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Diversity of *Vibrio* spp. at the Andaman Tarutao Island, Thailand

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ABSTRACT

Vibrios are halophilic bacteria and are ubiquitous in marine environments. The diversity of these bacteria in tropical areas is not clearly understood. In this study, *Vibrio* spp. including *Vibrio parahaemolyticus* were investigated at the Andaman Tarutao Island, Thailand. Water samples were collected at Son Bay and Ruesri Bay located on opposite sides of the island. The total numbers and the predominance of *Vibrio* spp. were determined by cultivation on thiosulfate citrate bile- salts sucrose agar. Enumeration of *V. parahaemolyticus* was performed on CHROMagar Vibrio. There was no correlation between the total numbers of vibrios, *V. parahaemolyticus*, temperature and salinity detected in both areas. By cultivation, *V. parahaemolyticus* was regularly observed at Son Bay except in December whereas it was detected only in April and November at Ruesri Bay. The diversity of *Vibrio* spp. was investigated using the Denaturant Gradient Gel Electrophoresis (DGGE) technique. The diversity of *Vibrio* spp. detected at Son Bay was homogeneous whereas it was more heterogeneous at Ruesri Bay. Interstingly, in December although *V. parahaemolyticus* was not detected by cultivation; it was present in this month according to the DGGE technique. Thus, the number of this bacterium was probably low or it was in a non-culturable phase. In this study, *V. harveyi* was detected in both areas every month by DGGE. This indicates that this bacterium is part of the normal microbiota and is present in the Andaman Sea throughout the year. Correlation between copepods and the numbers of *V. parahaemolyticus* was not observed in this study.

Key words: Vibrios, *Vibrio parahaemolyticus*, DGGE, non-culturable, copepods

INTRODUCTION

Marine ecosystems are complex and dynamic. Vibrios are Gram negative bacteria, highly abundant in marine environments and are found as free-living populations and in association with plankton or marine organisms e.g., coral, fish and shellfish (Vandenberghe *et al*., 1998; Ringo and Birkbeck, 1999; Rosenberg and Ben-Haim, 2002). Vibrios also play a role in aquatic food web by taking up dissolved organic matter and produce essential polyunsaturated fatty acids (Sherr and Sherr, 2002; Nichols, 2003). In addition, vibrios are able to break down chitin which is a large pool of amino sugars in the oceans (Riemann and Azam, 2002). Some *Vibrio* spp. are able to degrade toxic polycyclic aromatic hydrocarbons contaminated in marine sediments (Hedlund and Staley, 2001). However, some of them are human pathogens such as *Vibrio cholerae*,

the causative agent of cholera, *V. parahaemolyticus* and *V. vulnificus* which mostly cause gastroenteritis and septicemia respectively (Chiang and Chuang, 2003; Yeung and Boor, 2004; Shahcheraghi *et al*., 2009). Recently, *V. parahaemolyticus* has been reported to cause infections worldwide (Okuda *et al*., 1997).

The distribution of vibrios has been reported to relate to some environmental factors. A study in Chesapeake Bay indicated that *V. parahaemolyticus* and related *Vibrio* spp. were absent in the water column in winter but they reappeared in late spring and early summer when the water temperature increased. In the winter, they were detected in sediments (Kaneko and Colwell, 1973; Wright *et al*., 1996). Investigation of *Vibrio* community in Barnegat Bay revealed that abundance of vibrios was associated with increasing temperatures (Thompson *et al*., 2004). In addition, dynamic of *V. vulnificus* in this bay was less related to salinity than temperature (Randa *et al*., 2004). Association of vibrios to the external surface of zooplankton such as copepods has been demonstrated. Attachment of *V. cholerae* to live copepods made this bacterium survive longer in the water (Huq *et al*., 1983; Colwell, 1996). In addition, it has been speculated that planktonic blooms is associated with the cholera outbreaks (Lipp *et al*., 2002).

Most studies in vibrios have focused on temperate environments. However, the dynamics of *Vibrio* spp. in tropical environments where the water temperature is around 30°C all year round has not been fully investigated. The southern part of Thailand is a peninsula between the Andaman Sea and the Thai Gulf. Tarutao is the biggest Thai national park island off the west coast of Thailand in the Andaman Sea. The aims of this study were to investigate *Vibrio* spp. including *V. parahaemolyticus* and their correlation to environmental factors at Tarutao.

MATERIALS AND METHODS

Study areas and sample collection: Tarutao is located in Andaman Sea around 22 km away from the west coast of southern Thailand. Two areas, Son Bay and Ruesri Bay, located at opposite sides of Tarutao were selected for this study (Fig. 1). Samples were collected once a month for 6 months (January to April and November to December) during the pre-southwest monsoon period.

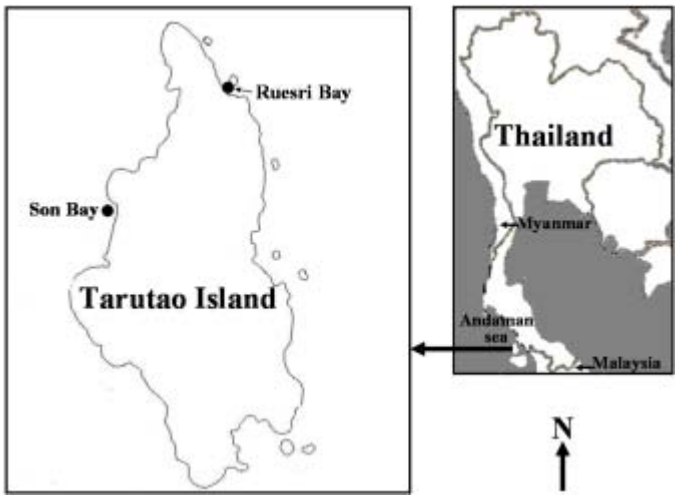

Fig. 1: Location of Son Bay and Ruesri Bay for sample collection

Three liters of water samples was taken 500 m away from the coast and at a depth of 50 cm. Plankton was collected by filtration of 10 L of water through 22 μm plankton net and preserved in 5% formaldehyde before analysis.

Environmental factors: Water temperature and salinity were detected using a thermocouple probe (Anritsu meter Co., Ltd., Japan) and salinometer (N.O.W., Japan), respectively.

Investigation of copepods: Copepods was identified and quantitated using a light microscope and a Sedgwich-Rafter (S-R) counting chamber (Pyser, UK). Each experiment was performed in triplicate.

Number of total vibrios and predominant *Vibrio* spp.: The number of total vibrios and predominant *Vibrio* spp. was determined by filtration of 100 mL of water sample through a 0.45 μm cellulose nitrate filter (Sartorious, Germany) and the filter was placed on Thiosulfate Citrate Bile-Salts sucrose agar (TCBS). In addition, 100 μL of water sample was also spreaded on TCBS. The agar plates were incubated at room temperature. The number of all visible colonies on the agar plates is referred as total vibrios.

Restriction Fragment Length Polymorphism technique (RFLP) has been demonstrated to determine genotypes of many bacteria including *Vibrio* spp. (Urakawa *et al*., 1997; De Vega *et al*., 2005). In this study, PCR-RFLP of 16S rDNA region was performed to identify predominant isolates of *Vibrio* spp. Briefly, colonies on TCBS with different morphological characteristics were selected and 16S rDNA sequences of those isolates were amplified by PCR using primers 27F 5' AGAGTTTGATC(A/C)TGGCTC AG 3' and 1492R 5' TACGG(C/T)TACCTTGTTACGACTT 3' as described previously by Lane *et al*. (1985) and Vergin *et al*. (1998). The amplification products were treated with *Bsa*HI, *Hin*fI and *Rsa*I restriction enzymes (Urakawa *et al*., 1997). After electrophoresis, the predominant isolates that exhibited different DNA fingerprints were selected and their species were confirmed by sequencing of a part of 16S rDNA using the Applied Biosystems 377 genetic analyzer. The search for sequence homology of the 16S rRNA was performed using the Basic Local Alignment Search Tool (BLAST) program. A >99% identity in the 16S rDNA gene sequence was the criterion used to identify an isolate to the species level.

Enumeration and identification of *V. parahaemolyticus*: A total of 100 mL of water sample was filtered through a 0.45 μm cellulose nitrate filter (Sartorious, Germany) then the filter was placed on CHROMagar Vibrio (CHROMagar Microbiology, Paris). In addition, 100 μL of water sample was also spreaded on CHROMagar Vibrio. The plates were incubated at 37°C overnight. Specific purple colonies of *V. parahaemolyticus* were counted (Hara-Kudo *et al*., 2003). Three to five colonies from each plate were selected to confirm them as *V. parahaemolyticus* by PCR targeted to the *toxR* gene (Vuddhakul *et al*., 2000).

Diversity of *Vibrio* spp.: Under stressful environmental conditions, some *Vibrio* spp. can enter Viable But Non-Culturable (VBNC) states (Colwell *et al*., 1985; Bates and Oliver, 2004). In this phase, a decrease in cell volume has been demonstrated (Denner *et al*., 2002). To identify *Vibrio* spp. including VBNC cells, 1,000 mL of water sample was filtered through a 0.22 μm membrane filter (Supor, Gelman). The filters were immediately placed in sterile micro centrifuge tubes and stored at -80°C until used.

DNA from these filters was extracted by a SDS-based DNA extraction method (Zhou *et al*., 1996) and was purified by a QIAquick Gel Extraction kit (QIAGEN, Germany). Nested PCR was performed to amplify 16S rDNA of *Vibrio* spp. using the universal primer pair 27F and 1492R for the first round as described above. The second round PCR analysis was performed using a reaction mixture containing 1 μL of the first-round PCR product, 2 μL of 10 x PCR buffer (Promega, USA), 2 mM $MgCl_2$, 1.25 μM of each GC567F (5'CGCCCGCCGCGCCCCGCGCCCGTCCCGCCGCCCCCGCCCGGGCGTAAAGCGCATGCAGGT3') and 680R (5'GAATTCTACCCCCCTCTACAG3') primers (Thompson *et al*., 2004), 0.025 U of Taq polymerase (Promega, USA) and 0.2 mM dNTPs in a total volume of 20 μL. The PCR reaction involved 95°C for 8 min, 35 cycles of 95°C for 1 min, 64°C for 3 min, and 72°C for 1 min, followed by a final extension at 72°C for 4 min. Amplified products were subjected to Denaturant Gradient Gel Electrophoresis (DGGE) (Eiler and Bertilsson, 2006).

RESULTS AND DISCUSSION

Number of vibrios and environmental factors: There is no single selective medium that can support growth of all species within the genus *Vibrio*. In this study, TCBS was used for enumeration of total numbers of vibrios because it is a selective medium and supports the growth of most environmental and clinical *Vibrio* isolates (Gharibi *et al*., 2010; Adeleye *et al*., 2011). The largest number of total vibrios at Son Bay and Ruesri Bay were detected (3.65×10^7 and 1.04×10^7 cfu 100 mL^{-1}, respectively) in April (Fig. 2a, b). Although the temperature at that time was high, there were no significant correlations observed between temperature and the number of vibrios over the full period of this study (Fig. 3a, b). The salinity at that time was 32.5 and 33.0 ppt at Son Bay and Ruesri Bay, respectively. Salinity did not change significantly during the full testing period but a combination of the salinity and high temperature at that time might support growth of the bacteria and resulted in the abundance of the Vibrio population detected in this month. This result supports the study of Eiler *et al*. (2007) who demonstrated that temperature alone did not significantly affect the abundance of total bacterioplankton, total *Vibrio* spp. or individual *Vibrio*.

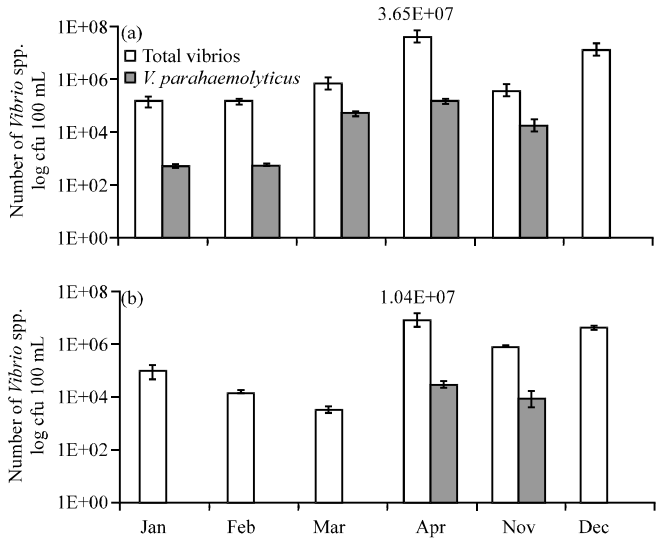

Fig. 2(a-b): Total vibrios and *V. parahaemolyticus* determined at (a) Son Bay and (b) Ruesri Bay

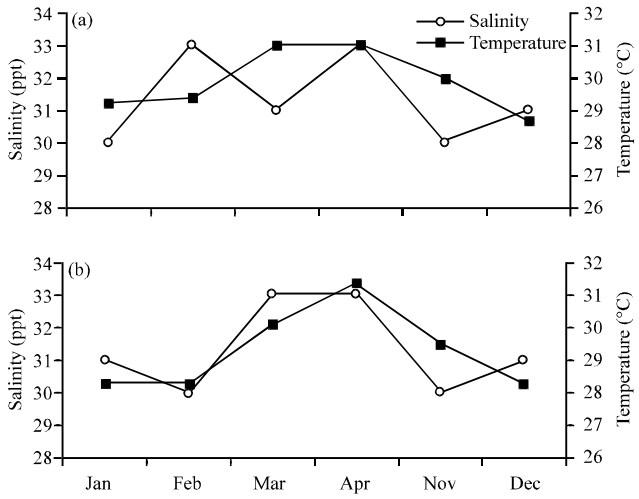

Fig. 3(a-b): Temperature and salinity investigated at (a) Son Bay and (b) Ruesri Bay

Dynamics of *V. parahaemolyticus*: CHROMagar is a selective and differential medium that has been widely used for detection many pathogenic bacteria (Rahbar *et al*., 2008; Taha *et al*., 2010). In this study, CHROMagar Vibrio was used for enumeration of *V. parahaemolyticus*. At Son Bay, *V. parahaemolyticus* was detected in every month except December (Fig. 2a) whereas at Ruesri Bay, it was detected only in April and November (Fig. 2b). The largest populations of *V. parahaemolyticus* were detected in those months that also had an abundance of *Vibrio* spp. in both areas. Son Bay is an open wide bay located at about the middle of the West Coast Shore of Tarutao Island thus water has a regular exchange and probably reflects the situation in the open sea. In this situation, the dynamics of *V. parahaemolyticus* does not significantly change. However, Ruesri Bay is a sheltered island bay at the top of the North East corner of Tarutao Island; a low exchange of water may affect the biological activity which influences the number of *V. parahaemolyticus*.

Predominant *Vibrio* spp.: In this study, we investigated only the variations of the predominant *Vibrio* spp. in each month by cultivation on TCBS. *V. harveyi* was the predominant isolate at Son Bay in 4 out of the 6 months (Table 1) whereas *V. harveyi* and *V. alginolyticus* were the predominant isolates at Ruesri Bay in 2 out of the 6 months. *V. brasiliensis*, a novel sp. of *Vibrio* was detected predominantly in April and March at Son Bay and Ruesri Bay, respectively. This bacterium was first isolated in Brazil which is in a tropical region (Thompson *et al*., 2003). Thus, its presence in these areas is not unpredictable.

Diversity of *Vibrio* spp. detected at Tarutao: The DGGE technique was used to evaluate the types of different Vibrio populations present in the community by generating fingerprints of PCR products from the DNA of different isolates (Rahman *et al*., 2008; Kutako *et al*., 2009). Although different DNA sequences that represent many of the dominant microbial organisms are amplified, this DGGE technique cannot quantify numbers of organism in the sample. DGGE analysis of *Vibrio* spp. revealed that similar *Vibrio* spp. including *V. harveyi*, *V. parahaemolyticus*, *V. proteolyticus*, *V. aestuarianus*, *V. neptunius*, *V. brasiliensis* were detected in every month at Son Bay (Table 1, Fig. 5) except that *V. hepatarius* was replaced by *V. parahaemolyticus* in

Table 1: Predominant and diversity of *Vibrio* spp. detected at Son and Ruesri Bays

	Son Bay		Ruesri Bay	
Month	Predominant *Vibrio* spp. on TCBS	Diversity of *Vibrio* spp. detected by DGGE	Predominant *Vibrio* spp. on TCBS	Diversity of *Vibrio* spp. detected by DGGE
Jan	*V. harveyi*	*V. harveyi, V. parahaemolyticus, V. proteolyticus, V. aestuarianus, V. neptunius, V. brasiliensis*	*V. harveyi*	*V. harveyi, V. alginolyticus*
Feb	*V. harveyi*	*V. harveyi, V. parahaemolyticus, V. proteolyticus, V. aestuarianus, V. neptunius, V. brasiliensis*	*V. alginolyticus*	*V. harveyi, V. alginolyticus*
Mar	*V. parahaemolyticus*	*V. harveyi, V. parahaemolyticus, V. proteolyticus, V. aestuarianus, V. neptunius, V. brasiliensis*	*V. brasiliensis*	*V. harveyi, V. alginolyticus, V. brasiliensis*
Apr	*V. brasiliensis*	*V. harveyi, V. parahaemolyticus, V. proteolyticus, V. aestuarianus, V. neptunius, V. brasiliensis*	*V. tubiashii*	*V. harveyi, V. alginolyticus, V. parahaemolyticus, V. tubiashii*
Nov	*V. harveyi*	*V. harveyi, V. parahaemolyticus, V. proteolyticus, V. aestuarianus, V. neptunius, V. brasiliensis*	*V. harveyi*	*V. harveyi, V. alginolyticus, V. parahaemolyticus*
Dec	*V. harveyi*	*V. harveyi, V. hepatarius, V. proteolyticus, V. aestuarianus, V. neptunius, V. brasiliensis*	*V. alginolyticus*	*V. harveyi, V. alginolyticus, V. parahaemolyticus, V. sinaloensis*

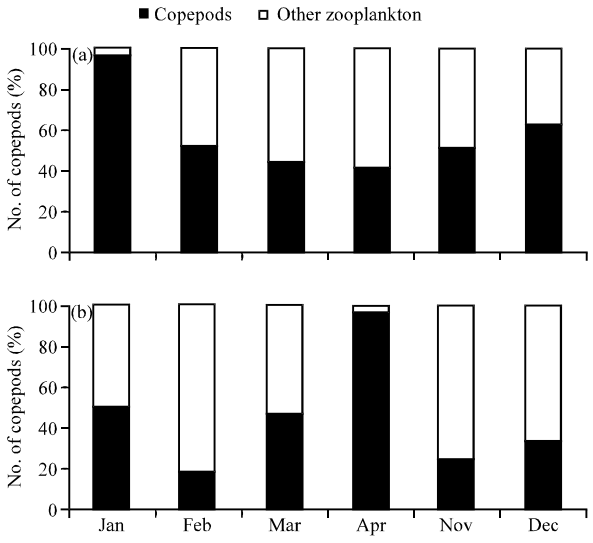

Fig. 4(a-b): Copepods detected at (a) Son Bay and (b) Ruesri Bay

December. This correlated to the result of an undetected this organism on the selective CHROMagar Vibrio in December (Fig. 2a). In Ruesri Bay, the detected *Vibrio* spp. was more heterogeneous (Table 1). It was of interest that in December although *V. parahaemolyticus* was not detected by cultivation on CHROMagar Vibrio, it was present in this month according to the DGGE technique. Thus, the number of this bacterium was probably low or it was in a VBNC phase for

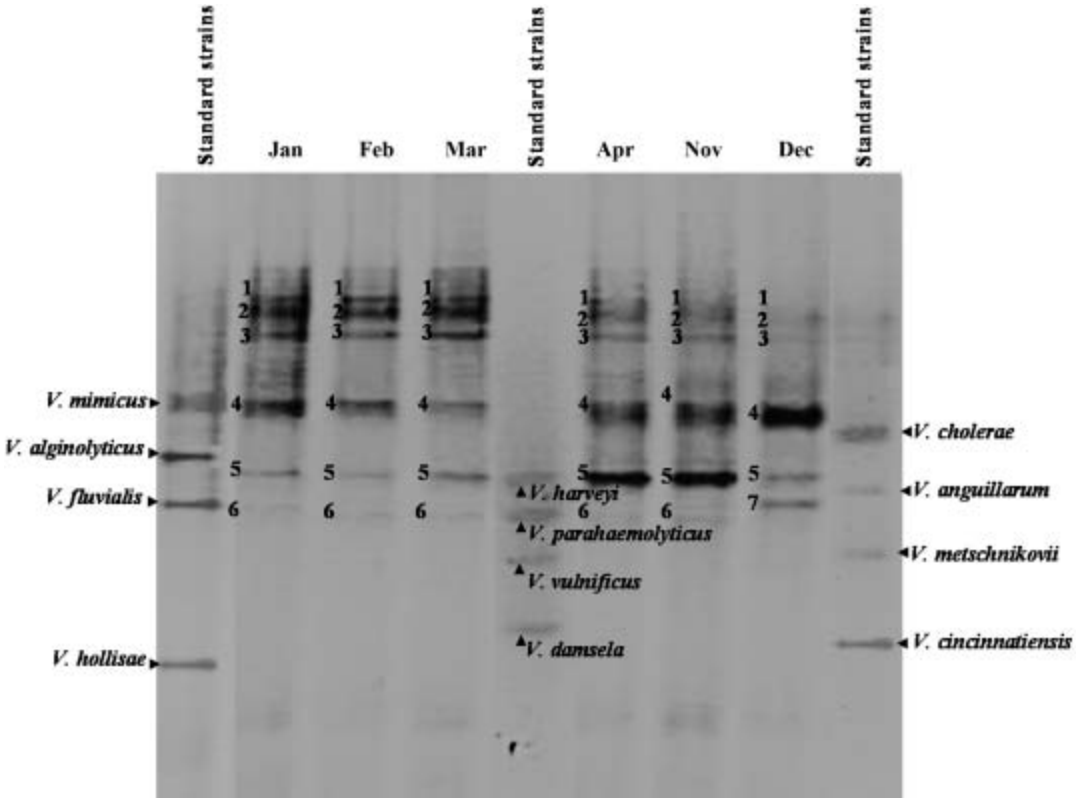

Fig. 5: DGGE profile of *Vibrio* spp. at Son Bay . (1) *V. proteolyticus*, (2) *V. aestuarianus*, (3) *V. neptunius*, (4) *V. brasiliensis*, (5) *V. harveyi*, (6) *V. parahaemolyticus* and (7) *V. hepatarius*

some unknown reason. In this study, *V. harveyi* was detected in both areas every month by DGGE. This indicates that this bacterium is normal microbiota and is present in the Andaman Sea throughout the year.

Correlation between copepods and *V. parahaemolyticus*: It has been suggested that there is a close partnership between *Vibrio* spp. and zooplankton. Large numbers of *Vibrio* including *V. parahaemolyticus* are known to attach to the external surface of zooplankton (Huq *et al.*, 1983; Heidelberg *et al.*, 2002). The association of *V. cholerae* with zooplankton, copepods, is assumed to cause cholera outbreaks during planktonic blooms (Lipp *et al.*, 2002). In this study, during January to April and November to December, the correlation between *V. parahaemolyticus* and copepods was investigated at both Son and Ruesri Bays (Fig. 4). Copepods were regularly detected at between 42-62% of the total zooplankton population in Son Bay except in January; copepods were predominant but their numbers did not correlate to the numbers of *V. parahaemolyticus* detected in this month (Fig. 2a, 4a). The distribution of this plankton was irregular at Ruesri Bay (between 20-50% of total plankton). There was no relationship between copepods and *V. parahaemolyticus* detected in this bay although both of them were detected in abundance in April (Fig. 2b, 4b). These results confirm the study of Huq *et al.* (1983) who demonstrated that in

the presence of live copepods, number of *V. cholerae* was increased but little change in number of *V. parahaemolyticus* was observed. Thus prediction of *V. parahaemolyticus* outbreaks cannot rely on the number of copepods.

CONCLUSION

An investigation of the *Vibrio* community in a tropical area, Thailand, indicated that the diversity of *Vibrio* spp. was dependent on the location. A homogeneous population of *Vibrio* spp. was detected in an area that was open to the sea whereas a more sheltered bay of the island, supported a much more heterogeneous population. Correlation between *V. parahaemolyticus* and copepods was not observed.

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