Diversity Study of Vibrios Isolated from Water and Sediment Samples at Khong-Jilhad, Krabi Province by Denaturing Gradient Gel Electrophoresis (DGGE) Technique

การศึกษาความหลากหลายของเชื้อวิบริโอที่แยกได้จากตัวอย่างน้ำและตะกอนดิน บริเวณคลองจิหลาด จังหวัดกระบี่ โดยเทคนิค Denaturing Gradient Gel Electrophoresis (DGGE)

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ABSTRACT

Vibrios, Gram-negative rod-shape bacteria are widespread in marine and estuarine environments. In this study, thirty-four isolates of *Vibrio* spp. were isolated from water and sediment samples in Khong-Jilhad, Krabi province, Thailand. To determine the diversity of vibrios, *16S rRNA* gene was amplified by PCR and denaturing gradient gel electrophoresis (DGGE) was performed. The results of this study suggested that *Vibrio alginolyticus* and *V. parahaemolyticus* were the dominant bacteria accounted for 37.5% and 25% in the water samples, 40% and 30% in sediment samples, respectively. Moreover, other vibrios were also found in these samples.

บทคัดย่อ

เชื้อวิบริโอเป็นแบคทีเรียแกรมลบรูปแท่ง ที่พบกระจายทั่วไปในสิ่งแวดล้อมทางทะเลและบริเวณปากแม่น้ำ ในการศึกษาครั้งนี้ ทำการแยกเชื้อวิบริโอจากตัวอย่างน้ำและตะกอนดินบริเวณคลองจิหลาด จังหวัดกระบี่ได้ทั้งสิ้น 34 ไอโซเลต จากนั้นทำศึกษาความหลากหลายของเชื้อวิบริโอ ที่แยกได้โดยวิธี Denaturing Gradient Gel Electrophoresis (DGGE) โดยทำการเพิ่มปริมาณ *16S rRNA* gene โดยวิธี PCR และทำ DGGE ผลจากการศึกษาในครั้งนี้ พบว่า เชื้อ *Vibrio alginolyticus* และ *V. parahaemolyticus* เป็นแบคทีเรียที่พบได้มากที่สุดโดยคิดเป็น 37.5% และ 25% ในตัวอย่างน้ำ และคิดเป็น 40% และ 30% ในตัวอย่างดิน ตามลำดับ นอกจากนี้ เชื้อวิบริโอสายพันธุ์อื่นๆ ก็สามารถพบได้ในตัวอย่างเหล่านี้

Key Words: Vibrios, Diversity, Denaturing gradient gel electrophoresis(DGGE) technique

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Introduction

Khong-Jilhad, Krabi province, Thailand is a canal that connect to the Andaman sea. This canal is an area with a large number of brackish water and coastal aquaculture. During June 2013, outbreak of vibriosis in fish cultured in this area was reported.

Vibrios, gram-negative rod-shaped bacteria are widespread in marine and estuarine environments. Vibrios occupies a diverse range of ecological niches including sediments, water and either associated with organisms as symbionts (Ruby *et al.*, 2005). Many vibrios such as *V. alginolyticus, V. anguillarum, V. parahaemolyticus, V. harveyi*, *V. vulnificus* are serious pathogen for marine animal both cultured and feral (Austin, 2010).

Cultivation methods and biochemical tests are normally used for bacterial identification. However, knowing that biochemical methods currently used to identify *Vibrio* spp. can be time-consuming, labour-intensive and hard to implement because of difficulties in distinguishing between closed related species due to the wide variety of biochemical profiles, especially, *Vibrio* spp. isolated from environments such as *V. mimicus* and *V. cholerae* (Davis *et al.*, 1981). Therefore, the application of molecular biological techniques to detect and identify bacteria by certain molecular markers, such as *16S rRNA* or its encoding gene (Amann *et al.*, 1995; Head *et al.*, 1998), is now more and more frequently used to explore the microbial diversity and to analyze the structure of microbial communities (Muyzer and Ramsing, 1995).

Denaturing Gradient Gel Electrophoresis (DGGE) is a technique widely used to study the diversity of bacteria in the environment. DGGE analyzes are employed for the separation of double stranded DNA fragments that are identical in length, but differ in sequence. This technique has the advantage of not requiring previous knowledge on microbial populations and can be used known standard for comparing the samples on different gels.

In this study, therefore, *Vibrio* spp. associated with water and sediment in Khong-Jilhad, and aquaculture farms were isolated and identified by using culture-dependent method and the diversity of vibrios was determined by DGGE. This information can subsequently be employed to elucidate possible pathways of *vibrio* contamination and identify putative pathogen.

Materials and methods

2.1 Bacterial isolation

Five samples of water and three samples of sediments were collected from fish aquaculture area at Khong-Jilhad, Krabi province, Thailand. The samples were enriched in alkaline peptone water (APW) and incubated at 37°C for 6-8 h. A loopful of culture broth was spreaded on CHROMagar Vibrio and incubated at 37°C for 18-24 h. After incubation, the bacterial colonies were divided into different type according to colony characteristics of shape, size, elevation, surface, edge, color and opacity. Representatives of each colony type were tested ability to growth on Thiosulfate-Citrate-Bile-Salts-Sucrose agar (TCBS) and streaked on corresponding plates repeatedly until pure cultures were obtained. All isolates were kept in 20% glycerol at -80°C until used.

2.2 Biochemical tests

All isolates were preliminary identified by the following biochemical tests; motility, oxidase, growth and colony color on TCBS and CHROMagar Vibrio, production of arginine dihydrolase, lysine and ornithine decarboxylase, glucose fermentation, indole, MR-VP, and growth at different salinities (0, 3, 6, 8 and 10%).

2.3 DNA extraction

The glycerol stock were re-streaked on Trytic Soy Agar (TSA) supplemented with 1% NaCl and incubated at 37°C for 18-24 h. After incubation, a single colony of each isolates was picked and inoculated into 3 mL of Luria Bertani (LB) broth supplemented with 1% NaCl and grown overnight with shaking at 150 rpm, 35°C. One mL of broth culture was boiled for 10 min to facilitate cell lysis and DNA release. The tube was immediately placed on ice for 10 min. Then the cell lysate was centrifuged and supernatant was transferred to a new tube. The supernatant was diluted 10-fold in distilled water to be used as template in PCR amplification.

2.3 PCR amplification

The *16S rRNA* gene of vibrios was amplified by PCR. The PCR reaction mixture containing 1X buffer, 1.5 mM $MgCl_2$, 1.25 µM of each GC567F and 680R primers (Thompson *et al.*, 2004), 0.2 mM dNTP, 0.5 unit *Taq* DNA polymerase and 10 ng of DNA template. 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. PCR product was used for sequence-specific separation by DGGE. To confirm the result of DGGE, PCR using species-specific primers for *V. alginolyticus*, *V. parahaemolyticus* and *V. vulnificus* was performed as described previously (Bej *et al.*, 1999; Cañigral *et al.*, 2010; Luo and Hu, 2008)

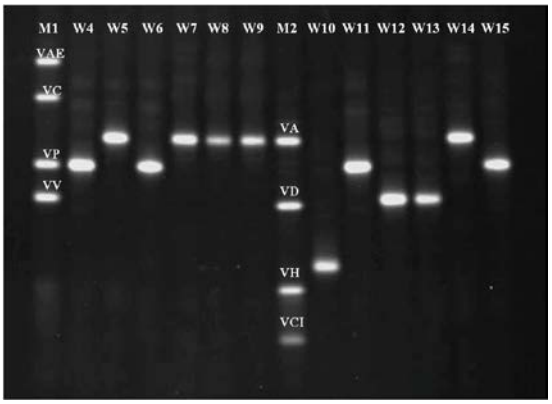
2.4 Denaturing gradient gel electrophoresis

DGGE was performed in 7.5% polyacrylamide gel containing 29:1 acrylamide-bis-acrylamide and 55 to 65 % urea and formamide denaturing gradient were used. Standard *Vibrio* spp. used in this study were *V. aestuarianus* ATCC 35048, *V. cholerae O1* PSU 368, *V. parahaemolyticus* PSU 578, *V. vulnificus* ATCC 27562, *V. damsela* RIMD 2222001, *V. hollisae* PSU 40 and *V. cincinnatiensis* ATCC 35912. The electrophoresis was performed. The gel was stained in ethidium bromide and examined under UV light. To determine the species of vibrios, the DGGE patterns of samples were compared to the standard marker of *Vibrio* spp.

Results

Thirty-four isolates of *Vibrio* spp. were obtained from water and sediment samples collected from fish aquaculture area at Khong-Jilhad in Krabi province, Thailand. The bacterial colony on CHROMagar Vibrio, TCBS and biochemical characteristics were showed in table 1. In this study, *Vibrio* spp. isolated from water and sediments showed the DGGE patterns corresponding to *V. alginolyticus, V. parahaemolyticus* and *V. vulnificus* for 13, 9 and 5 isolates, respectively. These isolates were clarified by PCR using species-specific primers for *V. alginolyticus*, *V. parahaemolyticus* and *V. vulnificus.* The identification results from PCR obtained were similar to those DGGE patterns (data not shown). However, 7 isolates of *Vibrio* spp. were unable to classified by comparison to the standard *Vibrio* marker used in this experiment. The example of DGGE patterns of *Vibrio* spp. isolated from water and sediment samples were showed in figure 1A and 1B, respectively.

1A



1B

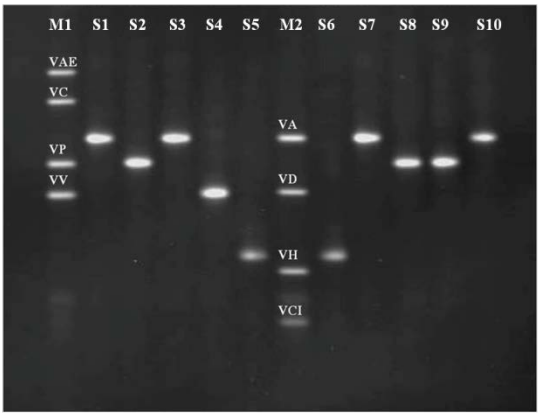
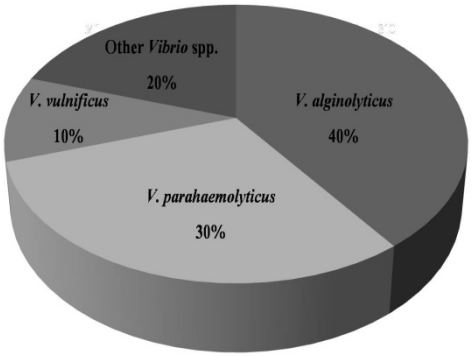


Figure 1 The DGGE patterns of *Vibrio* spp. isolated from water (A) and sediment samples (B). VAE; *V. aestuarianus* ATCC 35048, VC; *V. cholerae O1* PSU 368, VP; *V. parahaemolyticus* PSU 578, VV; *V. vulnificus* ATCC 27562, VA; *V. alginolyticus* PSU 6, VD; *V. damsela* RIMD 2222001, VH; *V. hollisae* PSU 40, VCI; *V. cincinnatiensis* ATCC 35912, W4- W15; *Vibrio* spp. isolated from water samples, S1- S10; *Vibrio* spp. isolated from sediment samples.

2A



2B

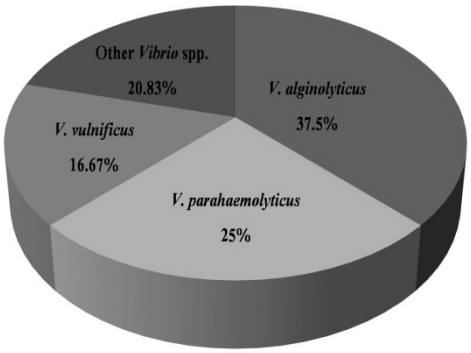


Figure 2 Composition of *Vibrio* spp. isolated from water (A) and sediment samples (B) identified by DGGE.

Discussion and Conclusions

In this study, 34 isolates of *Vibrio* spp. were isolated from water and sediment samples. All isolates were tested for biochemical properties and the diversity of vibrios was determined by DGGE. Although, highly reproducible DGGE patterns were observed in the water and sediment samples, a wide variety of biochemical profiles obtained made them difficult to identify

Table 1 The bacterial colony on CHROMagar Vibrio and TCBS agar, and biochemical characteristics of *Vibrio* spp. isolated from water and sediment samples.

Bacterial isolate no.	Colony color on CHROMagar Vibrio	TCBS	Oxidase	Motility	Indole	Arginine dihydrolase	Lysine decarboxylase	Ornithine decarboxylase	Methyl red	Voges- Proskauer	Glucose fermentation	growth at % NaCl					Related to
												0%	3%	6%	8%	10%	
W1	White	NG	+	-	+	-	-	-	+	-	+	-	+	-	-	-	*Vibrio spp.*
W2	Blue	G	+	+	-*	-	+	+	+	-	+	-	+	-*	-	-	*V. vulnificus*
W3	Yellowish white	NG	+	+	+	-	+	+	+	-	+	-	+	+	+	-	*Vibrio* spp.
W4	Purple	G	+	+	+	-	+	+	+	-	+	-	+	+	+	-	*V. parahaemolyticus*
W5	White	Y	+	+	+	-	+	+*	+	-*	+	-	+	+	+	+	*V. alginolyticus*
W6	White	Y	+	+	+	-	+	-	+	-*	+	-	+	+	+	+	*V. alginolyticus*
W7	White	NG	+	-*	+	-	-*	-	-	+	+	-	+	-	-	-	*Vibrio* spp.
W8	White	Y	+	+	+	-	+	+*	+	-*	+	-	+	+	+	+	*V. alginolyticus*
W9	White	Y	+	+	+	-	+	+*	+	-*	+	-	+	+	+	+	*V. alginolyticus*
W10	Yellowish white	NG	+	+	+	-	+	+	+	-	+	-	+	+	+	-	*Vibrio* spp.
W11	Purple	G	+	+	+	-	+	+	+	-	+	-	+	+	+	-	*V. parahaemolyticus*
W12	Blue	G	+	+	+	-	+	+	+	-	+	-	+	-*	-	-	*V. vulnificus*
W13	Blue	G	+	-*	+	-	+	-	+	-	+	-	+	+	-	-	*Vibrio* spp.
W14	White	Y	+	+	+	-	+	+*	+	-*	+	-	+	+	+	+	*V. alginolyticus*
W15	Purple	G	+	+	+	-	+	+	+	-	+	-	+	+	+	-	*V. parahaemolyticus*
W16	Yellowish white	NG	+	+	+	-	+	+	+	-	+	-	+	+	+	-	*Vibrio* spp.
W17	White	Y	+	+	+	-	+	+*	+	-*	+	-	+	+	+	+	*V. alginolyticus*
W18	Purple	G	+	+	+	-	+	+	+	-	+	-	+	+	+	-	*V. parahaemolyticus*
W19	Yellowish white	NG	+	-	+	-	+	+	+	-	+	-	+	+	+	-	*Vibrio* spp.
W20	White	Y	+	+	+	-	+	+*	+	-*	+	-	+	+	+	+	*V. alginolyticus*
W21	Purple	G	+	+	+	-	+	+	+	-	+	-	+	+	+	-	*V. parahaemolyticus*
W22	White	Y	+	+	+	-	+	+*	+	-*	+	-	+	+	+	+	*V. alginolyticus*
W23	Yellowish white	NG	+	+	+	-	+	+	+	-	+	-	+	+	+	-	*Vibrio* spp.
W24	Blue	G	+	+	+	-	+	+	+	-	+	-	+	+	-	-	*V. vulnificus*
S1	White	Y	+	+	+	-	+	+*	+	-*	+	-	+	+	+	+	*V. alginolyticus*
S2	Purple	G	+	+	+	-	+	+	+	-	+	-	+	+	+	-	*V. parahaemolyticus*
S3	White	Y	+	+	+	-	+	+*	+	-*	+	-	+	+	+	+	*V. alginolyticus*
S4	Blue	G	+	+	+	-	+	-*	+	-	+	-	+	+	-	-	*V. vulnificus*
S5	Yellowish white	NG	+	+	-	-	+	-	+	-	+	-	+	-	-	-	*Vibrio* spp.
S6	Yellowish white	NG	+	+	+	-	+	+	+	-	+	-	+	+	+	-	*Vibrio* spp.
S7	White	Y	+	+	+	-	+	+*	+	-*	+	-	+	+	+	+	*V. alginolyticus*
S8	Purple	G	+	+	+	-	+	+	+	-	+	-	+	+	+	-	*V. parahaemolyticus*
S9	Purple	G	+	+	+	-	+	+	+	-	+	-	+	+	+	-	*V. parahaemolyticus*
S10	White	Y	+	+	+	-	+	+*	+	-*	+	-	+	+	+	+	*V. alginolyticus*

Annotation : W; Water samples, S; Sediment samples, NG; no growth, G; Green, Y; Yellow, TCBS; Thiosulfate-Citrate-Bile-Salts-Sucrose agar, +; positive and –; negative *; result variation from the key identification scheme.

at the species level. The results of DGGE patterns demonstrated that *Vibrio alginolyticus* and *Vibrio parahaemolyticus* were the dominant bacteria accounted for 37.5% and 25% in the water samples and accounted for 40% and 30% in sediment samples, respectively (Figure 2), which was in line with other areas (Barbieri *et al.*, 1999; Dumontet *et al.*, 2000). To confirm the results of DGGE, species-specific PCR was used. This technique showed corresponding results to DGGE patterns.

From this study, *Vibrio* spp. isolated from environments showed some variations in biochemical characteristics compared to the species identification scheme which made it difficult to correctly identify these bacteria using only the conventional cultivation and biochemical tests. Therefore, the results of this study suggested that DGGE is a useful technique to identify the species of vibrios isolated from environments. This technique is very sensitive to variations in DNA sequence and can be used to analyze the microbial communities without any prior knowledge of the species by using universal primers for bacteria, while other methods such as PCR require an extended DNA sequence of microorganisms to design specific primers that target particular individuals. DGGE is also a less laborious technique and consumes a shorter time than cultivation method and biochemical tests. Therefore, DGGE is a suitable and reliable method for diversity study, monitoring and identification of *Vibrio* spp. which cause vibriosis in aquaculture.

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