Title: Virulence factors and molecular subtyping of *Vibrio vulnificus* isolated in Thailand

Name of researcher: Orn-Anong Ratchtrachenchai, Sriwanna Huttayananont, and Krongkaew Supawat

Affiliation: Enteric-Bacteria Laboratory, National Institute of Health, Department of Medical Sciences, Ministry of Public Health, Thailand

Summary: 300~500 words:

Vibrio vulnificus is the leading cause of death related to seafood consumption. *V. vulnificus* can cause disease in those who eat contaminated seafood or have an open wound that is exposed to seawater. *V. vulnificus* is grouped into three biotypes, biotypes 1 and 2 are capable of causing severe human infection, including necrotizing fascistic and septicemia; the death rate is substantial. During 1996–1997, a new biotype, *V. vulnificus* biotype 3, emerged as a cause of severe soft tissue infection and bacteremia in Israel. In this study, *V. vulnificus* strains isolated in Thailand during year 2006-2010 were characterized for virulence factors, molecular typing and antimicrobial susceptibility test. A total of 80 *V. vulnificus* isolates from clinical specimens of 67 patients with septicemia or wound infections, and from 8 sea food samples suspected the cause of infection in patients were used in this study. The bacterial strains were examined for biotypes, and characterized for virulence factors including detection of haemolysin genes (*vvh*A) and *V. vulnificus* potential hazard gene (*vvpdh*) by duplex PCR, and cytotoxicity test with HeLa cells. In addition, *V. vulnificus* strains were molecular subtyping by *NotI* digested PFGE patterns and examined for antimicrobial susceptibility test with 5 antimicrobial agents by E-test.

All 80 *V. vulnificus* isolates were biochemically confirmed as biotype 1. About 90% of *V. vulnificus* strains isolated from patients were positive for both *vvh*A and *vvpdh* genes; where as about 37.5% of *V. vulnificus* strains isolated from sea food samples were both genes positive. Though not all *V. vulnificus* strains were *vvhA* and *vvpdh* genes positive, all *V. vulnificus* strains showed rapid cytotoxic to HeLa cells upon incubation with cell lines for 30 minutes. In addition, the supernatant of bacterial cultures also showed cytotoxic to HeLa cells that indicated the secretion of cytotoxin that is high virulence. *V. vulnificus* strains isolated from 61 patients showed different *NotI* digested PFGE patterns

that indicated the genetic distinct among *V. vulnificus* strains in Thailand. Furthermore; all *V. vulnificus* strains were 100% susceptible to ampicillin, cephalothin, ciprofloxacin, co-trimoxazole and gentamicin.

Purpose:

Vibrio vulnificus is the leading cause of death related to seafood consumption. *V. vulnificus* is common in warm seawater and thrives in water temperature greater than 68° F (20° C). *V. vulnificus* can cause disease in those who eat contaminated seafood or have an open wound that is exposed to seawater. Among healthy people, ingestion of *V. vulnificus* can cause vomiting, diarrhea, and abdominal pain. In immunocompromised persons, particularly those with chronic liver disease, *V. vulnificus* can infect the bloodstream, causing a severe and life-threatening illness characterized by fever and chills, decreased blood pressure (septic shock), and blistering skin lesions. *V. vulnificus* bloodstream infections are fatal about 50% of the time. *V. vulnificus* can cause an infection of the skin when open wounds are exposed to warm seawater; these infections may lead to skin breakdown and ulceration. Persons who are immunocompromised are at higher risk for invasion of the organism into the bloodstream and potentially fatal complications. If *V. vulnificus* is suspected, treatment should be initiated immediately because antibiotics improve survival. The antibiotic doxycycline and a third-generation cephalosporin (e.g.,ceftazidime) is generally recommended. A single agent regimen with a fluoroquinolone such as levofloxacin, ciprofloxacin or gatifloxacin, has been reported to be at least as effective in an animal model as combination drug regimens with doxycycline and a cephalosporin. Children, in whom doxycycline and fluoroquinolones are contraindicated, can be treated with trimethoprim-sulfamethoxazole plus an aminoglycoside.

V. vulnificus biotypes 1 and 2 are capable of causing severe human infection, including necrotizing fascistic and septicemia; the death rate is substantial. During 1996–1997, a new biotype, *V. vulnificus* biotype 3, emerged as a cause of severe soft tissue infection and bacteremia in Israel. Several important features differentiate the illness caused by the new *V. vulnificus* biotype from previously described *V. vulnificus* infections.

The new *V. vulnificus* biotype differs from other *V. vulnificus* strains by its biochemical features (salicin-, cellobiose-, citrate-, and lactose-negative, plus delayed reaction for o-nitrophenyl-β-d-galactopyranoside [ONPG]). These biochemical differences initially prevented correct identification of the strain by routine laboratory methods. Furthermore, molecular analyses using several methods have shown that *V. vulnificus* biotype 3 is genetically distinct from biotypes 1 and 2.

In Thailand, though Thai National Institute of Health, Department of Medical Sciences, has received *V. vulnificus* isolated from patients and sea food samples from various provinces for confirmation every year, little is known about the *V. vulnificus* isolates in Thailand. There is no information regarding to the correlation between biotypes and virulence of the organisms as well as the genetic relationship of *V. vulnificus* isolated from various places of Thailand. Therefore, this study has been designed to determine biotypes, virulence factors, molecular subtyping, and antimicrobial resistance of *V. vulnificus* isolated from patients and sea food samples in Thailand.

Methods:

3.1 Bacterial isolates

Vibrio vulnificus isolates obtaining from patients (septicemia and wound infections) and sea food samples suspected the cause of disease in patients from various provinces of Thailand submitted to Thai National Institutes of Health from January 2006 to December 2010 were used in this study.

All isolates were grown on thiosulfate-citrate- bile-salts sucrose (TCBS) agar (Eiken Chemical, Co., Ltd., Tokyo, Japan) at 37°C for 18-24 hours. Sucrose-nonfermenting colonies appearing on the TCBS agar medium were then confirmed by biochemically tested as described by Farmer *et al.*

3.2 Biogroup

All *V. vulnificus* isolates were further identified for biogroups by its biochemical features on ornithine decarboxylase, indole production, and acid production

from sugar-base medium including, mannitol, sorbitol, salicin and cellobiose, respectively, as described by Farmer *et al.* (Table1).

3.3 Detection of Cytotoxin-hemolysin genes by PCR

Vibrio vulnificus were grown on LB agar plate at 37°C for 18 hours, then an over night grown culture was picked up and re-suspended in MilliQ water prior to boil in a water bath for 15 min. The DNA templates were then determined for the presence of hemolysin/Cytolisin gene (*vvh*A) and fragment of *pilF* gene (*vvpdh*) which is potentially dangerous for humans by duplex PCR. The primers sequences are shown in Table2. The amplification of DNA was performed with *i*Start *Taq* DNA polymerase for 25 cycles of denaturation at 94°C for 40 second, annealing at 54 °C for 55 second, and extension at 72°C for 55 second. A final extension at 72°C for 5 minutes was performed. The PCR products were electrophoresed on 2% agarose gel, and stained with ethidium bromide. Amplified fragments were visualized under UV illumination.

3.4 Cytotoxicity assay in cell cultures

V. vulnificus strains were tested for their cytotoxicity with HeLa cells. HeLa cells ($2x10^5$ cells) were grown to 70-80% confluence in DMEM with 10% fetal bovine serum and antibacterial-antimycotics (100U of penicillin, 100 μg of streptomycin, and 0.25 μg/ml of amphotericin B in 0.85% saline) in 24-well tissue culture plates (Nalge Nunc) at 37°C in 5%CO_2. HeLa cells in monolayer were washed twice with 500 μl of PBS, and 500 μl of fresh DMEM with alpha-D-mannoside at a final concentration of 1% was added into each well. *V. vulnificus* strains were cultured in 3 ml of LB broth at 37°C for 18 h. Two microlitre of bacterial suspension were inoculated into each tissue cultured well. In addition, 18 hours well grown bacterial cultures in LB broth were centrifugation at 15,000 rpm for 15 minutes, then the supernatants were collected and 50 microlitre of supernatants were incubated with HeLa cells monolayers. After 15, 30, 45 and 60 minutes, the tissue culture plates were then observed under a microscope (Nikon, Japan) at the magnification of 200 X.

3.5 Molecular subtyping by PFGE

V. vulnificus strains were determined for their DNA fingerprints by pulsed-field gel electrophoresis (PFGE) of *Not*I digested genomic DNA. PFGE was performed by rapid standardized laboratory protocol for molecular subtyping of *Vibrio parahaemolyticus* as described by PulseNet USA. PFGE profile was performed with a linearly ramped swithching time of 1second to 10 second for 14 hours and further at 20 second to 25 second for 6 hours. PFGE was run on CHEF-DR II and then PFGE patterns were analyzed by BioNumerics version 4.6

3.6 Antimicrobial susceptibility test

V. vulnificus strains were tested for antibiotic resistance with 6 kinds of antimicrobial including, ampicillin (AM), cephalothin (CE), ciprofloxacin (CI), co-trimoxazole (TS), gentamicin (GM) and tetracycline (TC) by the E-test method as described by Clinical and Laboratory Standards Institute (CLSI) for Enterobacteriaceae.

Results:

A total of 80 *Vibrio vulnificus* isolates were confirmed and used in this study. Seventy *V. vulnificus* isolates from clinical samples of 67 patients including 63, 6, 2, and 1 isolates from blood samples, pus, synorial fluid, and sputum, respectively. Eight *V. vulnificus* isolates were from sea food samples including 7 isolates from shrimp, and 1 isolates from ark shell. All 80 *V. vulnificus* strains showed biochemical characters fitted to biotype 1.

To determine for the virulence genes, all 80 *V. vulnificus* strains were examined for the presence of haemolysin/Cytolisin genes (*vvh*A) and a fragment of *pilF* potentially dangerous for humans genes (*vvpdh*) by duplex PCR. The PCR products are shown in Figure1. Among 80 *V. vulnificus* strains, 74 strains (91%) were positive for both *vvh*A and *vvpdh* genes as shown in Table3. More than 98% of *V. vulnificus* isolated from clinical samples were positive for both genes, in contrast about 37.5% of *V. vulnificus* isolated from sea food samples were both genes positive, 25% were *vvh*A positive, but

37.5% were both genes negative.

All 80 *V. vulnificus* strains caused cytotoxic to HeLa cells. After 30 minutes incubation of HeLa cells with *V. vulnificus*, HeLa cells monolayer were detached and showed cell lysis rapidly. In addition, the supernatant of *V. vulnificus* also showed cytotoxic to HeLa cell and caused cell lysis after 30 minutes of incubation.

Seventy strains of *V. vulnificus* including 65 strains from 61 patients, and 5 strains from sea food samples were analyzed for genetic relationship by *Not I* digested genomic DNA pulsed-field gel electrophoresis. *V. vulnificus* strains obtained from patients and sea food samples revealed different PFGE patterns indicated genetically distinct among *V. vulnificus* strains isolated in Thailand as shown in Figure 2.

V. vulnificus strains used in this study were 100% susceptible to ampicillin, cephalothin, ciprofloxacin, co-trimoxazole and gentamicin.

Discussion:

V. vulnificus is highly heterogenous and comprises strains virulent and avirulent for humans, shrimp, and fish, and it is grouped into 3 biotypes. Biotypes 1 and 3 are opportunistic human pathogens, while biotype 2 is pathogenic for aquatic animal. Roig F.J *et al* has develop the PCR-based method to identify the potentially hazard *V. vulnificus*. He demonstrated that haemolysin/Colistin genes, *vvh*A, is *V. vulnificus*-specific sequence that found in all biotypes, and *vvpdh* genes on the *pilF* fragment can discriminated potential hazard strains. In our study, all of *V. vulnificus* isolated from patients in Thailand during January 2006-December 2010 were biotypes 1, and almost all *V. vulnificus* isolated from patients were both *vvh*A and *vvpdh* genes positive that indicated the virulent strains. About 37.5% of *V. vulnificus* strains isolated from sea food samples were both genes negative indicated the avirulent strains in sea food. Our result also showed that not only the PCR-positive strains were toxic to HeLa cells, but the PCR negative for both genes were also cytoxic to HeLa cells and caused cell death rapidly. These results suggested that there might have some other virulence factors or toxins that significant related to cause the diseases. The *NotI* digested PFGE patterns revealed different PFGE patterns that confirmed the highly heterogeneous *V. vulnificus* in Thailand. Antibiotic

should be initiated immediately to patients that approved as *V. vulnificus* infections because antibiotics improve survival. In this study, *V. vulnificus* isolated in Thailand showed susceptibility to ampicillin, cephalothin, ciprofloxacin, co-trimoxazole and gentamicin that.

References

1. Farmer, J. J. III, J. M. Janda, and K. Birkhead 2003. *Vibrio*. p. 706-718. *In* : Murray, P. R., Baron, E. J., Jorgensen, J. H., Pfaller, M. A. and Yolken, R. H. (ed.), Manual of Clinical Microbiology, 8th *ed.* ASM Press, Washington, D.C.

2. Nataro JP, J.B. Kaper, R. Robins-Browne, V. Prado, P. Vial, and M.M. Levine. Patterns of adherence of diarrheagenic *Escherichia coli* to HEp-2 cells. *Pediatr. Infect. Dis. J.* 1987; 6: 829-831.

3. Roig FJ, Sanjuán E, Llorens A, Amaro C. pilF Polymorphism-based PCR to distinguish *Vibrio vulnificus* strains potentially dangerous to public health. Appl Environ Microbiol. 2010 Mar;76(5):1328-33.

4. Parsons MB, Cooper KL, Kubota KA, Puhr N, Simington S, Calimlim PS, Schoonmaker-Bopp D, Bopp C, Swaminathan B, Gerner-Smidt P, and Ribot EM. PulseNet USA standardized pulsed-field gel electrophoresis protocol for subtyping of *Vibrio parahaemolyticus*. Foodborne Pathog Dis. 2007 Fall;4(3):285-92.

5. Performance Standards for Antimicrobial Disks Susceptibility Test: Approved Standard 10th Edition. January 2009, Volume 6 (No1), Clinical and Laboratory Standard Institute.

Table1. Biochemical characters of *Vibrio vulnificus* biotype 1, 2 and 3.

Biochemical characters	Biotype 1	Biotype2	Biotype3
Ornithine decarboxylase	V	-	-
Indole production	+	-	+
Acid production from sugar-base medium			
- Mannitol	V	-	-
- Sorbitol	-	+	-
- Salicin	+	+	-
- Cellobiose	+	+	-

V: variable (11-89% strains are positive))

+: positive (90-100% positive)

-: negative (0-10% positive)

Table2. Oligonucleotide primer sequences used in this study.

Primers	5'..... Sequence .....3'	Target genes	Product size (bp)
*vvh*AF	CCGCGGTACAGGTTGGCGC	Hemolysin/ Cytolisin	521
*vvh*AR	CGCCACCCACTTTCGGGCC		
*vvpdh*F	TGTCGGTGAAAACGGCAAAGCTG	*pilF* (potentially dangerous for humans)	338
*vvpdh*R	GGTATCGATTTCCAACTTAGCGAGGTTG AGCACC		

Table 3. Virulence-associated genes among 80 *V. vulnificus* isolated in Thailand as determined by duplex PCR.

Origin of strains	Haemolysin genes		No. of strains
	*vvh*A	*vvpdh*	
Clinical (*n= 72*)	+	+	71 (98.6%)
	+	-	1 (1.4%)
Food (*n = 8*)	+	+	3 (37.5%)
	+	-	2 (25.0%)
	-	-	3 (37.5%)

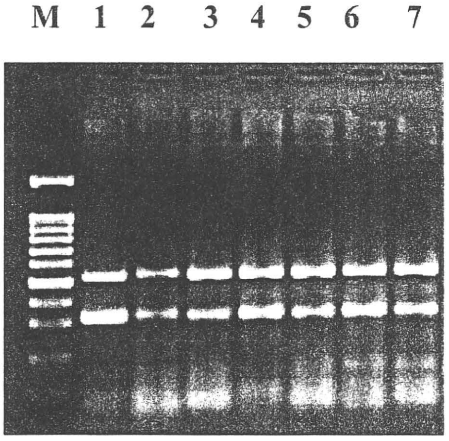
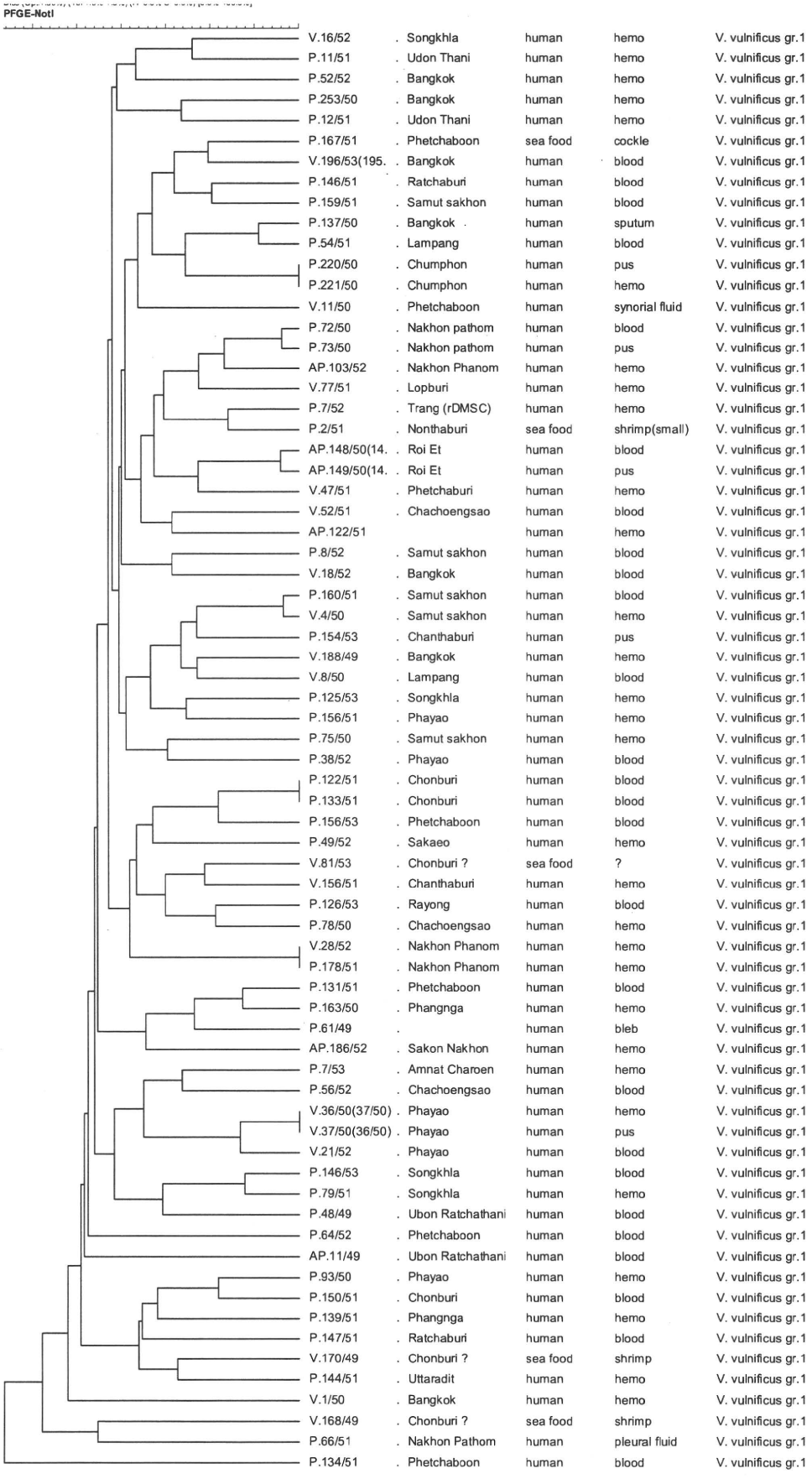


Figure1. PCR products of haemolysin genes of *V. vulnificus* on 2% agarose gel electrophoresis.

Lane M: 100 bp ladder DNA

Lane 1-7: PCR products from *V. vulnificus* isolated from patients revealed a 338 bp. of *vvpdh* gene (lower band) and a 521 bp. of *vvhA* gene (upper band).

Figure 2. Genetic relationship of *NotI* PFGE patterns of *V. vulnificus* strains.



Antimicrobial resistance of *Salmonella enterica* serovar Typhi from Bangladesh, Indonesia, Taiwan, Vietnam

Chien-Shun Chiou[1, 2]*, Munir Alam[3], Tsai-Ling Yang Lauderdale[4], Dac Cam Phung[5], Haruo Watanabe[6], Jun Terajima[6], Shiu-Yun Liang[1]

[1]The Central Region Laboratory, Center for Research and Diagnostics, Centers for Disease Control, Taichung, Taiwan

[2]Institute of Medicine, Chung Shan Medical University, Taichung, Taiwan

[3]The International Centre for Diarrhoeal Disease Research, Bangladesh, Dhaka, Bangladesh

[4]National Health Research Institute, Miaoli, Taiwan

[5]National Institute of Hygiene and Epidemiology, Hanoi, Vietnam

[6]National Institute of Infectious Diseases, Tokyo, Japan

E-mail:

Chien-Shun Chiou: nipmcsc@cdc.gov.tw

Munir Alam: munirul@icddrb.org

Tsai-Ling Yang Lauderdale: lauderdale@nhri.org.tw

Dac Cam Phung: cam@fpt.vn

Haruo Watanabe: aruwata@nih.go.jp

Jun Terajima: Terajima@nih.go.jp

Shiu-Yun Liang: sho9004018@yahoo.com.tw

Corresponding author: Chien-Shun Chiou

Mailing address: 5F 20 Wen-Sin South 3rd Road, Taichung City 40855, Taiwan.

Tel.: +886 4 24750452; fax: +886 4 24750474.

E-mail address: nipmcsc@cdc.gov.tw (C. S. Chiou).

Keywords: *Salmonella enterica* serovar Typhi; molecular typing; multilocus variable number tandem repeat analysis (MLVA); pulsed-field gel electrophoresis (PFGE); antimicrobial resistance

ABSTRACT

We compared the genetic relatedness and antimicrobial resistance profiles of *Salmonella enterica* serovar Typhi isolates from Bangladesh, Indonesia, Taiwan and Vietnam. The genetic relatedness determined using multilocus variable number of tandem repeat (VNTR) analysis (MLVA) showed that the Bangladeshi and Vietnamese isolates were very closely related. A few Taiwanese isolates shared the same MLVA profiles with Indonesian isolates that suggested a direct epidemiological relationship between the Taiwanese patients and the Indonesian migrant workers with carriage of *S.* Typhi. Taiwanese isolates displayed heterogeneous MLVA profiles, indicating that typhoid in Taiwan was caused by multiple sources of *S.* Typhi. Combination of the patterns of pulsed-field gel electrophoresis (PFGE) and the profiles of MLVA5, which was based on analysis of 5 slowly evolved VNTRs, identified 4 major clones, A to D. The 4 clones had distinct resistance profiles and country origins. All the isolates were susceptible to aztreonam, cefotaxime, ceftazidime, ceftriaxone, imipenem and kanamycin and only 1 isolate was resistant to gentamicin. Most isolates of clone B were from Bangladesh and Vietnam and were multiple resistant to the first line antimicrobials, including the ACSSuT (ampicillin, chloramphenicol, streptomycin, sulfamethoxazole and tetracycline), trimethoprim, trimethoprim/sulfamethoxazole and nalidixic acid. It should be noted that 39.5% of the Bangladeshi isolates, belonged to clone B, were ciprofloxacin-resistant. In contrast, isolates of clones A, C and D, most from Indonesia and Taiwan, had low resistance rate to the antimicrobials tested. The multidrug-resistant (MDR) clone B should have spread over the South and Southeast Asia in the study period. The emergence and spread of MDR *S.* Typhi and ciprofloxacin resistant *S.* Typhi strains have aggravated the disease burden over the public health.

Introduction

Salmonella enterica subspecies *enterica* serovar Typhi causes typhoid fever, a disease transmitted primarily through the fecal-oral route. Typhoid fever is most prevalent in South-Central and South-East Asia, causing an estimated 22 million cases and 0.22 million deaths in the year 2000 (Crump et al., 2004). In developed countries, the incidence of typhoid fever has declined markedly, and it is now predominantly associated with travelers returning from endemic areas (Connor and Schwartz, 2005).

Antimicrobials are the mainstay of therapy for typhoid patients. However, the intensive use of the first line antimicrobials, such as ampicillin, chloramphenicol, nalidixic acid, has led to emergence and spread of multidrug-resistant (MDR) *S.* Typhi strains (Kariuki et al., 2010; Mengo et al., 2010; Naheed et al., 2010; Holt et al., 2011). Due to the increasing resistance to the antimicrobials use traditionally for therapy, the use of fluoroquinolones, such as ciprofloxacin and ofloxacin, for the treatment of typhoid has become more common in the developing countries. The use of fluoroquinolones has also led rapidly to an increase of reduced susceptibility to fluoroquinolones in *S.* Typhi. MDR *S.* Typhi, nalidixic acid and ciprofloxacin resistant *S.* Typhi have been report in Africa, South and Southeast Asia (Le et al., 2007; Smith et al., 2010; Nobthai et al., 2011). Single-nucleotide polymorphism study of *S.* Typhi strains has revealed intercontinental spread of a single MDR clone (Kariuki et al., 2010) as typhoid in Kenya in Africa is associated with a dominant MDR *S.* Typhi haplotype that is also widespread in Southeast Asia.

Characterization by various genotyping methods provides useful information, to assess genetic relatedness among isolates for the investigation of short- and long-term epidemiology (Spratt, 2004). Pulsed-field gel electrophoresis (PFGE) is highly discriminatory for the differentiation of most bacterial pathogens and has been widely used to investigate disease outbreaks (Hyytia-Trees et al., 2007). However, in some cases, PFGE is insufficient for discriminating among epidemiologically unrelated *S.* Typhi isolates (Tien et al., 2011). Multilocus variable-number tandem repeat (VNTR) analysis (MLVA) is a well-known typing method that has been successfully developed for many bacterial species or serovars within a species (Lindstedt et al., 2003; Noller et al., 2003; Liao et al., 2006; Marsh et al., 2006; Liang et al., 2007). Based on the analysis of highly variable VNTRs, MLVA can have extremely resolving power in distinguishing between closely related isolates for the investigation of disease outbreaks (Noller et al., 2003; Liang et al., 2007; Torpdahl et al., 2007; Tien et al., 2011).

In this study, we compared the genetic relatedness and antimicrobial resistance profiles for *S.* Typhi isolates from four Asian countries, Bangladesh, Indonesia, Taiwan and Vietnam. The results indicate that the resistance rates to the antimicrobials are associated with *S.* Typhi clones with strong correlation to country origins.

Materials and methods

Bacterial isolates

Bacterial isolates were collected in 2007-2009 from Bangladesh, Indonesia, Taiwan, and Vietnam. The Indonesian isolates were recovered in Taiwan by Centers for Disease Control, Taiwan, from Indonesian migrant workers or travelers returning from Indonesia. Bangladeshi isolates and Vietnamese isolates were provided by the collaborators in the countries. In total, 184 *S.* Typhi isolates were obtained (Table 1).

PFGE

The PulseNet PFGE protocol for *Salmonella* and other enterobacteria was used for PFGE analysis (Ribot et al., 2006). A smaller amount of *Xba*I (10 U) was used for the digestion of each slice.

MLVA

The MLVA, based on analysis of 11 VNTRs described previously (Tien et al., 2011), was used to characterized *S.* Typhi isolates.

Antimicrobial susceptibility testing (AST)

S. Typhi isolates were subjected to antimicrobial susceptibility testing with 16 antimicrobials by the microbroth dilution method using Sensititre MIC panels. AST results were interpreted using the Clinical and Laboratory Standards Institute guidelines. The antimicrobials include ampicillin (penicillins), aztreonam (monobactams), cefotaxime (3rd cephalosporins), ceftazidime (3rd cephalosporins), ceftriaxone (3rd cephalosporins), chloramphenicol, ciprofloxacin (quinolones), gentamicin (aminoglycosides), kanmycin (aminoglycosides), imipenam (carbapenems), nalidixic acid (quinolones), streptomycin (aminoglycosides), sulfamethoxazole (sulfonamides), tetracycline (tetracyclines), trimethoprim (sulfonamides), trimethoprim/sulfamethoxazole (sulfonamides).

Data analysis

PFGE images were analyzed using the fingerprint analysis software BioNumerics version 4.5 (Applied Maths; Kortrijk, Belgium). The number of repeat units for each allele of VNTR was converted from the amplicon sizes and saved as "Character Type" in the BioNumerics database, for later cluster analysis using the Minimum Spanning Tree (MST) algorithm provided in the BioNumerics software. A dendrogram was constructed using composite data sets of PFGE-XbaI

patterns and MLVA5 profiles in 1:1 weight ratio. MLVA5 was based on analysis of 5 slower evolving VNTRs (Sty2, Sty3, Sty20, Sty39 and Sty42).

Results

Prevalence of antimicrobial resistance

S. Typhi isolates were subjected to antimicrobial susceptibility testing with 16 antimicrobials. All the 184 isolates were susceptible to aztreonam, cefotaxime, ceftazidime, ceftriaxone, imipenem, and kanamycin (Table 2). Only 1 (0.5%) isolate was resistant to gentamicin. The resistance rates for the remaining 9 antimicrobials tested were from 8.2% for ciprofloxacin to 51.1% streptomycin. The resistance rates were strongly associated with the countries from where the isolates was originated. Bangladeshi isolates and Vietnamese isolates had significantly higher resistance rates to the 9 antimicrobials than Indonesian and Taiwanese isolates. Resistance to nalidixic acid was observed on 81.6% Bangladeshi isolates, 19.6% Vietnamese isolates and only 1 Indonesian isolate. Ciprofloxcin-resistance was observed only on (39.5%) Bangladeshi isolates. Bangladeshi isolates and Vietnamese isolates had high resistance rates on the ACSSuT (ampicillin, chloramphicol, streptomycin, sulfamethoxazole and tetracycline) and two sulfonamide agents, trimethoprin and trimethoprin/ sulfamethoxazole. The rates of resistance to the 7 agents were from 78.4% to 84.3% for the Vietnamese isolates; from 21.1% to 76.3% for the Bangladeshi isolates. Compared to the Bangladeshi and Vietnamese isolates, Indonesian and Taiwanese isolates had low resistance rate to the 7 antimicrobial agents.

Genetic relationships determined by MLVA data

All isolates were subjected to MLVA which was based on analysis of 11 VNTRs. The MLVA11 profiles were used to evaluate the genetic relationships among the isolates. Because 7 of the 11 VNTR markers are highly variable, they are useful to construct accurate genetic relationships only among very closely related isolates. MST algorithm was applied to construct the genetic relationships among the 184 isolates (Figure 1). The MST tree contained 3 major clusters, I, II and III. Cluster I consisted of 28 Bangladeshi isolates, 50 Vietnamese isolates and 1 Taiwanese isolate. All the ciprofloxacin-resistant isolates belonged to this cluster. The isolates from Vietnam were quite homogenous as 49 of the 51 isolates linked tightly in the cluster II. The isolates from Bangladesh were less homogenous with 10 of the 38 separated distantly. Cluster II had 2 Indonesian isolates and

8 Taiwanese isolates. Cluster III contained 48 Indonesian isolates and 5 Taiwanese isolates; 4 Taiwanese isolates shared common MLVA types with Indonesian isolates. These 4 isolates were recovered from patients who were transmitted by their Indonesian housemaids. The Indonesian isolates were relatively homogenous, except that 8 isolates had distant relationships. The Taiwanese isolates were the most diverse, implying typhoid in Taiwan were caused by multiple pathogen sources.

Clonal relationships determined by PFGE and MLVA data

The clonal relationships among the *S.* Typhi isolates were established using PFGE patterns and MLVA5 profiles that based on analysis of 5 low and less variable VNTRs (Sty2, Sty3, Sty20, Sty39 and Sty42). A dendrogram was constructed using the composite of PFGE patterns and MLVA5 profiles in 1:1 weights ratios (Figure 2). Four distinct clusters, A, B, C and D, were identified for 175 of the 184 isolates. Nine isolates were designated as miscellaneous (M). Clusters B and D were highly homogenous; isolates in the clusters shared at least 81.38% and 90.15% genetic relatedness, respectively. The cluster B and cluster D contained the same panels of isolates as those in the cluster I and III of the MST established with the MLVA11 profiles (Figure 1). Clusters A and C were more diverse than clusters B and D. Cluster A comprised the same panel of isolates as those in the cluster II and a few separate branches of the MST. Cluster C was the most diverse; most of the MLVA types were distantly separate in the MST. The clusters (clones) A, B, C and D had distinct antimicrobial resistance profiles and country origins (Table 3). Isolates of clone A were mainly from Taiwan and they were susceptible to all the antimicrobial agents tested or resistant to only one single agent (Figure 2). Isolates of clone B were primarily from Bangladesh and Vietnam; most of them were resistant to several the first line antimicrobials. All isolates with ciprofloxacin resistance were from Bangladesh. Isolates of clone C and clone D were mainly from Taiwan and Indonesia. Most of the isolates were susceptible to all the antimicrobials or resistant to one or two agents; however, 5 isolates were resistant to 4-9 agents.

Prevalence of antimicrobial resistance among different clones

The *S.* Typhi clones, defined by the PFGE patterns and MLVA5 profiles, had distinct antimicrobial resistance patterns. Clone A had, respectively, 5.6% (1) isolate resistant to nalidixic acid, sulfamethoxazole and trimethoprin and 22.2% (4) isolates resistant to streptomycin (Table 4).

Clone B had 44.3% and 19.0% resistance rate to nalidixic acid and ciprofloxacin, respectively, and 63.3% to 84.8% resistance rates to the 5 ACSSuT antimicrobials and two triemethoprins. Most isolates of clone B were MDR; 54.4% and 22.8% isolates were resistant to 7 and 6 antimicrobials, respectively (Figure 2). Despite that, 4 isolates of clone B were susceptible to all 16 agents tested. Clones C and D had low resistance rate to nalidixic acid, 4 ACSSuT agents and two tremethoprins. A higher streptomycin resistance rate was noted to clones C and D.

Discussion

We collected *S.* Typhi isolates from four Asian countries, Bangladesh, Indonesia, Taiwan and Vietnam, to compare their genetic relatedness and antimicrobial resistance profiles. The results showed that the some Bangladeshi and most Vietnamese isolates were very closely related and the Indonesian isolates and some Taiwanese isolates showed a strong epidemiological link. All the isolates were susceptible to aztreonam, cefotaxime, ceftazidime, ceftriaxone, imipenem and kanamycin and only 1 isolates was resistant to gentamicin. Most Bangladeshi and Vietnamese isolates were MDR. It should be noted that 39.5% Bangladeshi isolates were resistant to ciprofloxacin, a fluoroquinolone. Compared to the Bangladeshi and Vietnamese isolates, the Indonesian and Taiwanese isolates displayed low prevalence of resistance to the 16 antimicrobials tested.

The *S.* Typhi isolates were characterized using an MLVA11 method (Tien et al., 2011). Because 7 of the 11 VNTRs for MLVA are highly variable, the MLVA11 profiles are only useful in establishing genetic relationships among very closely related isolates. It is very interesting that 50 of the 51 Vietnamese isolates and 28 of the 38 Bangladeshi isolates are located in a common cluster of the MST constructed using MLVA11 profiles (Figure 1). Bangladeshi and Vietnam have nearly two thousands of kilometers away but their isolates circulating in 2007-2008 are so closely related, implying that a major clone (clone B) with multi-drug resistance had been circulating in the geographic region in the collection period. This speculation is supported by some early Cambodian isolates. Our *Salmonella* fingerprint database contains 2 Cambodian *S.* Typhi isolates recovered in 1998 and 10 isolates recovered in 2004. The 10 isolates from 2004 are very close to some Vietnamese isolates in cluster B of the MST constructed using MLVA11 profiles (Figure 1). All the Cambodian isolates are MDR; the 2 isolates recovered in 1998 are resistant to 7 antimicrobial agents, including the 5 ACSSuT, trimethoprin and Trimethoprim /sulfamethoxazole, and the 10 isolates

from 2004 are resistant to additional nalidixic acid. The clonal spread of MDR *S.* Typhi strains in the South and Southeast Asian countries should have aggravated disease burden to the public health for the countries the geographic regions.

Of the 79 isolates in cluster B, 77.2% are resistant to 6 or 7 first-line antimicrobials (5 ACSSuT, trimethoprin and Trimethoprim /sulfamethoxazole), 44.3% resistant to nalidixic acid and 19.0% resistant to ciprofloxacin. The emergence of nalidixic-resistant and ciprofloxacin-resistant strains has been a big issue to concern. Quinolone resistance can be mediated by target (DNA gyrase and topoisomerase IV) changes and decreased intracellular accumulation resulting from enhanced active efflux pumps and/or a decrease in cell membrane permeability (Tran and Jacoby, 2002; Blondeau, 2004). Although the mechanisms for quinolone resistance are quite complicated (Piddock et al., 1998; Giraud et al., 1999; Cebrian et al., 2003; Smith et al., 2010), the most frequently reported mechanism for quinolone resistance is point mutations mutations in the gyrase gene (*gyrA* and *gyrB*) and the topoisomerase IV gene (*parC* and *parE*), especially in the quinolone resistance-determining regions of these genes ((Kanematsu et al., 1998; Piddock et al., 1998; Trees et al., 1999; Hsueh et al., 2004). Nalidixic acid is the primitive form of quinolone. Changes to either the targets or the proteins that control the accumulation and permeation of the antimicrobials could easily result in high levels of resistance to nalidixic acid (Piddock et al., 1998; Giraud et al., 1999; Hsueh et al., 2004). Ciprofloxacin belong to the fluoroquinolones. As the derivatives of quinolone, they confer better antimicrobial activity and have better pharmacokinetic performance than nalidixic acid (Kidwai et al., 1998). It has been demonstrated that the development of resistance to these modified quinolones is much more complex than that to nalidixic acid. High levels of fluoroquinolone resistance may require multiple amino acid changes in the gyrase and topoisomerase IV sequences, as well as combinations of changes in the specific components, such as active efflux pumps and porins (Kanematsu et al., 1998; Tran and Jacoby, 2002; Keddy et al., 2010). Ciprofloxacin resistance due to multiple mechanisms has been reported in *S.* Typhi (Keddy et al., 2010).

The results show that the Indonesian isolates and Taiwanese isolates have low prevalence of resistance to the antimicrobials tested. Only 3 Taiwanese isolates and 2 Indonesian isolates are MDR which are resistant to at least 4 of the 7 first-line antimicrobials (5 ACSSuT and trimethoprin and trimethoprin/sulfamethoxazole). Of the 5 isolates, 2 belonged to clone C and 3 to clone D (Figure 2). Accordingly, the isolates were very likely to acquire the resistance traits horizontally unlike those MDR isolates in cluster B which could have received the resistance trait vertically. Resistance to the

antimicrobials could be mediated by specific genes that are carried on movable elements such as integron and transposon (Tamang et al., 2007).

Of the 56 Indonesian isolates, 48 are closely related as they link tightly in the MST constructed using MLVA11 profiles (Figure 1). These isolates were recovered from Indonesian migrant housemaids most of whom came from the same district of Indonesia. Five Taiwanese isolates are distributed in the same MST cluster and 4 share common MLVA11 types with Indonesian isolates, suggesting a direct epidemiological link between the Taiwanese patients and the Indonesian housemaids with carriage of *S.* Typhi. The introduction of Indonesian workers did have brought an impact on typhoid in Taiwan.

The antimicrobial resistance has been studied with 17 clinical isolates of *S.* Typhi from Surabaya, Indonesia, in 2006 (9 strains) and 2008 (8 strains) (Yanagi et al., 2009). The 9 isolates recovered in 2006 are susceptible to all the antimicrobials (ampicillin, nalidixic acid, levofloxacin, ciprofloxacin, tetracycline, chloramphenicol, ceftriaxone, imipenem, trimethoprim/sulfamethoxazole). However, the 8 isolates from 2008 were resistant to nalidixic acid and ampicillin and had a gyrA mutation at codon 87. In addition, 3 of the 8 isolates from 2008 showed multiple drug resistance, including resistance to chloramphenicol, trimethoprim–sulfamethoxazole, and ciprofloxacin. Even though all the Indonesian isolates tested in this study are susceptible to ciprofloxacin, ciprofloxacin resistance could emerge in *S.* Typhi at any place. Clonal spread of ciprofloxacin-resistant and MDR *S.* Typhi strains could be a big public health issue in the world.

In conclusion, comparison of the MLVA genotypes of *S.* Typhi isolates revealed a close relationship between most Bangladeshi isolates and Vietnamese isolates and direct epidemiological relationship between some Taiwanese isolates and Indonesian isolates. All isolates are susceptible to the monobactam (aztreonam), 3 third-generation cephalosporins (cefotaxime, ceftazidime, ceftriaxone), the carbapenem (imipenem) and one aminoglycoside (kanamycin) tested. The prevalence of antimicrobial resistance is relatively different among the four Asian countries. Isolates from Bangladesh and Vietnam have high resistance rates to 8 first-line antimicrobials tested. It should be noted that 39.5% Bangladeshi isolates are ciprofloxacin-resistant. Taiwanese and Indonesian isolates have low resistance rates to the 8 first-line antimicrobials. The highly genetic diversity among the Taiwanese isolates indicates that Taiwan had no large scale typhoid outbreak in 2007-2009.