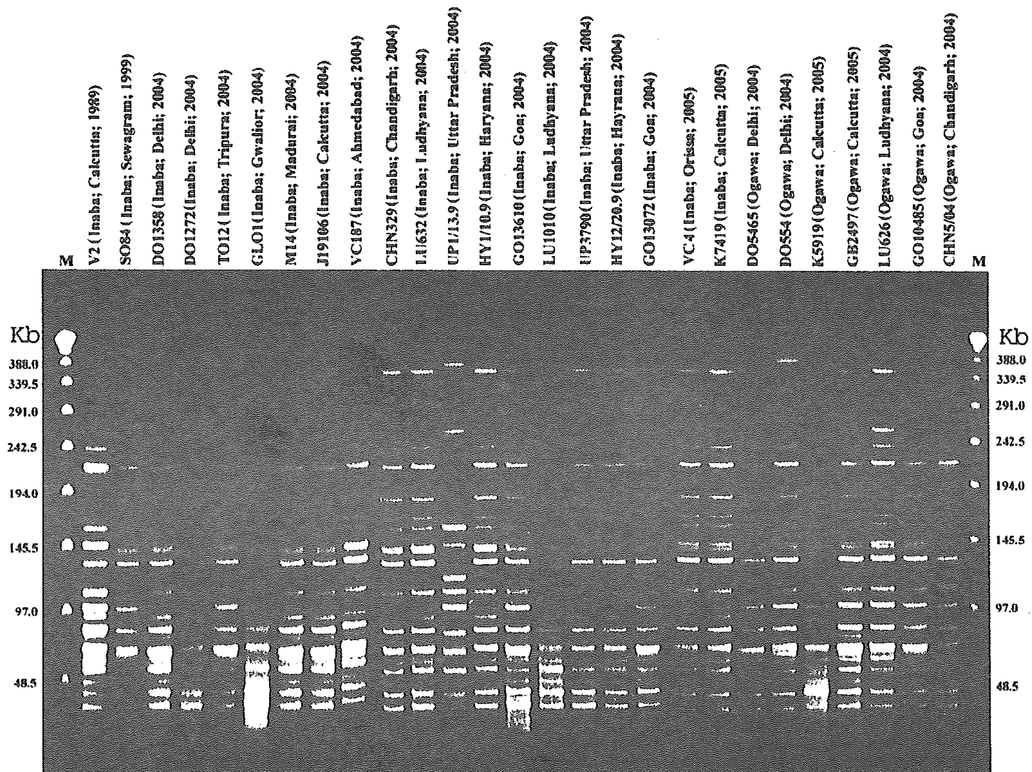

Fig. 3

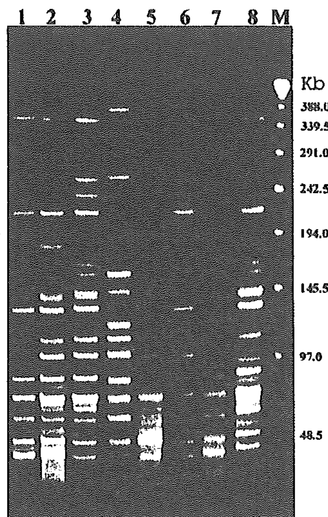

Fig. 4

10. List of publications

i) Chakraborty, R., S. Sinha, A. K. Mukhopadhyay, M. Asakura, S. Yamasaki, S. K. Bhattacharya, G. B. Nair, and T. Ramamurthy. 2006. Species-specificidentification of *Vibrio fluvialis* by PCR targeted to the conserved transcriptional activation and variable membrane tether regions of *toxR* gene. J. Med. Microbiol. (In press).

ii) Dutta, B., R. Ghosh, N. C. Sharma, G. P. Pazhani, N. Taneja, Raychowdhuri, B. L. Sarkar, S. K. Mondal, A. K. Mukhopadhyay, R. K. Nandy, M. K. Bhattacharya, S. K. Bhattacharya, and T. Ramamurthy. Spread of Cholera with new clone of *Vibrio cholerae* O1 El Tor, serotype Inaba in India. (Communicated to the Journal of Clinical Microbiology, March 2006).

iii) Saha, A., R. Deb, S. Shah, T. Ramamurthy, S. Shinoda, A. K. Mukhophadyay, and R. K. Bhadra. 2006. A PCR based identification of *Vibrio cholerae*, its biotypes and closely related *Vibrio mimicus* targeting the chromosomal origin of replication ($oriCI_{VC}$) of *V. cholerae*. FEMS Micrbiol. Lett. (In press).

Title: Virulence Factors and Molecular Epidemiology of Bacteria Causing Food-borne Poisoning Isolated in Thailand

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Summary: 300~500 words:

Food-borne pathogenic bacteria including *Staphylococcus aureus, Vibrio parahaemolyticus,* Shiga toxin-producing *E. coli* (STEC) and Enterotoxigenic *E. coli* (ETEC) isolated from diarrhea patients or food samples were characterized for their virulence factors by mean of phenotypic and genotypic.

S. aureus 122 isolates obtained from patients in year 2003-2005 were characterized for antimicrobial susceptibility, bacteriophage type, and the presence of enterotoxins genes (*sea, seb, sec, sed,* and *see*) by multiplex PCR. Methicillin-resistance (MRSA) and Methicillin-susceptible (MSSA) were found in 3 isolates (2.5%) and 119 isolates (97.5%) of *S. aureus*, respectively. MRSA and MSSA strains were susceptible to vancomycin, teicoplanin and fosfomycin. Untypable phage was found predominantly in 43.4% of *S. aureus.* About 19.7% of *S. aureus* harbored enterotoxins genes.

A total of 168 isolates of *V. parahaemolyticus* isolated from gastroenteritis patients during June to December 2005 were characterized for serotypes and virulence factors, including urease production and determination of two known virulence genes namely thermostable direct hemolysin (*tdh*) and TDH-related hemolysin (*trh*) by PCR. *V. parahaemolyticus* isolates were classified into 25 serotypes and the most predominant serotypes were O3:K6 (22.0%), O4:K55 (18.5%) and O4:K68 (17.9%). The detection of virulence factors were examined from 129 *V. parahaemolyticus* strains. Of the 129 *V. parahaemolyticus* strains, 94.6% were positive for *tdh* but negative for *trh* genes, 0.8% carried both genes and 4.6% were negative for both genes. All tested strains of serotypes O3:K6, O4:K55 and O4:K68 were *tdh* positive. Two strains (1.6%) were urease- positive.

A total of 1,611 *E. coli* isolates obtained from patients were submitted during January to December 2005. All isolates were identified as ETEC and STEC by

determination of heat-labile and heat-stable enterotoxin genes (*eltIA, stIA*) of ETEC and shiga toxin genes (*stx1/2*) of STEC. In addition, all isolates were determined for their O-serogroups by agglutinating with commercial available 43 O-antisera. Of the 1,611 *E. coli* isolates, 1.5% and 0.6% were identified as ETEC and STEC, respectively. Heat-labile and heat-stable enterotoxins genes were found in 41.7% and 45.8% of ETEC, respectively. About 58% of ETEC were distributed in 7 O-serogroups and 42% were O-untypable. STEC found in this study were O157:H non-motile and *stx1/2* positive.

This study demonstrated the presence of MRSA isolated from diarrheic patients and the emergence of *V. parahaemolyticus* serotypes O4:K55 and O4:K68, in addition to O3:K6, as the possible new pandemic clone in Thailand. Moreover, this study also demonstrates the emergence of pathogenic STEC O157: non-motile. The genetic relation among each pathogen will be studied in more details. The DNA fingerprints of each pathogen will be performed by pulsed-field gel electrophoresis.

Purpose:

Food-poisoning is still one of the major health problems in Thailand. In year 2003, 131,561 cases of food-poisoning with infection rate of 209.04 per 100,000 populations were reported to Bureau of Epidemiology Division, Department of Diseases Control, Ministry of Public Heath, Thailand [1]. During the past ten years (1994-2004) the infection rate of food poisoning becomes increasing from 113.61/100,000 populations in year 1994 to 209.04/100,000 populations in year 2004. Though, the exact number of causative agents of food-poisoning is not available, many outbreaks of food poisoning were caused by *Staphylococcus aureus* and *Vibrio parahaemolyticus*. *S. aureus* is highly vulnerable to destruction by heat treatment and nearly all sanitizing agents. Since 1997, infections cause by Methicillin-resistance *S. aureus* (MRSA) with intermediate susceptibility to vancomycin (VISA)(MIC 8-16 μg/L) have been reported from Japan, France, the United states, Korea, Germany and Thailand. *V. parahaemolyticus* is one of the most important food-borne pathogen in Thailand, causing approximately half of the food-poisoning outbreaks. Since first observed in India in 1996, the pandemic O3:K6 clone of *V. parahaemolyticus* have been reported to cause outbreaks in many regions such as India, Russia, Southeast Asia, Japan and North America [11]. In addition, Shiga

toxin-producing *E. coli* (STEC) O157:H7 caused large outbreaks of food poisoning in many countries. Recently, food-poisoning due to STEC non-O157:H7 such as O26, O111, and etc, have been reported in many countries. Enterotoxigenic *E. coli* (ETEC) cause infantile diarrhea in many developing countries and traveler's diarrhea in developed countries. Recently, an attention of food-borne pathogens are raised that may due to the emergence changes of non-pathogenic to more severe pathogenic strain. In addition, the rapid transportation between country and country support the spread of an emergence pathogen through out the world. Therefore, well characteristic of pathogens are needed for prevention and control of an outbreak of an emergence food-borne pathogen. In Thailand, characteristic of food-borne pathogens is limited and not enough for making warning sign to public. Therefore, this study was designed to cover epidemiology and virulence characteristics of *S. aureus*, *V. parahaemolyticus*, STEC, and ETEC obtained from diarrheic patients or food samples.

Methods:

1.1. Bacterial strains

Rectal swabs (one per patient) were collected from patients with acute diarrhea who attended hospitals across Thailand. Rectal swab samples collected in Cary-Blair transport medium were inoculated directly onto selective media including Sorbitol Mac-Conkey agar (SMAC), Mac-Conkey agar, Thiosulfate-Citrate-Bile salt-Sucrose (TCBS) agar, *Salmonella-Shigella* agar, Xylose Lysine Desoxycholate agar, mannital salt agar, selenite broth, and alkaline peptone water for culture overnight at 37°C. In addition, food sample or swabs from food samples were cultured as the same manner. Sorbitol non-fermenting colonies were presumptively screened for STEC O157 by agglutinating with *E. coli* O157 antiserum. One to three lactose-fermenting and any lactose non-fermenting colonies with typical *E. coli* morphology were initially selected. *S. aureus, V. parahaemolyticus,* and *E. coli* isolates, which had been presumptively identified at the hospitals concerned, were submitted to Enteric-Bacteria Laboratory, National Institute of Health, Thailand, for confirmation and further investigation.

S. aureus isolates were identified by gram staining, coagulase production, and latex agglutination test for identification of *S. aureus* (Pastorex Staph-Plus, BioRad). *V.*

parahaemolyticus and *E. coli* isolates were confirmed by standard methods[4,5]. In addition, *V. parahaemolyticus* isolates were tested with Urea agar supplemented with NaCl at a final concentration of 1% (Christensen's method) for detection of urease production [7,12].

1.2 Anti-microbial susceptibility test

S. aureus were screened for Methicillin-resistance (MRSA) by using latex agglutination kit (MRSA screen kit, Denka Seiken, Japan) and then tested for antimicrobial susceptible with 14 kinds of antimicrobial disk including amoxicillin/clavulinic acid (AMC), chloramphenicol(CHL), clindamycin(CLI), cefoxitin (CEF), co-trimoxazole (SXT), erythromycin (ERY), fosfomycin (FOS), gentamycin (GEN), ofloxacin (OFL), oxacillin (OXA), penicillin G (PEN), tetracycline (TET), teicoplanin (TEI) and vancomycin (VAN) by disk diffusion agar method as described by CLSI (Clinical and Laboratory Standards Institute) .

1.3 Bacteriophage typing

S. aureus were examined for bacteriophage typing by using the international phage typing set issued by PHLS Central Public Health Laboratory, Colindale, UK[8,9] .

1.4 Serotyping

V. parahaemolyticus and *E. coli* were determined for O and K typing with antiserum kit product of Denka Seiken, Japan, as described by the manufacturer.

1.5 Determination of virulence-associated genes by PCR

1.5.1 *S. aureus* were determined for enterotoxin genes (*sea*, *seb*, *sec*, *sed*, and *see*) by multiplex PCR [10].

1.5.2 *V. parahaemolyticus* were examined for thermostable direct hemolysin (*tdh*) and TDH-related hemolysin (*trh*) genes by duplex PCR [3] .

1.5.3 *E. coli* were examined for the presence of enterotoxins genes (*eltIA, stIA*) of ETEC and Shiga toxin genes (*stx1/2*) of STEC by multiplex PCR [12] .

Results:

S. aureus

A total of 122 *S. aureus* isolates submitted in year 2003 (31 isolates), 2004 (68 isolates) and 2005 (23 isolates) were screened for Methicillin-resistance (MRSA) and then

tested for susceptible to 14 kinds of antimicrobial disk. Of the 122 *S. aureus* isolates, 3 (2.5%) and 119 (97.5%) were identified as MRSA and MSSA, respectively. All MRSA strains were resistance to almost all drugs, except fosfomycin, teicoplanin, and vancomycin. Of the 119 MSSA strains, 8 (6.6%) were susceptible to all drugs, 111 (93.4%) were susceptible to almost all drugs except penicillin G. Of the 122 *S. aureus*, 53 (43.4%) were phage-untypable, 5 (4.1%), 15(12.3%), 13(10.7%), 2(1.6%), 3(2.5%) and 31(25.4%) were phage type lytic group I, II, III, V, miscellaneous group, and mixed group, respectively (shown in Table 1). Twenty-four (19.7%) of the 122 *S. aureus* were enterotoxins genes positive. Among enterotoxin genes–positive strains, 13 strains were isolated from stools and 11 strains were isolated from foods. Enterotoxins A, B and C genes (*sea, seb, sec*) were found in 14 (11.5%), 3 (2.5%), and 2 (1.6%) strains, respectively. In addition, 4 strains (3.3%) were *sea* and *sec* genes positive, and 1 strain (0.8%) were *seb* and *sec* positive (Table 2.)

V. parahaemolyticus

A total of 168 isolates of *V. parahaemolyticus* isolated from gastroenteritis patients during June to December 2005 were confirmed and classified into 25 serotypes as shown in Table 3. The most predominant serotypes were O3:K6 (37, 22.0%), O4:K55 (31, 18.5%), and O4:K68 (30, 17.9%), respectively. About 129 strains of *V. parahaemolyticus* were selected and examined for virulence factors by determination of virulence-associated genes (*tdh* and *trh*) and urease production. Of the 129 strains, 122 (94.5%) were positive for *tdh* but negative for *trh* genes, 1 (0.8%) carried *tdh* and *trh* genes, and 6 (4.7%) were negative for both genes. Two out of the 129 strains (1.6%) were urease positive and belonged to O1:K56 serotype. One of the urease- positive strains was *tdh* and *trh* positive, the other was *tdh* positive but *trh* negative as shown. in Table 4. All of the most predominant serotypes of O3:K6 (19 strains), O4:K55 (31 strains), and O4:K68 (25 strains) were *tdh* positive as shown in Table 4.

E. coli

A total of 1,611 *E. coli* isolates obtained from patients were submitted during January to December 2005. All isolates were identified as ETEC and STEC by determination of heat-labile (*eltIA*) and heat-stable (*stIA*) enterotoxin genes of ETEC and

shiga toxin genes (*stx1/2*) of STEC. Of the 1,611 *E. coli* isolates, 24 (1.5%) and 10 (0.6%) were identified as ETEC and STEC, respectively. Among 24 ETEC strains, 10(41.7%) were *eltIA* genes positive, 11 (45.8%) were *stIA* genes positive, and 3 (12.5%) were *eltIA* and *stIA* genes positive. About 58% of ETEC were distributed in 7 O-serogroups and 42% were O-untypable as shown in Table 5. Ten strains of STEC isolated from a child with watery diarrhea were O157:H non-motile and stx1/2 positive.

Discussion:

MSSA was the major cause of food poisoning in this study. Most of MSSA were susceptible to almost all drugs. About 19.7% of MSSA were enterotoxins genes positive, enterotoxin A was found the most frequent. Furthermore; 8.2% of the enterotoxin A genes positive were untypable phage type. This study indicates that MSSA producing enterotoxin A is widely spread among the general population. Enterotoxin B and C genes were found in 2.5% and 1.6% respectively. Enterotoxin D and E genes were not found in this study but found common in several countries. The emergence of MRSA (2.5%) was found during year 2003 -2005, all MRSA were enterotoxins genes negative and susceptible to vancomycin. MRSA were emerged indicating drug resistance of *S. aureus* in food-poisoning. About 53% of *S. aureus* were phage typable and distributions of phage types were unspecific in each year. Because of the low susceptible to bacteriophages shown by *S. aureus* isolated in Thailand, comparing with the reports from several countries, other methods of typing should be used in conjunction with phage typing in epidemiological investigations concerning this organism.

V. parahaemolyticus are diverse serotypes, 75 different combinations of O and K serotypes are recognized. After year 1996, besides O3:K6, other serotypes including O4:K68, O4:K8, O4:K12, O4:KUT, O1:K25, O1:K41, O1:K56, O3:K75, O1:KUT, and O5:KUT have emerged and been shown to belong to the pandemic clone by molecular typing techniques [2]. In this study, 25 serotypes were found, with the most frequent in O3:K6, O4:K55 and O4:K68, during June-December 2005. Serotypes O3:K6 and O4:K68 associated with small outbreaks in July and O4:K55 caused an outbreak in October. Though many serotypes involved in outbreaks in Thailand, our previous study found that O3:K6 and O4:K8 associated with numerous outbreaks during year 2000-2004

(unpublished data). This study shows that, in addition to O3:K6, the predominance of O4:K55 and O4:K68 indicate the emergence of other pandemic strains in Thailand.

V. parahaemolyticus strains that produce thermostable direct hemolysin (TDH) and TDH-related hemolysin (TRH), which are encoded by *tdh* and *trh* genes, respectively, are considered pathogenic [6]. In this study more than 95% of *V. parahaemolyticus* strains were *tdh* genes positive. All strains of serotypes O3:K6, O4:K55 and O4:K68 were *tdh* genes positive indicating the presence and the emergence of pathogenic pandemic strains in Thailand. In common with other *Vibrio* species, generally only a small population of clinical *V. parahaemolyticus* strains produces urease. The relatively rare urease-positive phenotype of *V. parahaemolyticus* is always associated with the possession of the *trh* gene, making urease production a reasonably good clinical diagnostic marker for virulent (*trh* positive) *V. parahaemolyticus* [14]. In this study, 1.6% of the 129 *V. parahaemolyticus* strains were urease positive and they belonged to O1:K56 serotype. The prevalence of urease-positive *V. parahaemolyticus* strains in this study is lower than previous reports that 4% and 7.5 % of urease-positive *V. parahaemolyticus* were found in Taiwan during 1992 and 1995 [15] and in Thailand in 1995 [16] . In this study, the difference between urease-positive strains was observed in that one of the 2 strains was *trh* negative, thus genetic variation between the 2 strains needs to be further investigated. In addition, molecular epidemiology of the emergence pandemic strains of serotypes O3:K6, O4:K55 and O4:K68 needs to be more studied in details.

The prevalence of ETEC (1.5%) in this study shows that the prevalence of ETEC becomes decreasing comparing to three previous studies from Thailand. In1985 and 1986 [16, 17], ETEC (6% and 7%) and STEC (0% and 0%), as determined by probe hybridization, were recovered from 393 children in 16 district hospitals [16] and 278 children in the Children's Hospital in Bangkok [17], respectively. Moreover; ETEC (3%), and STEC (0.04%), as determined by PCR, were isolated from 2,100 Thai children during year 1996-2000 [18]. By the way, the virulence of ETEC in Thailand do not change much since the heat-stable enterotoxin probes/genes-positive ETEC and both the heat-labile and the heat-stable enterotoxins probes/genes-positive ETEC are still the major and the minor groups, respectively. STEC are very rare in Thailand, only few STEC non-O157:H7 were

found during the past ten years [18]. This study shows an emergence of non-motile STEC O157.

In conclusion this study demonstrates the presence of MRSA isolated from diarrheic patients and the emergence of *V. parahaemolyticus* serotypes O4:K55 and O4:K68, in addition to O3:K6, as the possible new pandemic clone in Thailand. Moreover, this study also demonstrates the emergence of pathogenic STEC O157: non-motile. The genetic relation among each pathogen will be studied in more details. The DNA fingerprints of each pathogen will be performed by pulsed-field gel electrophoresis.

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Table 1. Prevalence of Phage types of 122 *S. aureus* strains in year 2003-2005

	No. of strains (%)			
Group	**2003**	**2004**	**2005**	**Total**
I	1	3	1	5 (4.1)
II	9	4	2	15 (12.3)
III	5	7	1	13 (10.7)
V	0	1	1	2 (1.6)
Miscellaneous	0	2	1	3 (2.5)
Mixed	8	15	8	31 (25.4)
Typable	**23 (74.2)**	**32 (47.1)**	**14 (60.9)**	**69 (56.6)**
Untypable	8 (25.8)	36 (52.9)	9 (39.1)	53 (43.4)
Total	**31 (100)**	**68 (100)**	**23 (100)**	**122 (100)**

Table 2. Prevalence of Enterotoxin genes of 122 strains of *S. aureus* in year 2003-2005

Enterotoxin genes	**2003 (*n=31*)**	**2004 (*n=68*)**	**2005 (*n=23*)**	**Total (%) (*n=122*)**
sea	3	10	1	14 (11.5)
seb	3	0	0	3 (2.5)
sec	0	2	0	2 (1.6)
sea + sec	1	3	0	4 (3.3)
seb + sec	1	0	0	1 (0.8)
Toxin producing	8	15	1	24 (19.7)
None	23	53	22	98 (80.3)

Table 3. Prevalence serotypes of 168 *Vibrio parahaemolyticus* strains isolated from patients in Thailand (June –December 2005).

O Group	O:K serotypes (No. of strains)				
O1 (18)	O1:K25 (2)	**O1:K56 (7)**	O1:KUT (9)		
O2 (5)	O2:K3 (1)	O2:K28 (3)	O2:KUT (1)		
O3 (50)	O3:K5 (1)	**O3:K6 (37)**	O3:K29 (9)	O3:K58 (1)	O3:KUT (2)
O4 (80)	**O4:K8 (12)**	O4:K9 (2)	O4:K11 (1)	O4:K12 (1)	**O4:K55 (31)**
	O4:K63 (1)	**O4:K68 (30)**	O4:KUT (2)		
O5 (9)	O5:K15 (1)	O5:KUT (8)			
O8 (2)	O8:KUT (2)				
O10 (1)	O10:KUT (1)				
O11 (1)	O11:KUT (1)				
OUT (2)	OUT:KUT (2)				

Table 4. Serotypes and Virulence factors of 129 *V. parahaemolyticus* strains (June –December 2005).

	Virulence factors			
		Haemolysin genes		No. of
Serotypes	Urease production	*tdh*	*trh*	Strains
O1:K25	-	+	-	2
O1:K56	-	+	-	4
O1:K56	+	+	+	1
O1:K56	+	+	-	1
O1:KUT	-	+	-	8
O2:K3	-	+	-	3
O2:K28	-	-	-	1
O3:K6	-	+	-	**19**
O3:K29	-	+	-	4
O3:K29	-	-	-	2
O3:K58	-	+	-	1
O3:KUT	-	+	-	1
O4:K8	-	+	-	**10**
O4:K9	-	+	-	2
O4:K11	-	+	-	1
O4:K12	-	-	-	1
O4:K55	-	+	-	**31**

O4:K68	-	+	-	**25**
O4:KUT	-	+	-	1
O5:K15	-	+	-	1
O5:KUT	-	+	-	7
O8:KUT	-	+	-	1
O10:KUT	-	-	-	1
OUT:KUT	-	-	-	1

Table 5. Serogroups and enterotoxin genes of Enterotoxigenic *E. coli* isolated from patient with diarrhea symptoms in year 2005

	No. of positive strains by PCR (%)			
O-serogroup	*eltIA*	*stIA*	*eltIA* + *stIA*	**Total**
O169	1	1	-	2
O167	-	2	-	2
O78	-	1	-	1
O18	2	-	-	2
O15	1	2	-	3
O8	1	-	-	1
O6	-	-	3	3
Untypable	5	5	-	10
Total	**10 (41.7)**	**11 (45.8)**	**3 (12.5)**	**24**

Development of Multilocus Variable-Number Tandem Repeat analysis (MLVA) method for molecular subtyping of *Shigella* spp.

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Summary

Sixteen and fifteen variable number tandem repeat (VNTR) locus candidates have been found for *Shigella flexneri* and *S. sonnei*, respectively. Multiplex PCR methods have also been developed successfully for analysis of the two *Shigella* species. A preliminary result showed that the 16 VNTR loci could exist in all serotypes of *S. flexneri* and the 15 VNTR in all *S. sonnei* isolates tested. Our results indicate that successful development of MLVA methods can be expected. Since MLVA method can be used as a tool for phylogenetic analysis, it can be applied to investigate the distribution of clonal groups in different countries or regions.

Purpose

Shigella species including *Shigella dysenteriae*, *S. flexneri*, *S. boydii* and *S. sonnei* are the causative agents of shigellosis, one of the major diarrheal diseases in developing countries. Transmission of the disease is frequently associated with international traveling. Characterization of *Shigella* strains by various subtyping methods provides useful information for cluster detection, outbreak investigation and tracking the global spread of clones. Among the subtyping methods, PFGE is the most discriminatory tool for *Shigella* spp. The PFGE protocol for *Shigella* spp. has been standardized in the PulseNet laboratories, so the image-type data generated by PFGE can be exchanged and compared between laboratories [1]. PFGE is a powerful tool for subtyping of *Shigella* isolates; a high level of discriminatory power, in other hand, is disadvantageous to connecting the relationship between strains evolved over few years. Another drawback to PFGE is that result of PFGE is useful for comparison of genetic relatedness but not for study of clonal relationship. Therefore, it is needed to develop a subtyping method with similar level of discriminatory power to PFGE and with data exchangeable, comparable and useful in constructing clonal relationship between *Shigella* strains. This study aims to develop a multilocus variable-number tandem repeat (VNTR) analysis (MLVA) for molecular subtyping of *S. flexneri* and *S. sonnei*. The MLVA protocol will be standardized and transferred to all the participants in the PulseNet Asia Pacific network.

Methods

Searches of VNTR candidates. The genomes of *S. flexneri* 2a strain 2457T (GenBank accession no. AE014073), *S. flexneri* 2a strain 301 (GenBank accession no.

AE005674), *S. sonnei* strain Sso46 (GenBank accession no. CP000038) and *S. sonnei* strain G53 (accessed from http://www.sanger.ac.uk/Projects/*Escherichia_Shigella*/) were explored for potential VNTR loci using unpublished VNTRDB computer software developed by Kao et al. in National Taiwan University. The program, which incorporates the algorithm of the Tandem Repeat Sequence Finder software [2], searches tandem repeat loci from one of the three genomic sequences and then locates the positions of each of the loci at the other two compared genomes. The genomic sequences are used in turn as the "parent" sequence, so that a locus with only one repeat unit at a genome, but with two or more repeat units at other genomes, will not be missed. Repeat loci with numbers of repeat units different among the genomes or with identical number of repeat units and more than 3 copies of repeat unit but sharing more than 85% nucleotide identity were selected for further PCR evaluation. The nucleotide sequences of the invasive plasmid pCP301 (GenBank accession no. AF386526) from *S. flexneri* strain 301 and the invasive plasmid pSS (GenBank accession no. CP000039) from *S. sonnei* strain Ss046 were also explored for potential VNTR loci using the Tandem Repeat Sequence Finder software.

Preparation of crude bacterial DNA. *Shigella* isolates, stored at –70 °C, were plated onto tryptic soy agar and incubated overnight at 37 °C. A loopful (10 μl) of bacterial growth was removed from the plate, suspended in 100 μl of TE buffer (10 mM Tris-Cl, 1 mM EDTA, pH 8.0) in an Eppendorf tube, and boiled for 10 min. After centrifugation at 3700 g for 10 min, the supernatant was transferred to a new tube and used for PCR amplification.

PCR amplification and analysis of VNTR regions. The primer sets specific to the selected VNTR loci for *S. flexneri* and *S. sonnei* are listed on Table 1 and Table 2, respectively. The primers were designed using the free program available at http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi. A primer of each primer set was labeled on 5' end with an ABI-compatible dye, 6-FAM, NED, VIC or PET by the manufacture (Applied BioSystems, Foster City, CA, USA). Each 10-μl PCR mixture contained 1x PCR buffer, 3 mM $MgCl_2$, 0.1 μM each primer, 200 μM each deoxyribonucleotide, 1.0 unit of the recombinant SuperNew Taq DNA polymerase (Jier Sheng Company, Taipei, Taiwan), and 1 μl of DNA template prepared as above-mentioned. A multiplex PCR was developed for *S. flexneri* and *S. sonnei*, respectively, with primer combinations as shown in Table 3. The samples were placed on a GeneAmp PCR System 9600 (Applied BioSystems) and the PCR reaction was performed with a denaturing step at 94 °C for 5 min, followed by 30 cycles of amplification step at 94 °C for 30 s, at 54 °C for 45 s, and at 72 °C for 45 s, and by an extension step at 72 °C for 10 min. Three microliters of each PCR products was electrophoresed in 2% SeaKem LE agarose (Cambrex Bio Science, Rockland, ME, USA) to check the sizes of amplified DNA products and the quality of PCR amplification.

Before size analysis the fluorescent amplicons were diluted in water, usually at a

1:100 or 1:200 ratio, then separated by capillary electrophoresis on an ABI Prism 3130 Genetic Analyzer with GeneScan 500 LIZ Size Standard (cat # 4322682; Applied BioSystems). Data were collected and lengths of amplicons were determined with GeneScan Data Analysis Software, ver 3.7 (Applied BioSystems).

Results

VNTR candidates for *S. flexneri*. The searches for VNTR candidates were conducted using VNTRDB computer software on the released genomic sequences of the two *S. flexneri* 2a strains (301 and 2457T). DSC1, a locus with only on repeat unit at plasmid pCP301, was identified from a finding of locus with 9 repeat units on the plasmid pSS in *S. sonnei* strain Ss064. After preliminary test on 5 *S. flexneri* isolates, a total of 16 VNTR candidates were chosen for further evaluation (Table 1).

VNTR candidates for *S. sonnei*. The searches for VNTR candidates were conducted using VNTRDB computer software on the two genomic sequences of *S. sonnei* strains (Ss046 and G53). The nucleotide sequence of plasmid pSS was searched for repeat loci using the Tandem Repeat Sequence Finder. After preliminary test on 5 *S. sonnei* isolates, a total of 15 VNTR candidates were therefore chosen for further evaluation (Table 2). Among the 15 VNTR candidates, the DSC1 locus was identified from plasmid pSS.

Multiplex PCR for MLVA analysis of *S. flexneri* and *S. sonnei*. Since four ABI-compatible dyes (6-FAM, NED, VIC or PET) can be used to label primers, a multiplex PCR can be set to amplify up to four loci. A multiplex PCR was successfully developed. Five multiplex PCR combinations were achieved for analysis of *S. flexneri* and *S. sonnei* (Table 3). Up to date, the multiplex PCR combinations had used to evaluate 20 *S. flexneri* isolates. The final multiplex PCR combinations will be determined after evaluation for more than 100 isolates.

Evaluation of VNTR loci in *S. flexneri* serotypes. Twenty *S. flexneri* isolates of serotypes 6, 1a, 1b, 2a, 2b, 3a, 3b, 4a, y variant and nontypable were characterized by MLVA analysis to determined the presence of VNTR loci in various *S. flexneri* serotypes. Except DSC1 locus that is present in an invasive plasmid, the 15 VNTR loci were detected in the 20 isolates of various serotypes (Table 4). This evaluation indicated the 16 VNTR loci could be applied for MLVA analysis for all the serotypes of *S. flexneri* species. The evaluation showed that the SSTR-C18 locus could not be a VNTR locus for *S. flexneri*.

Discussion

At the beginning, we were trying to found a set of universal VNTR loci that could be applied to analyze the four *Shigella* species. After further evaluation on strains of the four species, we found that some loci were VNTR for a species but were not variable on the other species. Our preliminary evaluation indicated that VNTR loci could be

species-specific. To date, two *S. flexneri* and two *S. sonnei* genomic sequences are available that is very helpful to compare the tandem repeats and find out VNTR candidates. The second genomic sequence for *S. dysenteriae* and *S. boydii* are being determined. Therefore, the VNTRDB program can be applied to identify potential VNTR loci. Since *S. dysenteriae* and *S. boydii* are still important in many developing countries, to develop MLVA methods for the two species is still in need.

Our preliminary results showed that development of MLVA method was workable for *S. flexneri* and *S. sonnei*. Although PFGE has been shown be highly discriminatory to subtyping of *Shigella* species, the results can only be used to evaluate the genetic relatedness but not phylogenetic relationship between *Shigella* strains. Many studies have indicated that MLVA exhibits higher level of discriminatory power than PFGE and MLVA results can be used to construct phylogenetic relationship between bacterial strains using the eBurst algorithm [3, 4]. For example, a study conducted in our laboratory shows that MLVA is much more discriminatory than PFGE for distinguishing *Neisseria meningitidis* strains. In the study, a total of 31 serogroup W135 meningococcal isolates were discriminated into 4 PFGE genotypes but 30 MLVA genotypes [5]. In Taiwan, *S. flexneri* 2a is indigenous and circulating in aboriginal tribes in mountainous area [6], while most *S. sonnei* are imported. By the successful development of MLVA, we can study the transmission route of *S. flexneri* 2a in the aboriginal tribes. MLVA can also be a powerful tool for international surveillance of *S. sonnei*, a pathogen usually transmitted via traveling.

The VNTRDB program was developed for searching tandem repeat loci in two or more genomic sequences. The program incorporates the algorithm of the Tandem Repeat Sequence Finder program to find out tandem repeat loci and then locates the positions of each of the loci at the other compared genomes. The genomic sequences compared are used in turn as the "parent" sequence, so that a locus with only one repeat unit at a genome, but with two or more repeat units at other genomes, can be found. This function helps to find out more VNTR candidates. For example, RSS1 locus has only one repeat unit in *S. sonnei* strain 53G but 10 repeat units in *S. sonnei* strain Ss046. The more genomic sequences of a bacterial species are available, the more existing VNTR loci can be found.

The first year of study was focusing on searching and confirming VNTR loci. The second year of study will be focusing on MLVA analysis of *S. flexneri* and *S. sonnei* to evaluate the discriminatory power and usefulness of the MLVA methods. The MLVA methods will be released to members of PulseNet Asia Pacific. In the future, the MLVA methods can be used to conduct an international research project to see the distribution of *Shigella* clonal groups in Asian countries.