. It was of interest that two isolates were urease positive. The highest number of *V. parahaemolyticus* was detected in mussel (4.7 × 10<sup>4</sup> cfu/g average) and the lowest number was detected in crab (1.0 × 10<sup>2</sup> cfu/g average).

Interestingly, proportion of *V. parahaemolyticus* relative to the total number of bacteria was very high for some molluscan bivalves (10.9% for mussel, 10.6% for hard clams) and very low for some molluscan bivalves (0.07% for bloody clam) (Table 1).

### Diversity of serotype and DNA fingerprints

Five mauve colonies were selected from a culture plate for each of six selected single shellfish samples and their O:K serotypes were determined (Table 2). Three to five different serotypes of *V. parahaemolyticus* were demonstrated in each of those six single shellfish samples (Table 2). Two to three identical serotypes of *V. parahaemolyticus* were observed in shrimp (O8:KUT), crab (O4:K4), mussel (O1:KUT), and hard clam (O8:KUT) (Table 2). DNA fingerprints of those isolates, including *V. parahaemolyticus* isolates that

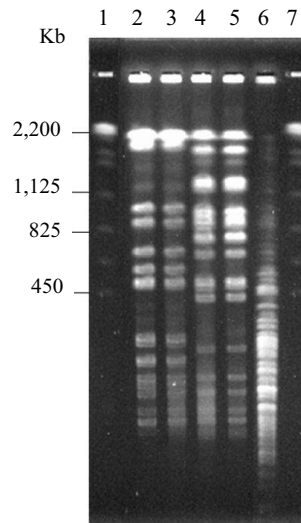


**Fig. 1** AP-PCR profile of *V. parahaemolyticus* (VP) isolates from shellfish. DNA fingerprints of identical or nearly identical serotypes of VP isolates from shellfish were determined. The results were obtained with primer 2.

- Lanes 1 and 21:  $\lambda$  HindIII + 100 bp ladders.
- Lane 2: VP PSU 3185, O8:KUT, shrimp<sup>a</sup>.
- Lane 3: VP PSU 3192, O8:KUT, shrimp.
- Lane 4: VP PSU 3242, O4:K4, crab.
- Lane 5: VP PSU 3244, O4:K4, crab.
- Lane 6: VP PSU 3245, O4:KUT, crab.
- Lane 7: VP PSU 3098, O4:K63, bloody clam.
- Lane 8: VP PSU 3103, O4:KUT, bloody clam.
- Lane 9: VP PSU 3100, O3:K48, bloody clam.
- Lane 10: VP PSU 3106, O3:KUT, bloody clam.
- Lane 11: VP PSU 3195, O4:KUT, squid.
- Lane 12: VP PSU 3200, O4:K8, squid.
- Lane 13: VP PSU 3201, O10:K52, squid.
- Lane 14: VP PSU 3202, O10:KUT, squid.
- Lane 15: VP PSU 5312 O1:KUT, mussel.
- Lane 16: VP PSU 5313 O1:KUT, mussel.
- Lane 17: VP PSU 5314 O1:KUT, mussel.
- Lane 18: VP PSU 5309 O8:KUT, hard clam.
- Lane 19: VP PSU 5310 O8:KUT, hard clam.
- Lane 20: VP PSU 5311 O8:KUT, hard clam.

<sup>a</sup> Sample from which *V. parahaemolyticus* was isolated.

exhibited the same O antigen but different K antigens (one possessed a known K antigen and another was KUT) were examined next. AP-PCR showed that strains with identical serotypes, O8:KUT (strains PSU 3185 and PSU 3192) and O4:K4 (PSU 3242 and PSU 3244) isolated from shrimp and crab, respectively, produced different DNA fingerprints (Fig. 1 and Table 2). In addition, the *V. parahaemolyticus* strains isolated from a single shellfish with the same O antigen but with different K antigens (O4:K4, O4:KUT isolated from crab; O4:K63 and O4:KUT, O3:K48 and O3:KUT isolated from bloody clam; O4:KUT and O4:K8, O10:K52 and O10:KUT isolated from squid) also exhibited different DNA profiles



**Fig. 2** PFGE confirmation of *V. parahaemolyticus* (VP) isolates from mussel and hard clam. Both isolates of each serotype (O1:KUT and O8:KUT) of VP obtained from mussel and hard clam were investigated.

- Lanes 1 and 7: *Saccharomyces cerevisiae* PFGE marker.
- Lane 2: VP PSU 5312 O1:KUT, mussel.
- Lane 3: VP PSU 5313 O1:KUT, mussel.
- Lane 4: VP PSU 5310 O8:KUT, hard clam.
- Lane 5: VP PSU 5311 O8:KUT, hard clam.
- Lane 6: *Pseudomonas aeruginosa* control strain.

(Fig. 1). However, DNA profiles of two of three identical serotypes, O1:KUT (PSU 5312–5314) and O8:KUT (PSU 5309–5311) of *V. parahaemolyticus* isolated from mussel and hard clam, respectively, were identical. Confirmation by PFGE revealed that both isolates of each serotype were indistinguishable (Fig. 2).

## DISCUSSION

In the control of seafood safety, the most probable number (MPN) technique is currently used to estimate total number of *V. parahaemolyticus*. In this study, shellfish, especially three kinds of mollusc, were selected to evaluate the total number of bacteria and that of *V. parahaemolyticus*. CHROMagar Vibrio was used as the selective isolation mainly medium because it is suitable for detection of *V. parahaemolyticus*<sup>20</sup>. This agar also supports growth of bacteria belonging to most species of the genus *Vibrio*<sup>20</sup>. These include enteropathogenic *Vibrio* species such as *V. cholerae*, *V. mimicus*, and *V. vulnificus*. If the number of total bacterial colonies on CHROMagar Vibrio well reflects the number of colonies belonging to enteropathogenic *Vibrio* species in general and if the proportion of

virulent to total population of enteropathogenic *Vibrio* species, e.g., *tdh*<sup>+</sup> and/or *trh*<sup>+</sup> population versus total population of *V. parahaemolyticus*, is almost constant in a specific sampling site during the sampling season, the relative number of total bacteria on CHROMagar Vibrio may be used as an indicator for enteropathogenic *Vibrio* species including *V. parahaemolyticus*. If so, the total bacterial count on CHROMagar Vibrio can be an alternative to quantification of enteropathogenic *Vibrio* species by the MPN estimation-based method. The former method is easier than the latter. However, correlation does not seem to exist between the relative frequencies of total bacterial count and *V. parahaemolyticus* because the percentage of *V. parahaemolyticus* in total number of bacteria ranged from sample to sample and varied greatly among various seafood species (Table 1).

Bloody clam, mussel and hard clam which are filter feeding shellfish seem to harbour greater numbers of *V. parahaemolyticus* than other shellfish except for shrimp (Table 1). The O3:K6 pandemic strains are accumulated in these molluscan bivalves<sup>8,13</sup>. *Vibrio* spp. have been demonstrated in various tissues of molluscan bivalves such as clams, oysters and mussels to levels sometimes 100 times that of the overlying water<sup>21</sup>. Wang et al<sup>22</sup> demonstrated that *V. parahaemolyticus* was accumulated in digestive glands, gills, adductor muscle and mantle cilia of oysters after artificial inoculation. In this study, the percentage of *V. parahaemolyticus* in total number of bacteria detected in bloody clam was lowest (0.07%) but the average number of this bacterium in this shellfish was not much different from those detected in hard clam and shrimp (Table 1). It seems that the number of *V. parahaemolyticus* presented in each kind of single shellfish is high. Consuming a few shellfish can reach an infective dose. Hence enough attention needs to be paid to proper cooking before consumption.

It has been demonstrated that around 3–6% of *V. parahaemolyticus* isolated from the environment carry the virulence genes (*tdh* or *trh*)<sup>7,8</sup>. Thus it is not easy to isolate *tdh*<sup>+</sup> and/or *trh*<sup>+</sup> strains from seafood. In this study, we did not obtain any virulent strains of *V. parahaemolyticus* although we usually detect the presence of *tdh*<sup>+</sup> and/or *trh*<sup>+</sup> strains by examining the boiled supernatant of the enrichment culture in our similar studies (V.V. and M.N., unpublished observations). This could be due to the small number of colonies examined per sample in this study. However, two isolates of *V. parahaemolyticus* (PSU 3103 and PSU 3200) obtained from bloody clam and squid, respectively, exhibited urease activity. It has been demonstrated that the presence of *trh* gene is close

to the *ure* gene on the chromosome of *V. parahaemolyticus*<sup>23</sup>. Hence Southern blot hybridization with *trh* probes was performed; those two isolates (PSU 3103 and PSU 3200) did not produce any positive band for *trh* (data not shown). Park et al<sup>24</sup> demonstrated an insertion sequence-like element present at the end of the DNA region containing the *trh* and *ure* genes. Thus it seems possible that the absence of *trh* gene in these isolates may be associated with an insertion sequence mediated deletion mechanism.

Serotype diversity of this bacterium was demonstrated in all six single shellfish samples examined (Table 2). Two to three identical serotypes of *V. parahaemolyticus* were detected in shrimp, crab, mussel and hard clam. However, DNA fingerprinting revealed those exhibiting identical serotypes were not identical except both isolates of each serotype obtained from mussel (O1:KUT) and hard clam (O8:KUT) (Fig. 1 and Fig. 2). DNA profiles of those isolates obtained from a single shellfish and possessing the same O but different K antigens were also different. In addition, the DNA profiles of the isolates that possessed the same serotypes (O4:KUT) but were obtained from different kinds of shellfish (PSU 3103 from bloody clam; PSU 3195 from squid; and PSU 3245 from crab) were different. These indicate that most of the single shellfish harbour heterogeneous population of *V. parahaemolyticus*. The shellfish responsible for *V. parahaemolyticus* infection must contain a pathogenic strain-bearing heterogeneous population of *V. parahaemolyticus*.

In conclusion, the number of *V. parahaemolyticus* detected in shellfish does not correlate with the total number of bacteria present in each shellfish species. Most of the single shellfish responsible for *V. parahaemolyticus* infection possibly contain a pathogenic strain-bearing heterogeneous population of *V. parahaemolyticus*; the concentrations of pathogenic strains are low, but they may propagate to infectious doses in shellfish before consumption. If the exact mechanism involved in preferential growth of pathogenic strains in molluscan bivalves is elucidated, it will be useful in detection of potentially hazardous, pathogenic strains in molluscan bivalves.

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