2) PFGE analysis

In this year, we have changed the electrophoresis conditions for *NotI* and *SfiI*, because the gel ran by previous method have the difficulty of band discrimination, especially at the lower part. Therefore we have changed the electrophoresis condition for the *NotI* digestion of *V. vulnificus* to 3.5~20seconds pulse time and 22hrs run(fig.1) and for *SfiI* , to 2~10 seconds pulse time and 13hrs run (block 1) and 20~25 sconds pulse time and 6hrs run (block 2)

Fig. 1. Representative *NotI*-digested PFGE patterns

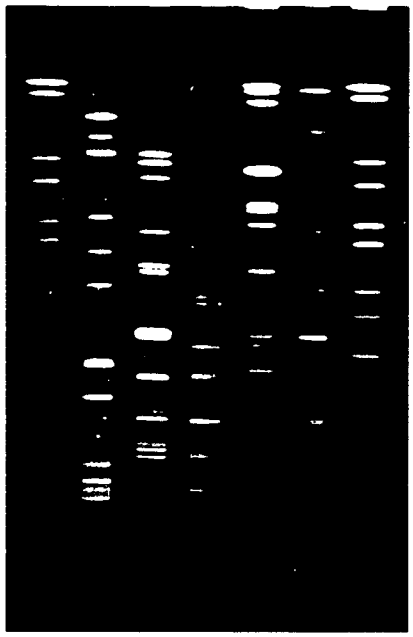

Fig. 2. Representative *SfiI*-digested PFGE patterns

We have tested total 193 isolates (101 clinical isolates, isolated between 2000~2007 and 92 environmental isolates, isolated between 2005~2007). For PFGE with *Not*I digestion, most of the clinical strains were grouped into cluster NA and NB, while the environmental strains were grouped into cluster NE. Clinical strains showed a higher level of genetic homogeneity than environmental strains. But, No other genetic relation was found to the isolated region and isolation time. These strains with similarity indices of 63.5% or more were arbitrarily grouped into three clusters designated NA, NB, and NC. Cluster NB was further subdivided into three subtypes (NB1-3). Most of the strains (60.5%) were comprised of cluster NB, while clusters NA and NC comprised 17.1% and 14.5% of the strains, respectively. About 7.9% of the strains belonged to other unnamed clusters. The subtypes (NB1-3) of cluster NB comprised 13.2, 28.9, and 18.4% of the strains, respectively. Thirteen strains were discriminated in a single cluster (similarity > 97%) with *Not*I types NA, NB2, and NC.

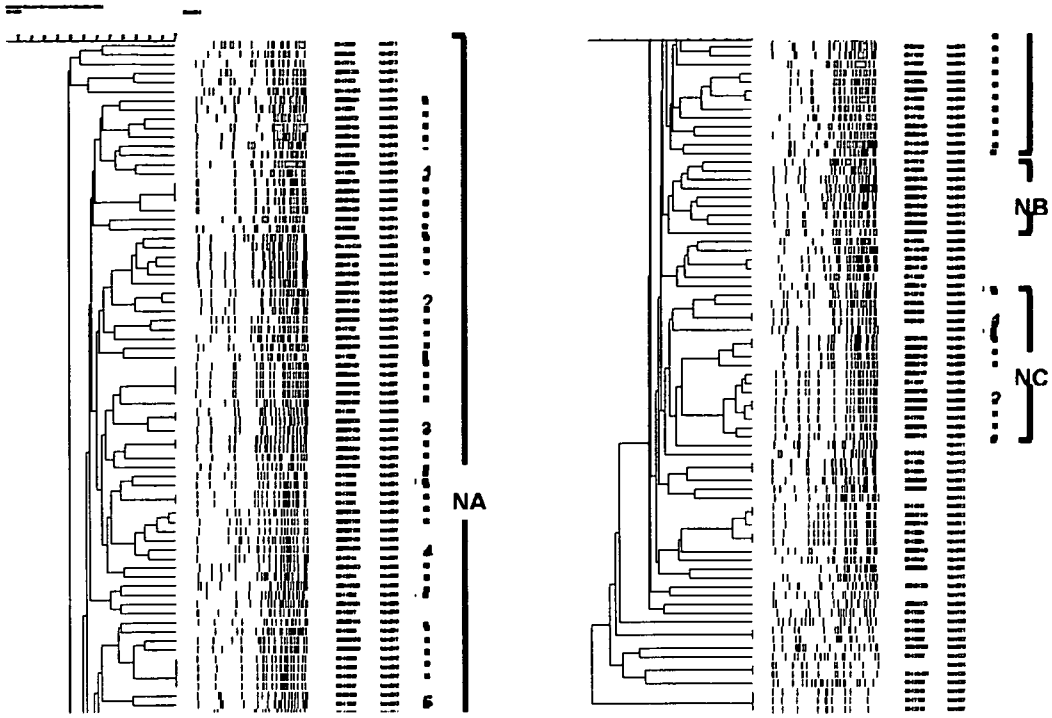

Fig. 3. Dendrogram of the *NotI* digested PFGE patterns of *V. vulnificus*.

For PFGE with *SfiI* digestion, Strains with similarity indices of 66.0% or more were also grouped into five clusters (SA to SE). Most of the clinical strains were grouped into cluster SC, while the environmental strains were grouped into cluster SB and SD. Cluster SB and SC was further subdivided into subtypes (SB1, 2) and subtypes (SC1-3). Cluster SB and SC was the major types and comprised 22.9% and 35.5% of the strains, while clusters SA, SD, and SE comprised 6.6% to 11.8% of the strains. The subtypes SB1 and SB2 comprised 25% and 3.9% of the strains, respectively. All of the subtypes, SC1, SC2, and SC3, comprised 11.8%. Twelve strains were discriminated in a single cluster (similarity > 97%) with *SfiI* types SB1, SC2, SC3, and SE.

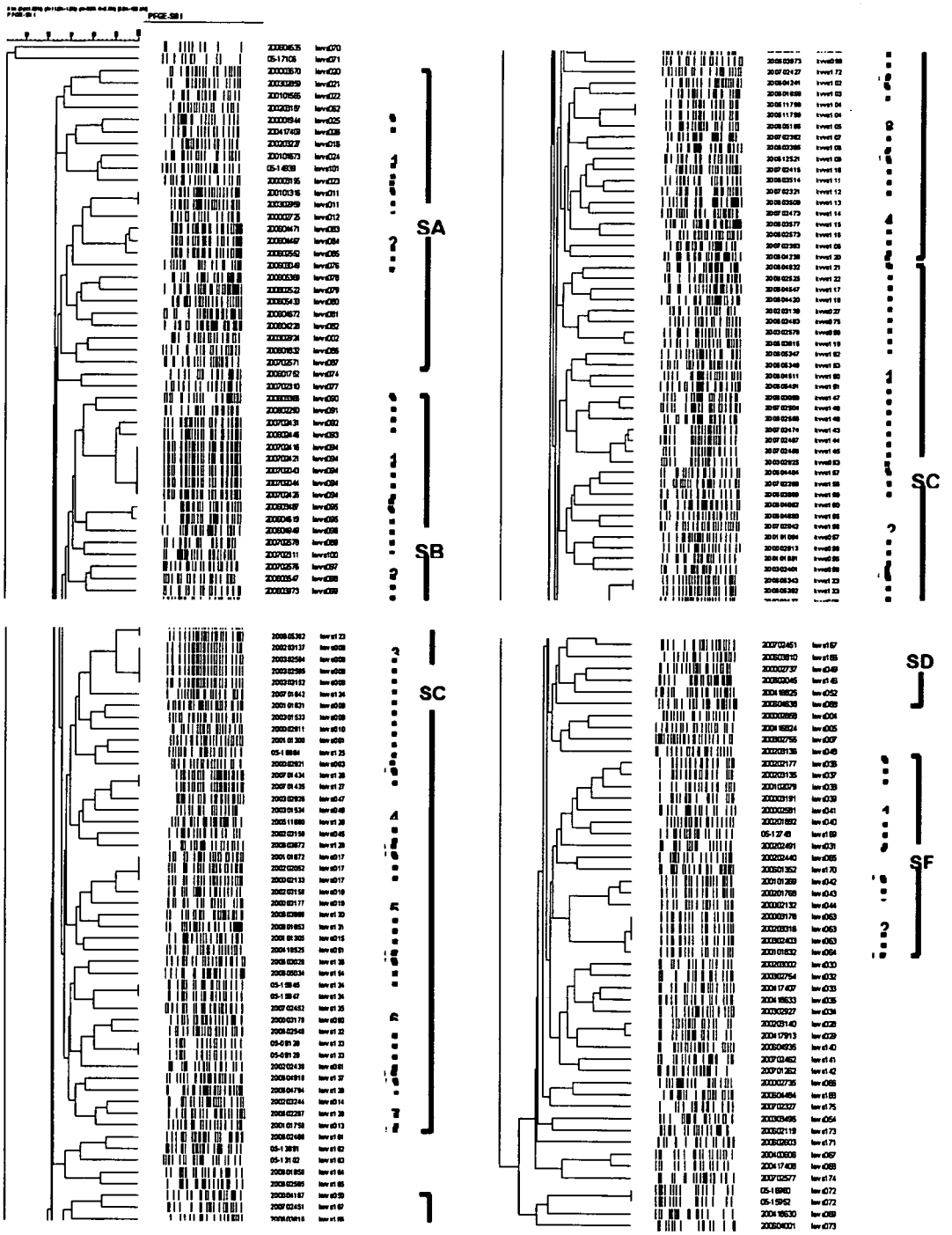

Fig. 4. Dendrogram of the *SfiI* digested PFGE patterns of *V. vulnificus*.

In *SfiI* digested PFE patterns, some relation between clinical and environmental isolates was found (table 2).

Table 2. Subgroup with high similarities (mora than 90%)

Subgroup	Year	Region	No.	
			Clinical	Environmental
SA-2	2001	BS	1	
	2003	GN	1	
	2006	CN		3
SC-1	2002	GN	1	
	2003	GN	1	
		BS	2	
	2006	CN	2	
	2007	GN		2
SC-6	2003	GN	1	
	2006	GG	1	
	2007	US		1
		BS		2
		IC		1
SE-1	2002	GN	2	
SE-2	2000	GN	2	
		JB	1	
	2001	GN	1	
		JB	1	
	2002	GN	1	
		JN	1	
	2003	GN	1	

Discussion:

Compared to RAPD-PCR and ribotyping, PFGE, with its higher degree of discrimination, is often unsuccessful in demonstrating a correlation between genotype and phenotype, and thus it is used relatively less often for environmental research. In this year, we tested ribotyping to replace PFGE for *V. vulnificus*. We have found four shred ribotypes, among these, one ribotype was shared with four clinical and one environmental isolates. But the ribotype of *V. vulnificus* was as diverse as *NotI* digested PFGE patterns. Therefore we can conclude that ribotyping is not a good tool for the classification of *V. vulnificus*.

The new electrophoresis conditions for the *NotI* digested PFGE offered an easy distinction of bands than the previous condition. But, it has too higher degree of discrimination, showed same problem as previous methods. The new electrophoresis conditions for the *SfiI* digested PFGE is same as the PulseNet standard condition for *V. cholerae*, and it is now under the evaluation of standard protocol for *V. vulnificus*. *SfiI* digested PFGE patterns, showed also high degree of discrimination, but, in some *SfiI* digested PFGE pattern, genetic similarity related to isolated region was found. The *SfiI* digested PFGE patterns grouped into different clusters showed that clinical strains of *V. vulnificus* from the southeastern Korea have a higher level of genetic homogeneity compared with ones from the southwestern part. So This method can be used for subspecies typing of *V. vulnificus* strains isolated from distant geographic regions.

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Title: Study of the Relatedness of Quinolone resistant Nontyphoidal Salmonella isolated from 2002 onwards in Metro Manila

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Summary:

Background. Infection with quinolone-resistant nontyphoidal Salmonella has been uncommon in the Philippines until 1992 when the first strains were identified in Metro Manila. Treatment of extraintestinal nontyphoidal Salmonella infections may be complicated by the emergence of strains resistant to antimicrobials. We describe some of the quinolone-resistant isolates obtained from sentinel sites of the antimicrobial resistance surveillance program of the Philippines from 2002 to present and will determine the genetic relatedness of quinolone resistant nontyphoidal Salmonella utilizing pulse field gel electrophoresis (PFGE).

Methods We reviewed patients' charts with quinolone-resistant nontyphoidal Salmonella for demographic, epidemiologic factors, and clinical signs and symptoms. Laboratory tests included antimicrobial susceptibility testing, serotyping with a plan to perform PFGE on the isolates ***once the technology is established at the antimicrobial resistance surveillance reference laboratory (ARSRL).***

Results. A total of 16 patients with culture-confirmed quinolone-resistant nontyphoidal Salmonella were identified. Ninety three percent (93%) of all patients came from various cities in Metro Manila. Mean age was 21 years old (range: 0.33-75 years). Eight (50%) of the patients were children, all of whom were less than or equal to 1 year old. There was no food implicated as a possible vehicle of transmission nor was any of the patients associated with a foodborne illness outbreak. 4 patients had been administered fluoroquinolones prior to availability of culture and sensitivity results. All patients eventually recovered from their illness.

Nine (9) serotypes were identified with Salmonella *choleraesuis var Kunzendorf* accounting for 25% of the isolates. Salmonella *schwarzengrund* was the serotype associated with ciprofloxacin resistance. All the other serotypes were associated only with nalidixic acid resistance. PFGE had been done on all isolates and final analysis will be performed after the staff undergo PFGE data analysis training scheduled on February 19-25, 2008 in the Hongkong Public Health Laboratory.

Conclusions. Quinolone-resistant nontyphoidal Salmonella appear to affect mainly children than adults with no identifiable food vehicle or specific serotypes except for Salmonella *schwarzengrund* which was associated with ciprofloxacin resistance. Several of the isolates were associated with multi-drug resistance. Conclusions cannot be drawn on genetic relatedness of the strains while analysis of PFGE results are still pending.

Purpose:
Fluoroquinolone-resistant(FqRS) *Salmonella* has been isolated in the Philippines since 1992. Molecular and epidemiological investigations have traced two outbreaks of fluoroquinolone-resistant *Salmonella* Schwarzergrund in the U.S[1] to an identical strain isolated in one of the hospitals in Metro Manila, Philippines. Additional cases were detected by requiring all ARS sentinel sites to refer all the nontyphoidal *Salmonella* isolates to ARSRL for confirmation starting 2002 where identification, antimicrobial susceptibility testing to ampicillin, chloramphenicol, cotrimoxazole, ciprofloxacin and nalidixic acid by disc diffusion method (Kirby-Bauer method)[2], quantitative susceptibility testing by antibiotic gradient method (Etest by ABBiodisk)[3], and serotyping of all non-typhoidal isolates utilizing Kauffman-White scheme for somatic factor and Sven Gard method for flagellar typing[4] are performed. From 2002 to 2004, 12 nalidixic acid and 2 ciprofloxacin resistant nontyphoidal *Salmonella* were confirmed at the ARSRL, with all patient sources coming from Metro Manila.

Most non-typhoidal *Salmonella* infections do not require treatment but treatment can be life saving in patients with co-morbid medical conditions and extra-intestinal infections. Treatment is complicated with the emergence of strains that are resistant to multiple antimicrobials, including fluoroquinolones, which is frequently used for treatment of patients infected with isolates resistant to the first line antibiotics.

Because of the potentially large burden of resistant *Salmonella*, any enhancement of the surveillance system should also include genotyping for laboratory confirmation of both sporadic and outbreak associated cases. For this purpose, genotypic methods of characterizing bacteria through microbial subtyping (such as through pulse field gel electrophoresis or PFGE) are one of the best existing methods for subtyping and tracking of bacteria relevant to infection prevention and control. The aim of subtyping is to indicate whether two or more isolates are indistinguishable, and therefore potentially from the same source.

Objectives:

This proposal aims to establish PFGE as a genotypic subtyping method in the ARSRL and to utilize this method to determine genetic relatedness of quinolone resistant nontyphoidal Salmonella.

Methods:

Epidemiologic Methods. Information about the patients was obtained through reviews of medical charts and included demographic data, clinical signs and symptoms, information on food history, associated medical problems, and outcome.

Laboratory Investigation.

Bacterial Strains
All non-typhoidal Salmonella isolates referred from sentinel sites from 2002 onwards of the ARS were included in the study. Antimicrobial susceptibility testing was performed by both disc diffusion method and quantitative method using antibiotic gradient (Etest-ABbiodisk) in order to

determine the minimum inhibitory concentration (MIC). Serotyping was performed utilizing Kauffman White Scheme for somatic factor and Sven Gard method for flagellar typing,

Pulse Field Gel Electrophoresis
The one-day standard laboratory protocol for subtyping of nontyphoidal Salmonella by PFGE developed by the U.S. Centers for Disease Control will be the laboratory procedure followed. The technology for performing PFGE was acquired by attending a PFGE course at the Hongkong Public Health Laboratory which was held February 7-10, 2006. Purchase of reagents for the PFGE procedure is ongoing and initial PRGE procedures will follow once all laboratory reagents are purchased. Purchase of reagents for the PFGE procedure is on-going and initial PFGE runs will follow once all laboratory reagents are purchased.

Results

1) Epidemiologic Investigation. A total of 16 patients with culture-confirmed quinolone-resistant nontyphoidal Salmonella were identified. Ninety three percent (93%) of patients came from various cities in Metro Manila but 2 patients with ciprofloxacin-resistant Salmonella came from the district of Sampaloc (Table 1). There were 11 males and 5 females with a mean age of 21 years old (range: 0.33-75 years). Eight of the patients were children, all of whom were less than or equal to 1 year old. One patient was HIV infected. There was no food implicated as a possible vehicle of transmission nor was any of the patients associated with a foodborne illness outbreak based on limited information available from the patient's charts. Three patients had concomitant medical problems which were: cardiac, pulmonary and genitourinary in nature. 4 patients had been administered fluoroquinolones prior to availability of culture and sensitivity results. The twelve (12) other patients were given antimicrobials of other classes, 11 (91%) of which were Beta-lactams. 13 patients were febrile, 6 had diarrhea and 4 had vomiting. All patients eventually recovered from their illness.
2) Laboratory Investigation. Nine (9) serotypes were identified with Salmonella choleraesuis var Kunzendorf accounting for 25% of the isolates (Table 2). Salmonella schwarzengrund was the serotype associated with ciprofloxacin resistance. All the other serotypes were associated only with nalidixic acid resistance. Table 3 shows the minimum inhibitory concentrations of the 16 isolates. Several of the isolates were associated with multi-drug resistance.

Conclusions. Quinolone-resistant nontyphoidal Salmonella appear to affect mainly children than adults with no identifiable food vehicle or specific serotypes except for Salmonella *schwarzengrund* which was associated with ciprofloxacin resistance. Several of the isolates were associated with multi-drug resistance. Conclusions cannot be drawn on genetic relatedness of the strains while PFGE results are still pending.

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Table 1. Characteristics of patients with FqR Salmonella, Philippines

Characteristics	N=16
1. Age (years)	
Mean	21.2
Range	0.33-75
2. Female sex – no. (%)	5(31%)
3. Treatment with antimicrobial agent	
a. Fluoroquinolones	4
b. Others	12
4. Concomittant medical problems	
a. HIV and pulmonary	1
b. cardiac and pulmonary	1
c. genitourinary	1

Table 2. Serotypes of nontyphoidal Salmonella isolates, N = 16

Serotype	No. (%)
Salmonella choleraesuis var. Kunzendorf	4 (25)
Salmonella Hissar	3 (18)
Salmonella Schwarzengrund	2 (12)
Salmonella Typhimurium	2 (12)
Salmonella Lomita	1 (6)
Salmonella Stanley	1 (6)
Salmonella Oritamerin	1 (6)
Salmonella Irumu	1 (6)
Salmonella Enteritidis	1 (6)
	16 (100.00)

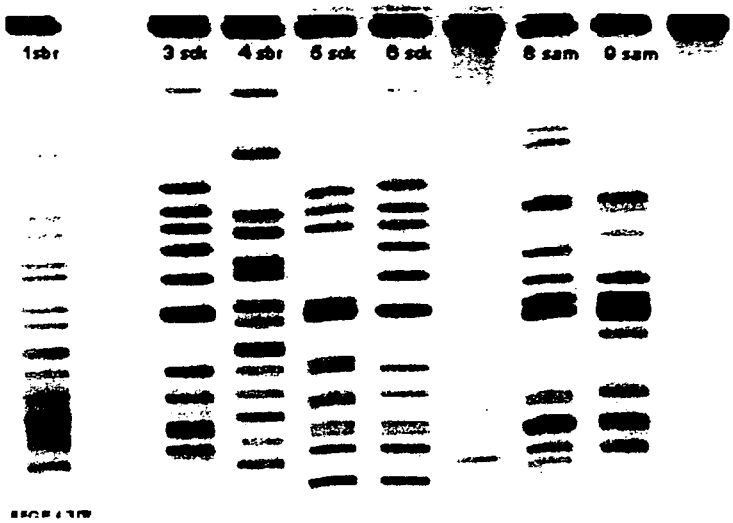
Table 3. Minimum inhibitory concentration (ug/ml) of each of the quinolone resistant nontyphoidal Salmonella isolates.

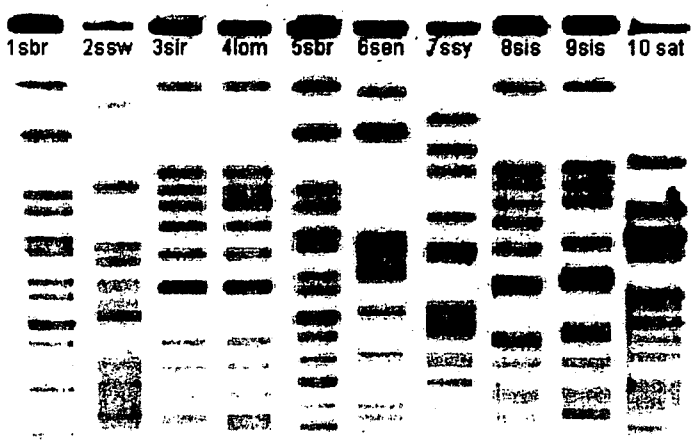
IDNUM	SEROTYPE	MIC Results				
		AMP	CIP	CHL	SXT	NAL
500001	SALMONELLA SCHWARZENGRUND	>256	>32	≤8	<2	>256
500002	SALMONELLA SCHWARZENGRUND	>256	>32	>256	>32	>256
200003	SALMONELLA LOMITA	>256	<1	192	>32	>256
300004	SALMONELLA STANLEY	≤8	0.094	≤8	<2	>256
400005	SALMONELLA ORITAMERIN	>256	<1	>256	>32	>256
400006	SALMONELLA HISSAR	>256	0.25	>256	>32	>256
400007	SALMONELLA CHOLERAESUIS VAR. KUNZENDORF	≤8	<1	192	>32	>256
400008	SALMONELLA TYPHIMURIUM	>256	<1	>256	>32	>256
400009	SALMONELLA CHOLERAESUIS VAR. KUNZENDORF	>256	<1	≤8	>32	>256
400010	SALMONELLA CHOLERAESUIS VAR. KUNZENDORF	>256	<1	≤8	>32	>256
400011	SALMONELLA HISSAR	>256	<1	≤8	>32	>256
200017	SALMONELLA HISSAR	>256	<1	≤8	<2	>256
500018	SALMONELLA CHOLERAESUIS VAR. KUNZENDORF	>256	<1	≤8	>32	>256
500019	SALMONELLA IRUMU	>256	0.125	≤8	<2	>256
700020	SALMONELLA TYPHIMURIUM	>256	<1	≤8	<2	>256
800021	SALMONELLA ENTERITIDIS	>256	<1	≤8	<2	>256

Legend:

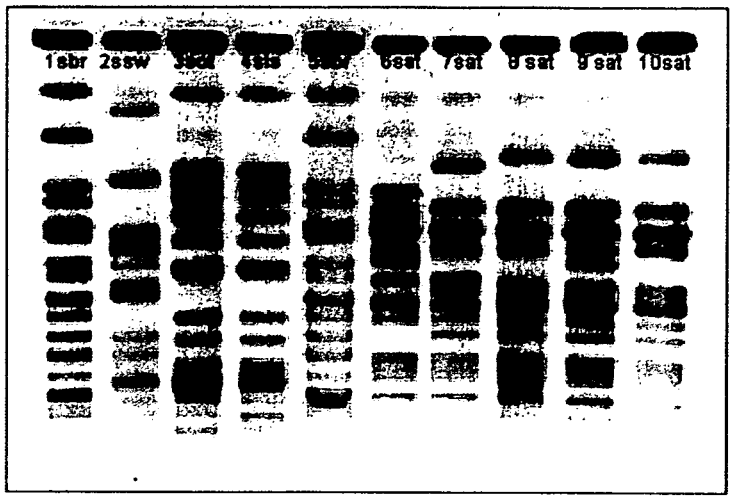
Amp - Ampicillin
Cip - Ciprofloxacin
Chl - Chloramphenicol
Sxt - Cotrimoxazole
Nal - Nalidixic Acid

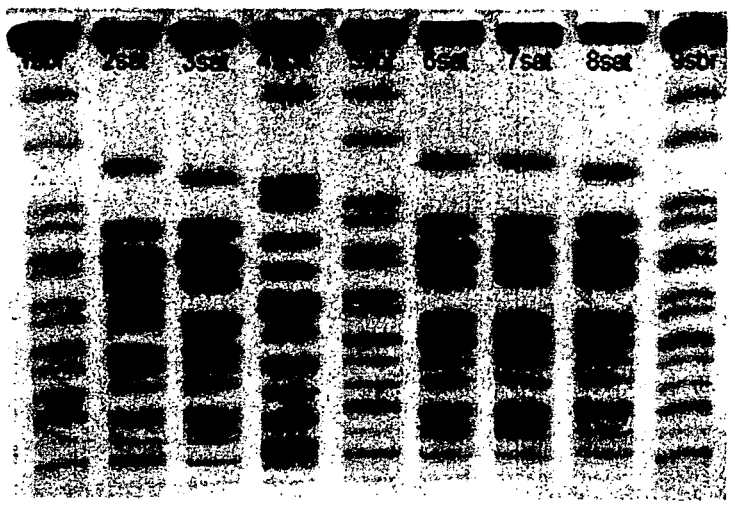
The following are the TIFF files of the isolates.





PFGE 5.4.07





sbr – Braenderup
Sam - typhimurium
Sck – Kunsendorf
ssw – Schwarzengrund
sir – Hissar
lom – lomita
sen- enteritidis
ssy – Stanley
sis - Hissar
sot - Oritamerin

Clonal diversity of Shiga toxin-producing *Escherichia coli* isolated from slaughtered animals in Bangladesh

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Introduction:

Shiga toxin (Stx)-producing *Escherichia coli* (STEC) is one of the most important groups of food-borne pathogens (4, 8, 10). They can cause gastroenteritis that may be complicated by hemorrhagic colitis (HC) or the hemolytic–uremic syndrome (HUS), which is the main cause of acute renal failure in children. STEC strains causing human infections belong to a large, still increasing number of O:H serotypes. Most outbreaks and sporadic cases of HC and HUS have been attributed to STEC O157 strains (13). Domestic ruminants, mainly cattle, sheep and goats have been established as major natural reservoirs for STEC (11) and play a significant role in the epidemiology of human infections (6). During the processing of the carcasses, fecal contamination or transfer of bacteria from the animal's hide to the carcass can facilitate transmission of pathogenic *E. coli* to the meat (2, 3, 5). STEC infections are major concern in developed countries. However, human infections associated with STEC strains have also been described in Latin America, India and other developing countries (7, 9). Recently we have isolated 45 strains of STEC O157 from rectal contents of slaughtered animals in Bangladesh. This study described the clonal diversity among the strains as performed by using pulsed-field gel electrophoresis (PFGE) technique.

Methodology

STEC O157 strains were isolated and identified previously from rectal contents of slaughtered animals including buffalo, cows, and goats following standardized method (1). PFGE was performed following the standardized protocol developed by PulseNet for *E. coli* O157:H7 (12). Total genomic DNA was isolated in an agarose-embedded form and was subjected to enzymatic digestion with 50 U of *Xba*I. Electrophoretic separation of the DNA fragments was achieved through the contour-clamped homogenous electric field (CHEF) method on a CHEF Mapper system (Bio-Rad) using 0.5x TBE buffer (45 mM Tris-HCl, 45 mM boric acid, 1.0 mM EDTA, pH 8.0). Run conditions were generated by the autoalgorithm mode of the CHEF Mapper PFGE system for the sizes ranging between 30 and 600 kb, and PFGE was performed for 20 h. Analysis of the TIFF images was carried out by the BioNumerics software package (Applied Maths, Belgium) using the Dice coefficient and UPGMA to generate dendrograms with 1.0% tolerance values.

Results and conclusion:

Digestion of genomic DNA from the 45 isolates with *Xba*I and analysis by CHEF-PFGE revealed 37 distinct restriction profiles suggesting a heterogeneous clonal diversity (Fig. 1).

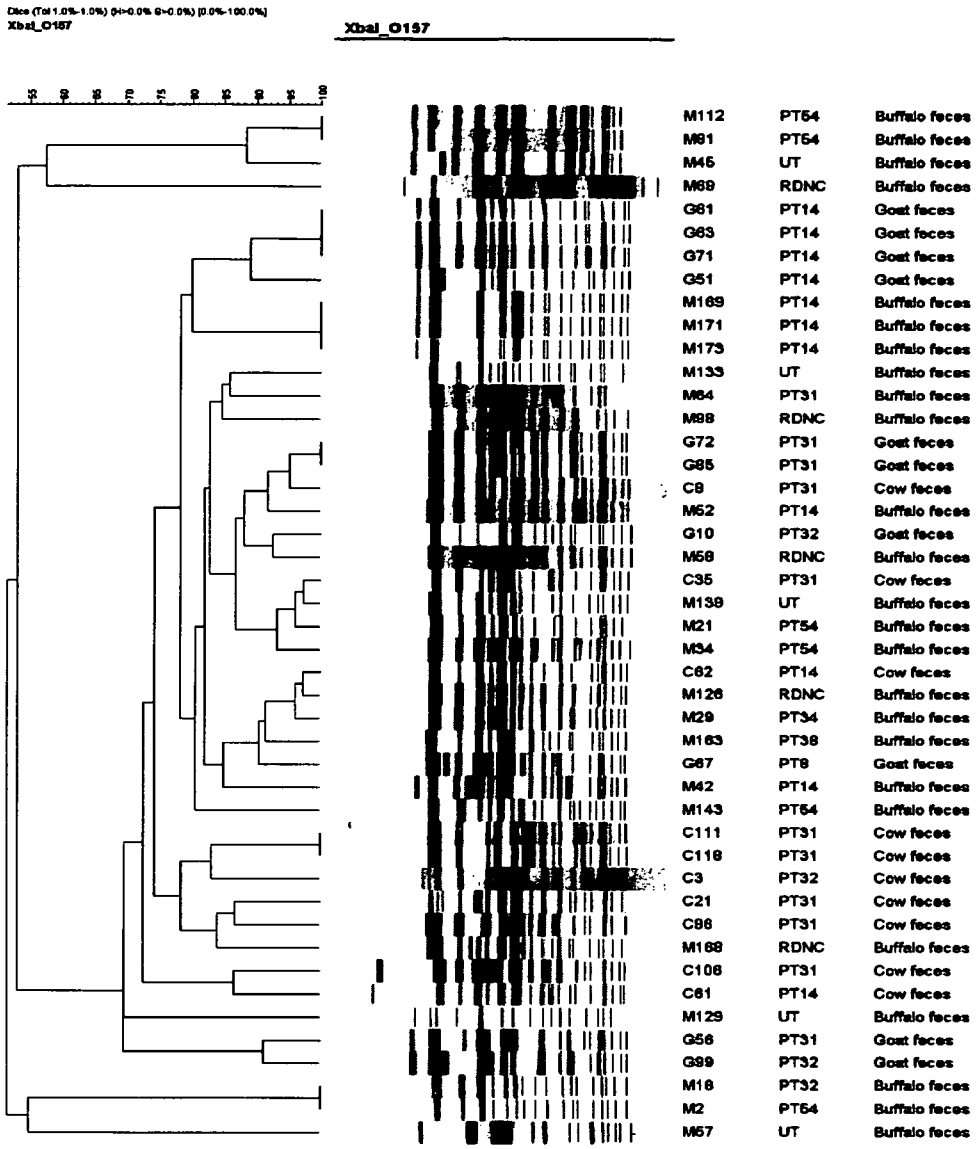

Fig 1. Dendrogram generated by Bionumeric software, showing distance calculated by the Dice similarity index of PFGE *Xba*I profiles for 45 STEC O157 isolates isolated from rectal contents of slaughter animals. The degree of similarity (%) is shown on the scale.

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Molecular Traits of *Vibrio parahaemolyticus* Strains of Diverse Geographical Origins Including Bangladesh as Determined by Pulsed-field Gel Electrophoresis

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Summary:

Vibrio parahaemolyticus causes seafood-related gastroenteritis worldwide, which is also reported from sporadic cases in the coastal villages of Bangladesh and India, although the molecular traits of predominant strains occurring in this region and their clonal relationship with others of different geographical origins are as yet unknown. In our 'PulseNet AP-progress report 2007', we indicated that despite the *V. papahaemolyticus* strains were characterized extensively for phenotypic and molecular traits, we did not have standard protocol for analyzing those strains by PFGE. Thus, we (ICDDR,B, Bangladesh) proposed at the 3rd PulseNet Asia Pacific Meeting, held in NIID, Tokyo in 2005, the need for optimizing the PFGE protocol by its participating laboratories. Accordingly, in the next phase, we optimized the PFGE protocol for *V. papahaemolyticus* strains in our laboratory by using genomic DNA digested with both *NotI* and *SfiI* restriction enzymes. For this standardization purpose, the Hong Kong Public Health Laboratory (PHL) supported us with the protocol and *V. parahaemolyticus* strains that were used by all the PulseNet Asia Pacific participating laboratories. We then submitted the PFGE images of both *NotI* and *SfiI*-digested *V. parahaemolyticus* DNA to the coordinating lab and the images were accepted as optimally performed ones. In the next phase, the *V. parahaemolyticus* strains isolated from coast of Bay of Bengal, which awaited to be analyzed using optimized PFGE protocol, were tested. As we showed that *V. parahaemolyticus* strains isolated from four distinct areas covering the coastal aquatic