Drug Resistant Strains of *Vibrio vulnificus* from Sea Mud in Prince of Songkla University, Pattani Campus and Some Shells in Pattani Seafood Markets

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Abstract

*Vibrio vulnificus* (Vv) is an opportunistic human pathogen that may cause primary septicemia, necrosis and gastroenteritis. It can be isolated from marine animals and marine environment. This research aimed to isolate and characterize the drug resistant strains of *Vibrio vulnificus* from sea mud within Prince of Songkla University, Pattani campus and closed areas and from green mussel (*Perna viridis*) and bloody cockle (*Tegillarca granosa*) in seafood markets. The result found that from 43 sea mud samples, the total of 4 strains of *Vibrio vulnificus* were isolated from Pattani estuary 1 strain, Pattani estuary 2 strains and Rusamilae estuary 1 strain. The 3 strains of *Vibrio vulnificus* were detected in 10 samples of green mussel and 1 strain in bloody cockle. The antimicrobial susceptibilities of *Vibrio vulnificus* isolates recovered from sea mud and shells were determined. All isolates were sensitive to ceftazidime and resistant to ampicillin and clindamycin.

**Keywords:** *Vibrio vulnificus*, Antimicrobial susceptibility, Green mussel, Bloody cockle,

1. Introduction
**Vibrio** spp. are ubiquitous along estuaries and coastal waters throughout the world. While the majority of these bacteria are harmless, several species can potentially infect humans or other animals and cause serious disease such as *Vibrio vulnificus* and *Vibrio parahaemolyticus* are the most important pathogens associated with seafood, especially shellfish (Drake *et al.*, 2007). *Vibrio vulnificus* and *Vibrio parahaemolyticus* are gram-negative, mesohalophilic curved rod-shaped bacterium with a single polar flagellum. *Vibrio vulnificus* and *Vibrio parahaemolyticus* are green colony on TCBS agar. *Vibrio vulnificus* is distinguished from *Vibrio parahaemolyticus* in its ability to ferment lactose, and it naturally occurs in estuarine and marine environments of warm and temperate regions (Martinez-Urtaza, Bowers, Trinanes, & DePaola, 2010). *V. vulnificus* is an opportunistic human pathogen that causing primary septicemia, having a mortality rate of more than 50% (Linkous & Oliver, 1999), gastroenteritis and wound infection in susceptible individuals with liver diseases, immune disorder, diabetes and alcoholism (Vickery, Nilsson, Strom, Nordstrom, & DePaola, 2007) through consumption of raw or undercooked bivalves molluscan shellfish.

In addition to ingestion of raw shellfish, skin cuts or wounds exposed to *V. vulnificus* in marine water are also a well-documented route of infections (Hsueh *et al.*, 2004; Strom & Paranjpye, 2000). In Thailand, *V. vulnificus* exhibited in seafood, marine sediment and sea water, especially oysters, in coastal provinces such as Chonburi, Songkhla and Surat Thani (Sangsricome, 2012; Homtong & Wongput, 2006). Pattani is a coastal province in the southern of Thailand and has been known as aquatic farming especially for green mussels and bloody cockles. These bivalves are feeding organisms that pump seawater through digestive system to obtain oxygen and food. In this process are accumulation and concentration of microorganism that can serve as passive carriers.
of foodborne diseases. This research aimed to isolate and characterize the drug resistant strains of *Vibrio vulnificus* from sea mud within Prince of Songkla University, Pattani campus and closed areas and from green mussel (*Perna viridis*) and bloody cockle (*Tegillarca granosa*).

2. Materials and Methods

2.1 Sample collection and preparation

All samples were collected from January to March, 2016. A total of 43 sea mud samples were collected from mangrove trail in Prince of Songkla University: at Pattani campus (Area A), Pattani estuary (Area B), and Rusamilae estuary (Area C). Area A is located within 1,500 m sampling 5 sites, Area B is located within 210 m sampling 3 sites, and Area C is located within 1,000 m sampling 5 sites. All sea mud samples were collected in sterile plastic bags from a depths of the sampling site 15 cm and 1-5 m beyond the seashore. The samples were transported to the laboratory in an ice chamber. The inoculation into selective medium were made within 24 h after the collection of sea mud samples. Temperature, pH and salinity were recorded at each sampling site by thermometer, pH meter and salinity refractometer, respectively.

While green mussel (*Perna viridis*) and bloody cockle (*Tegillarca granosa*) samples were purchased from Pithan market, Makrut market and Nakrue seafood market in Pattani province. The 15 grams of each shellfish meat samples were placed on sterile plastic bag with 135 mL of alkaline peptone water (APW).

2.2 Bacteriological analysis

For the analysis of *Vibrio* spp., sea mud samples were 10-fold serially diluted into APW. While 15 grams of each clam flesh samples of green mussel (*Perna viridis*) and bloody cockle (*Tegillarca granosa*) were placed in a sterile stomacher bag with 135
mL of alkaline peptone water (10-fold dilution) and were stomached (Colworth Stomacher 400; A.J. Seward, London, United Kingdom) for 2 min. The clam homogenates were prepared into appropriate dilution gradient and spread on Thiosulfate Citrate Bile Salt Sucrose (TCBS) agar in triplicate. Inoculated plates were incubated at 37 ºC for 24 h and followed the population of Vibrio spp. and the greenish colonies by manual count to calculate the density of viable cells in the samples (colony forming unit per gram, CFU/g). The analysis results were reported the mean of three replicates. Gram-negative bacilli and catalase-positive colonies were cultured in MR-VP medium, LIM, nutrient broth containing 0, 3, and 8% NaCl and phenol red broth containing sucrose, lactose, and cellobiose for 24 h at 37 ºC (Holt et al., 1994).

2.3 Antibiotic susceptibility

The antimicrobial susceptibility of the isolated strains was determined with disk diffusion method on Mueller Hinton agar (MHA) containing 1% NaCl. The following antimicrobial discs were Ampicillin (10 µg), Ciprofloxacin (5 µg), Ceftazidime (30 µg), Gentamycin (30 µg), Clindamycin (10 µg) and Rifampicin (5 µg). The inoculum was prepared in 5 mL of sterilized 0.85% NaCl solution and the turbidity was adjusted to 0.5 MacFarland’s standard. Inoculated plates were inverted and incubated at 37 ºC within 24–48 h. The diameter of the inhibitory zones was measured twice at right angles with a ruler graduated to 0.5 mm and expressed by referring to the manufacturer standard table and reported as susceptible, intermediate or resistant (CLSI, 2009).

2.4 Statistical analyses
The analysis results were obtained through the average of three replicates. Statistical analyses were performed using Microsoft Excel 2010. The comparison among three sampling sites was done with One-Way ANOVA test.

3. Results and Discussion

3.1 Vibrio concentration in the sea mud

From 43 sea mud samples, the total of 4 strains of *V. vulnificus* were isolated from Area A (1 strain), Area B (2 strains) and Area C (1 strain) and the study shown that *V. vulnificus* (9.30%) lower than *V. parahaemolyticus* (90.70%) (Table 1). At all three area, the number of *V. vulnificus* were no statistically significant differences (p = 0.297). The means of temperature were 31.4±0.5 °C in Area A, 32.7±0.6 °C in Area B and 33.0±0.0 °C in Area C. The means of pH were 7.4±05 in Area A, 7.3±0.6 in Area B and 7.6±0.5 in Area C. And the means of salinity were 22±1 part per thousand (ppt) in Area A, 24±0 ppt in Area B and 25±1 ppt in Area C and could be explained with possible effect of temperature, pH and salinity of sea mud from three area were no statistically significant differences (p > 0.05). According to WHO (2005) and Sangsricome (2012) that found *V. vulnificus* in sea water/sea mud and marine sediment (20.5%), respectively.

3.2 Vibrio in the clam samples

*V. vulnificus* were detected from green mussels (*Perna viridis*) (30%) and Bloody cockle (*Tegillarca granosa*) (10%) of the 20 examined clams, respectively (Table 2). The study shown that *V. vulnificus* were found in Green mussel higher than in Bloody cockle. However, *V. parahaemolyticus* were detected from green mussels (*Perna viridis*) (20%) and from Bloody cockle (*Tegillarca granosa*) (30%), respectively.
(Table 2). According to Sangsricome (2012) and Drake et al. (2007) that found *V. vulnificus* in some shells.

**Table 1** Occurrence of *V. vulnificus* and *V. parahaemolyticus* in sea mud samples from mangrove trail (Area A), Pattani estuary (Area B), Rusamila estuary (Area C).

| Locations                | No. of positive samples/total No. of samples | *p*-Value |
|--------------------------|---------------------------------------------|-----------|
|                          | *V. vulnificus*                              | *V. parahaemolyticus* |
| Mangrove trail           | 1/10                                        | 9/10      |
| (Area A)                 |                                             |           |
| Pattani estuary          | 2/9                                         | 7/9       |
| (Area B)                 |                                             |           |
| Rusamila estuary         | 1/24                                        | 23/24     |
| (Area C)                 |                                             |           |
| Total                    | 4/43 (9.30%)                                | 39/43 (90.70%) |

**Table 2** Occurrence of *V. vulnificus*, *V. parahaemolyticus* and *Vibrio* spp. in green mussel (*Perna viridis*) and bloody cockle (*Tegillarca granosa*) from Pithan market, Makrut market and Nakru海鲜市场 in Pattani province.

| Samples                  | No. of positive samples/total No. of samples |
|--------------------------|---------------------------------------------|
|                          | *V. vulnificus*                             | *V. parahaemolyticus* | *Vibrio* spp. |
| Green mussel             | 3/10                                        | 2/10                  | 5/10          |
| (*Perna viridis*)        |                                             |                       |
| Bloody cockle            | 1/10                                        | 3/10                  | 6/10          |
| (*Tegillarca granosa*)   |                                             |                       |
3.3 Antimicrobial susceptibility

The antimicrobial susceptibilities of *Vibrio vulnificus* isolates recovered from sea mud and shells were determined. The results shown that all *V. vulnificus* isolates were susceptible to Ciprofloxacin, Ceftazidime and Rifampicin. Whereas, resistance was recorded for Ampicillin and Clindamycin (Table 3). According to Sangsricome (2012), who reported that all *V. vulnificus* were sensitive to Doxycycline, Ceftazidime and Cefotaxime but resisted to Carbenicillin (17.6%), Ampicillin (9.8%), Gentamicin (2.8%) and Erythromycin (0.8%) among *V. vulnificus* isolates from seafood, sea water and marine sediment samples in Songkla and Surat Thani provinces (Sangsricome, 2012). Traditionally, *V. vulnificus* were susceptible to the majority of antibiotic agents. However, several recent studies have reported that *V. vulnificus* showed a multiple antibiotic resistance or intermediate sensitivity test reaction to one or more antibiotic agents (Elmahdi, DaSilva, & Parveen, 2016). Monitoring the hygiene status of seafood and using new strategies other than antibiotics (e.g. probiotics and phage therapy) to control infections in aquaculture are urgently needed to avoid threats to public health caused by massive misuse of antibiotics (Elmahdi, DaSilva, & Parveen, 2016).
**Table 3** Antimicrobial susceptibility of *V. vulnificus*.

| Antimicrobial agent       | Isolated strains |
|---------------------------|------------------|
|                           | AG6 | BG3 | BG4 | CG6 |
| Ampicillin (10 µg)        | I   | R   | R   | R   |
| Ciprofloxacin (5 µg)      | S   | S   | S   | S   |
| Ceftazidime (30 µg)       | S   | S   | S   | S   |
| Gentamicin (30 µg)        | S   | S   | I   | I   |
| Clindamycin (10 µg)       | R   | R   | R   | R   |
| Rifampicin (5 µg)         | S   | S   | S   | S   |

S – Sensitive, R – resistant, I – intermediate.

4. Conclusions

The results of this experiment conclude that from 43 sea mud samples, the total of 4 strains of *V. vulnificus* were isolated from Area A (1 strain), Area B (2 strains) and Area C (1 strain). The 3 strains of *V. vulnificus* were detected in 10 samples of green mussel and 1 strain in bloody cockle. The antibiotic susceptibility was tested on 6 different drugs by disc diffusion method. The results found that all *V. vulnificus* strains were sensitive to Ceftazidime and resistant to Ampicillin and Clindamycin.

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