การตรวจ *Vibrio parahaemolyticus* สายพันธุ์ก่อโรคที่แยกได้จากตัวอย่างทางการแพทย์และสิ่งแวดล้อมด้วยวิธี Multiplex polymerase chain reaction

Detection of Pathogenic *Vibrio Parahaemolyticus* Isolated from Clinical and Environmental Samples by Multiplex Polymerase Chain Reaction

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บทคัดย่อ

ในการศึกษาครั้งนี้ คณะผู้วิจัยได้ตรวจหายีนที่เป็นปัจจัยก่อความรุนแรงของโรค คือ *tdh* และ *trh* จากเชื้อ *Vibrio parahaemolyticus* (VP) ที่แยกได้จากตัวอย่างทางการแพทย์ และสิ่งแวดล้อม ด้วยวิธี multiplex polymerase chain reaction โดยนำเชื้อ VP จำนวน 100 สายพันธุ์ ที่แยกได้จากอุจจาระของผู้ป่วย (n=16), ผู้เป็นพาหะ (n=19), หอยแครง (n=49), และหอยแมลงภู่ (n=16) ผลการศึกษาพบสายพันธุ์ก่อโรค ร้อยละ 17 และสายพันธุ์ที่ไม่ก่อโรค ร้อยละ 83 สายพันธุ์ก่อโรค พบยีน *tdh*$^+$*trh*$^-$ มากที่สุด ร้อยละ 10 ตามด้วย *tdh*$^-$*trh*$^+$ และ *tdh*$^+$*trh*$^+$ ร้อยละ 5 และร้อยละ 2 ตามลำดับ สำหรับสายพันธุ์ก่อโรคพบจากผู้ป่วยมากที่สุด (ร้อยละ 10) พบยีน *tdh*$^+$*trh*$^-$(ร้อยละ 37.5), *tdh*$^-$*trh*$^+$ (ร้อยละ 12.5) และ *tdh*$^+$*trh*$^+$(ร้อยละ 12.5) สายพันธุ์จากพาหะ ให้ผลบวกกับยีน *tdh*$^+$*trh*$^-$ (ร้อยละ 21) และ *tdh*$^-$*trh*$^+$ (ร้อยละ 5.3) เท่านั้น ในทางตรงกันข้าม สายพันธุ์ก่อโรคจากตัวอย่างอาหาร (หอยแครงและหอยแมลงภู่) พบเฉพาะยีน *tdh*$^-$*trh*$^+$ ในเปอร์เซ็นต์ที่ต่ำที่สุด (ร้อยละ 3)

ABSTRACT

In this study, we analyzed virulence genes; *tdh* and *trh* in *Vibrio parahaemolyticus* (VP) strains from clinical and environmental samples using multiplex polymerase chain reaction. A total of 100 VP strains were obtained from diarrheal patients (n=16), human carriers (n=19), blood cockles (n=49), and Asian green mussels (n=16). The result showed that 17% of strains were determined as pathogenic strains and 83% were non-pathogenic strains. The pathogenic strains with *tdh*$^+$*trh*$^-$ were found at the highest prevalence (10%) followed by *tdh*$^-$*trh*$^+$(5%), and *tdh*$^+$*trh*$^+$(2%). Pathogenic strains were predominantly isolated from diarrheal patients (10%) virulence gene profiles of these isolates were *tdh*$^+$*trh*$^-$(37.5%), *tdh*$^-$*trh*$^+$(12.5%), and *tdh*$^+$*trh*$^+$(12.5%). The strains isolated from human carriers were positive for only *tdh*$^+$*trh*$^-$ (21%), and *tdh*$^-$*trh*$^+$ (5.3%). Conversely, pathogenic strains from food samples (blood cockles and Asian green mussels) possessed only *tdh*$^-$*trh*$^+$ in the lowest percent (3%).

คำสำคัญ: ยีน *ldh*, ยีน *tdh,* ยีน *trh,* มัลติเพล็กซ์ พีซีอาร์

Keywords: *ldh*, *tdh*, *trh*, multiplex PCR

INTRODUCTION

Vibrio parahaemolyticus (VP) is a major cause of food-borne gastroenteritis. This disease has an impact on the public health concern in worldwide including Thailand. The VP is a halophilic bacterium that is commonly found in marine environment and contaminated seafood (Fujino *et al*., 1953). Generally, VP are divided into two groups; pathogenic and non-pathogenic strains depending on the presence of virulence genes namely thermostable direct hemolysin (*tdh*) and *tdh*-related hemolysin (*trh*) (Honda *et al*., 1988; Nishibuchi *et al*., 1992). Pathogenic strains carry either *tdh* or *trh*, or both genes while non pathogenic strains do not carry *tdh* and *trh* (Honda and Iida 1993). Majority of pathogenic strains recovered from clinical samples (91.5%) while non pathogenic strains found in environmental samples (98-99%) (Sakazaki *et al*., 1968). In the present study, we examined pathogenic VP strains in our collection. These strains were reidentified by biochemical tests. Then, all strains were analyzed the presence of virulence genes by multiplex PCR.

MATERIALS AND METHODS

1. Bacterial strains

A total of 100 VP strains were obtained from Professor Dr. Orasa Suthienkul, Department of Microbiology Faculty of Public Health, Mahidol University, Bangkok. The strains include 16 diarrheal patients, 19 from human carriers, 49 from blood cockles, 16 from Asian green mussels. The VP strains VP902 (tdh^+trh^+), AQ4613 (tdh^+trh^-), AQ4020 (tdh^-trh^+), and BG26 (tdh^-trh^-) were used as positive and negative reference strains in the study.

2. Determination of purity of V. parahaemolyticus strains identifying by biochemical tests

All studied VP strains from semisolid stock medium containing 1% NaCl were restreaked on thiosulfate-citrate-bile-salt-sucrose (TCBS, Eiken, Tokyo, Japan) agar for checking purity of the culture.

3. DNA preparation

Each VP strains was inoculated into Luria-Batani (LB) broth supplemented with 3% (w/v) NaCl, incubated at 37°C for 18 – 24 h. After incubation, 1 ml of cell suspension was heated at 100°C for 10 min and then immediately placed on the ice for 5 min. The gDNA of VP was harvested by centrifugation at 16,000 x g for 10 min, and kept at -20°C for further analysis.

4. Primers and PCR condition

Primers of VP are shown in table 1. The PCR mixture and condition for detecting virulence genes were followed the method of Athajariya (2004).

Table 1 PCR primers used to detect virulence genes.

Target gene	5'-3'primer sequence	Amplicon size (bp)	Tm (°C)	References
ldh-F	AAA GCG GAT TAT GCA GAA GCA CTG	450	61	Taniguchi *et al*. (1985)
ldh-R	GCT ACT TTC TAG CAT TTT CTC TGC		59	
tdh-F	GTA CCG ATA TTT TGC AAA	382	48	Nishibuchi *et al*. (1985)
tdh-R	ATG TTG AAG CTG TAC TTG A		53	
trh-F	CTC TAC TTT GCT TTC AGT	276	50	Nishibuchi *et al*. (1989)
trh-R	TAC CGT TAT ATA GGC GCT TA		56	

RESULTS AND DISCUSSION

A total of 100 VP strains were analyzed for the presence of *ldh*, *tdh*, and *trh* by multiplex PCR analysis. The gDNA amplification are shown in Figure 1. These isolates were classified into 4 patterns based on the presence of virulence genes *tdh* and *trh* (Table 2). Moreover, VP strains that were positive for *tdh* or *trh*, or both *tdh* and *trh* represent 17% (17/100), while the strains that were negative for *tdh* and *trh* represent 83% (83/100). VP strains positive for $tdh^{+}trh^{-}$ were found the highest percent 10% (10/100), followed by $tdh^{-}trh^{+}$ 5% (5/100), and $tdh^{+}trh^{+}$ 2% (2/100). All three and two patterns of pathogenic VP strains were found in diarrheal patient samples, and human carriers. However $tdh^{-}trh^{+}$ were found 2% (1/49) in blood cockles, and 6.2% (1/16) in Asian green mussels samples. The previous study revealed that not only *tdh* positive were strongly associated with gastroenteritis but also *trh* positive strains (Kishishita *et al*., 1992; Nishibuchi *et al*., 1990). Furthermore, some clinical isolates were found to carry *tdh* and/or *trh*, while environmental isolates possessed either *tdh* or *trh* (Suthienkul *et al*., 1995).

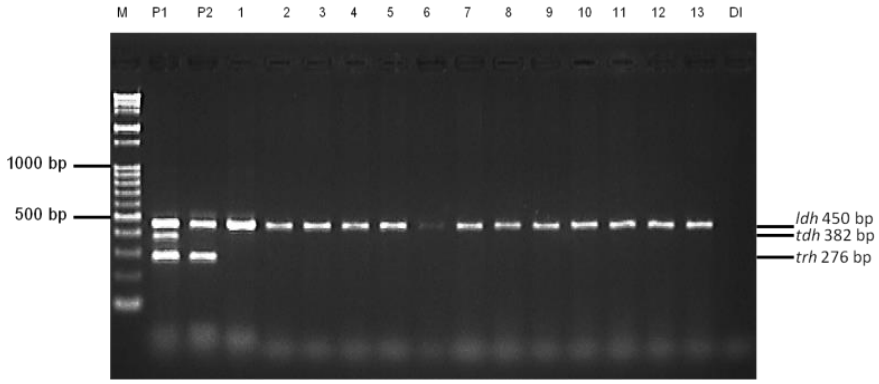

Figure 1 The detection of virulence genes (*tdh* and *trh*) in VP. Lane M, DNA ladder as a marker (Nippon Gene-Wako, Tokyo, Japan); lane 1, VP 902 ($tdh^{+}trh^{+}$); lane 2, VP 4020 ($tdh^{-}trh^{+}$); lane 3-5, VP strains isolated from clinical samples; lane 6-15 *V. parahaemolyticus* strains isolated from environmental samples, lane 16, negative control

Table 2 Detection of virulence genes of *Vibrio parahaemolyticus* isolated from diarrheal patients, human carriers, blood cockles and Asian green mussels by multiplex PCR

Hemolysin gene	No. (%) of strains				Total No.(%) of strains (N=100)
	Diarrheal patients (n=16)	Human carriers (n=19)	Blood cockles (n=49)	Asian green mussels (n=16)	
Pathogenic					
$tdh^{+}trh^{+}$	2 (12.5)	0 (0.0)	0 (0.0)	0 (0.0)	2 (2.0)
$tdh^{+}trh^{-}$	6 (37.5)	4 (21.0)	0 (0.0)	0 (0.0)	10 (10.0)
$tdh^{-}trh^{+}$	2 (12.5)	1 (5.3)	1 (2.0)	1 (6.2)	5 (5.0)
Total	**10 (62.5)**	**5 (26.3)**	**1 (2.0)**	**1 (6.2)**	**17 (17.0)**
Non -Pathogenic					**83 (83.0)**
$tdh^{-}trh^{-}$	6 (37.5)	14 (73.7)	48 (98.0)	15 (93.8)	83 (83.0)

The previous study showed that 98% of VP isolated from oyster carrying *trh* (Gonzalez-Escalona *et al*., 2006). Therefore, mollusc and marine environment are the source of pathogenic *V. parahaemolyticus.* However, there is evidence that nontoxigenic *V. parahaemolyticus* cause acute gastroenteritis (Ottaviani *et al*., 2012).

There are many reports of food-borne disease outbreak which found to be associated with the consumption of raw or under cooked seafood (DePaola *et al*., 2000). In Thailand, the proportion of gastroenteritis cases caused by *V. parahaemolyticus* is more than 50% annually (Chobkatanyoo, 2011).

CONCLUSION

In this study, virulence genes were detected not only in clinical samples but also in environmental samples. Therefore, the surveillance of pathogenic VP in seafood is necessary to evaluate the risk of contamination. However, other virulence factors such as Type III secretion system and antimicrobial susceptibility test should be tested in further study.

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