Discussion

We expanded *V. vulnificus* isolates obtained in 2000-2004 year in this 2nd year period. As described in 1st year report, we performed PFGE experiment with *V. vulnificus* clinical and environmental isolates obtained in 2005 year and the cluster analysis results were very various and heterogeneous. Although we analyzed PFGE patterns of only clinical isolates in this 2nd period, we couldn't any clonal lineage. For the best discrimination of PFGE patterns, we need to separate the low-size PFGE bands.

According to RAPD results, we could find a common PCR product in clinical isolates. We have cloned and sequenced the amplicon. The gene was turned out, *nusA-IF2*. Interestingly, we found that this PCR product was only positive in clinical isolates rather than environmental isolates. We can hypothesize that there can be any sequence variable region(s) or difference in *nusA-IF2* between clinical and environmental isolates.

Contrary to PFGE results, RAPD could differentiate clinical isolates from environmental isolates. Although the reproducibility of RAPD is lower than that of PFGE, our RAPD primer and PCR condition is promising to cluster pathogenic *V. vulnificus* isolates. So, *nusA-IF2* gene analysis, for example prevalence, existence, pathogenic role of the gene, will be very important to understand molecular epidemiological analysis as well as its virulence.

In this project, we will use other molecular epidemiological tools to tune finely our data. Because of the variety of clonality, we need to find proper tools to cluster and chase the origin of infection. And, two step PFGE running condition for discrimination of lower bands should be considered.

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Publication list for this work:

1) **Molecular Epidemiological analysis of *Vibrio Vulnificus* isolated from patients and environment (in preparation).**

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

Name of Researcher: Dr. Celia C. Carlos
Affiliation: Research Institute for Tropical Medicine, Alabang, Muntinlupa, Metro Manila, Philippines

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 results are pending.

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.

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

Progress Report

1. Salmonella:

Salmonellosis in Bangladesh: Using Pulsed-field gel electrophoresis to identify the outbreak causing strain(s)

Name of Researcher: **Kaisar Ali Talukder &**
Ishrat Jahan Azmi

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Abstract:

In total, 5500 strains of salmonella species were isolated at Clinical Microbiology Laboratory from patients attending the Dhaka treatment center of ICDDR,B between 1997-2004. Of these, the isolation rate of *S.* Typhi was dominant except the year 1998 when the isolation of Salmonella group B strains was the highest (68%). Among the Salmonella group B strain, *S.* typhimurium was the predominant serotype. Twenty-four strains of these *S.* typhimurium were characterized extensively using serotyping, antibiotic resistance analysis, plasmid profile analysis, and pulsed-field gel electrophoresis (PFGE). PFGE of these strains revealed that all strains were clonal. Recently, an outbreak caused by *Salmonella* Typhimurium was identified in a rural area named Matlab in Bangladesh when the outbreak-affected people were taken food attending in a religious festival at Loknath Asrom of Matlab in June 2006. All the strains isolated from patients and foods were *S.* Typhimurium, were susceptible to the entire antibiotic tested, and PFGE of these strains was clonal but different from that of clinical strains between 1997-2004.

Introduction:

Non typhoidal salmonella are recognized as one of the main causes of food poisoning worldwide (1, 2, 3), with an estimated annual incidence of 1.3 billion cases and 3 million deaths (4). Furthermore, they can cause severe infections, such as septicemia and endocarditis, empyema, and meningitis (5), especially immuno-compromised hosts.

Salmonella typhimurium, which cause a variety of clinical manifestations from mild gastroenteritis to septicemia, are one of the leading causes of foodborne illness worldwide (6-7). *S.* Enteritidis and S. Typhimurium produced human diseases approximately 50% of cases infected by salmonellae (8). In a retrospective study of 5500 salmonella species isolated from 1996-2004, we found that the isolation rate of *S.* Typhi was dominant except the year 1998 when the isolation of Salmonella group B strains was the highest (68%) isolated from patients attending the Dhaka treatment center operated by ICDDR, B. Among the salmonella group B strain, *S.* Typhimurium was the predominant serotype. In July 01, 2006, a distinct outbreak of Salmonellosis was identified in a rural area named Matlab in Bangladesh. Symptoms in outbreak included acute diarrhoea, severe abdominal cramps, nausea, vomiting and fever. The outbreak-affected people attending a religious festival at Loknath Asrom of Matlab were taken proshad (holly food) a proshad made of milk, rice flour, sugar, and different types of fruit mixtures. One hundred thirty nine patients taking the proshad came down with acute diarrhoea and were admitted in diarrhoeal treatment centre of ICDDR,B, Matlab. Salmonella Typhimurium were isolated from stool of the patients and also from the foods that was served on the festival. All the strains isolated in 2006 were characterized extensively using serotyping, antibiotic resistance analysis, plasmid profile analysis, and pulsed-field gel electrophoresis (PFGE) in order to find some important clues for the outbreak and compared with *S.* Typhimurium strains isolated in 1998.

Methodology:

Stool samples from the patients were examined for *Shigella, Salmonella* and *Vibrio* by standard microbiological and biochemical techniques. Salmonella were isolated from stool samples of the patients and were identified as *S.* Typhimurium by serology. The food sample was enriched in APW and then spreaded on a selective plates (XLD, SS). Typical colony was confirmed as Salmonella typhimurium biochemically and serologically. Susceptibility to antimicrobial agents was determined by the disk diffusion method as recommended by the Clinical and Laboratory Standards Institute (CLSI) with commercial antimicrobial discs (Oxoid, Basingstoke, United Kingdom). Molecular

typing such as plasmid and pulsed-field gel electrophoresis were done using standard method.

Results and discussion:

Of 139 patients, 93 (71%) *S.* Typhimurium strains were isolated from their stools and 2 strains of *S.* Typhimurium were isolated from 2 food samples (prosad). All the isolates were susceptible to all first line antibiotics tested. No plasmid was found among the isolated strains whereas MDR strains isolated in 1998 harbored 140 and or 90 MDa plasmid. To establish the relationship between the isolates from human and foods, the strain were analyzed by Pulse-field gel electrophoresis. The rapid pulsed-field gel electrophoresis (PFGE) procedures developed for Salmonella typhi strains were used for typing of the outbreak strains. The strains were analyzed by digesting DNA with *X*baI restriction enzyme. Macro restriction patterns generated by PFGE showed that all strain tested had an identical PFGE pattern, which suggests close genetic relatedness. Strain isolated from food sample showed the same PFGE pattern indicating possible route of transmission of the strain from food to patients.

The PFGE pattern of twenty-four *S.* Typhimurium strains isolated in 1998 belonged to a single PFGE type, which was completely different from the recent food borne outbreak strains. All the strains isolated in 1998 were resistant to ampicillin, Chloramphenicol, Sulphamethoxazole-trimethoprim, Nalidixic acid and cotrimexazole (ACSxtNalCro) and plasmid profile of these strains were also identical.

Conclusion:

Epidemiologically, the outbreak had obvious connection. In addition a common source of infection was identified. However the observation suggest that the outbreak was caused by the same serotype of Salmonella, were associated with severe diarrhea. Therefore, we used molecular typing techniques to characterize the salmonella strain isolated from the

patients and from the food. Our observation suggests that this outbreak was caused by a distinct clonal strain of *S.* Typhimurium. The common source of infection and transmission route for the outbreak causing salmonella strain in food. Isolating *S.* Typhimurium from the stool of the patients confirmed the clinical diagnosis of salmonellosis.

S. Typhimurium, in contrast to *S.* Enteritidis, which is a highly clonal organism, is a farely diverse serotype. Therefore, detection of closely related *S.* Typhimurium strains in geographically distinct loci may signal worldwide spread or emergence of closely related clonal groups of salmonella having increased virulence. This possibility may be confirmed by worldwide (or nationwide) standardization of molecular typing protocols and further strengthening of data sharing capabilities between laboratories involved in the molecular typing of pathogenic bacteria.

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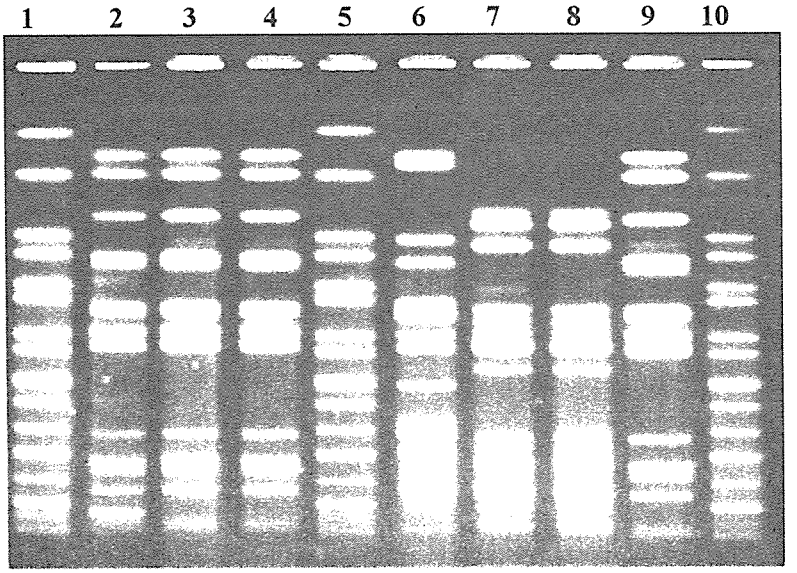


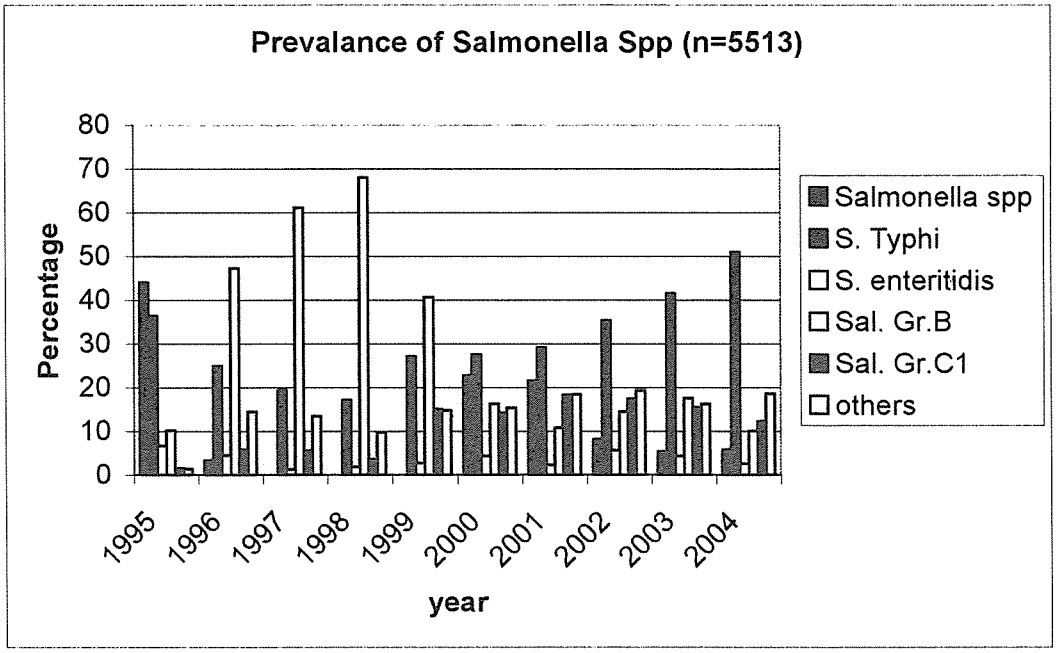
Figure: Outbreak investigation: same clone of Salmonella Typhimurium was isolated from patients and food source

Lane: 1,5,10: Salmonella braenderup (Marker)

Lane: 2-4: S. Typhimurium (isolated from patients in 2006)

Lane: 6-8: S. Typhimurium (isolated in 1998)

Lane: 9: S. Typhimurium (isolated from food sample in 2006)


Prevalance of Salmonella Spp (n=5513)
Percentage
80
70
60
50
40
30
20
10
0
1995
1996
1997
1998
1999
2000
2001
2002
2003
2004
year
Salmonella spp
S. Typhi
S. enteritidis
Sal. Gr.B
Sal. Gr.C1
others

2.*Vibrio parahaemolyticus*

Adoption of PulseNet Approved PFGE Protocol for Molecular Typing of *Vibrio parahaemolyticus* Occurring in The Coastal Ecosystem of Bangladesh

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Summary:

Vibrio parahaemolyticus, a pandemic pathogen causing seafood-related gastroenteritis, is often reported from sporadic cases in the coastal villages of Bangladesh and India, although molecular characteristics of predominant types of strains existing in this region remain as yet unknown. *V. parahaemolyticus* strains (n=29) isolated by using standard culture methods from four distinct areas covering the coastal aquatic environments were isolated and and studied extensively for serotyping, antibiogram, hemolytic activity (Kanagawa phenomenon) followed by PCR for important epidemic marker genes such as *toxR*, *tdh*, *trh*, *tlh*, group specific (GS), and ORF 8. Also, the DNA fingerprinting analysis employing RAPD, and ERIC-PCR was performed, although further analysis for DNA fingerprinting using pulsed-field gel electrophoresis (PFGE) could not be done even after repeated efforts because of the lack of existing standard protocol for analyzing *V. papahaemolyticus* strains by PFGE. So, we (ICDDR,B, Bangladesh) proposed in the 3rd PulseNet Asia Pacific Meeting held in NIID, Tokyo in 2005, the need for jointly standardizing the PFGE protocol for analyzing *V. papahaemolyticus* strains by the participating laboratories. The meeting unanimously decided on the issue and finally we have been able to optimize the PFGE protocol for *V. papahaemolyticus* in our laboratory by using genomic DNA digested with both *NotI* and *SfiI* restriction enzymes. For this standardization purpose, the PulseNet Asia-Pacific coordinating Public Health Laboratory Centre (PHLC) at Hong Kong supported us with

the protocol and *V. parahaemolyticus* strains that were used by all the PulseNet Asia Pacific participating laboratories. We carried out PFGE extensively using genomic DNA digested with *NotI* and *SfiI* restriction enzymes and finally succeeded in optimizing and adopting the PFGE protocol using both *NotI* and *SfiI* restriction enzymes in our laboratory conditions. Our results also showed the *NotI* to be the restriction enzyme for *V. parahaemolyticus* DNA that gives better seperation of the bands. We have submitted the PFGE images of both *NotI* and *SfiI*-digested *V. parahaemolyticus* DNA to the PulseNet Asia-Pacific coordinating PHLC at Hong Kong and the images were accepted as optimally performed ones. Finally, the *V. parahaemolyticus* test strains were then analyzed using the optimized PFGE protocol. The PFGE of *NotI*-digested genomic DNA revealed *V. parahaemolyticus* strains to be diverse clonally, although further PFGE studies comprising more environmental and clinical strains appear very crucial.

Purpose:

V. parahaemolyticus naturally inhabits marine water and is present in higher concentrations during summer; it is a halophilic organism which causes gastrointestinal illness in humans and are the cause of approximately half of the food borne outbreaks in some Asian countries. V. *parahaemolyticus* gastroenteritis is a multi-serogroup affliction, and as many as 75 different combinations of O and K serotypes of *V. parahaemolyticus* are so far recognized and known to be associated with gastroenteritis. In Bangladesh, *V. parahaemolyticus* associated diarrhea and gastroenteritis has recently been reported from various coastal villages but no study has so far been conducted on the molecular characterization of *V. parahaemolyticus* in this regions. On the other hand, pandemic serogroup strains of *V. parahaemolyticus*, namely O3:K6, have been reported to be disseminating rapidly in recent years, although the molecular characteristics namely the DNA fingerprinting analysis of the *V. parahaemolyticus* strains occurring in the coastal aquatic environments of Bangladesh were limited because of the lack of standardized pulsed-field gel electrophoresis (PFGE) protocol in use. This study was, thus, designed to optimize the PulseNet Asia Pacific-proposed PFGE protocol for *V. parahaemolyticus* strains using both *NotI* and *SfiI* restriction enzymes in our laboratory conditions to be able

to investigate the DNA fingerprinting of the *V. parahaemolyticus* strains that prevail in the seawater samples collected from the coastal areas of the Bay of Bengal.

Methodology:

Optimization and Adoption of Pulse Net Asia Pacific Protocol for analysis of *Vibrio parahaemolyticus* :

Pulse Net Protocol

1. Streak the test strain on selective media (TCBS) & incubate it at 37°C for O/N
2. Pick a single colony and streak on blood agar & incubate it at 37°C for O/N
3. Take 2 ml of CSB (Cell Suspension buffer) (100mM Tris, pH 8, 100mM EDTA, pH 8) in the cuvette, remove some of the growth from agar plate & suspend in the CSB & mix up properly (after each and every addition) and measure the Turbidity with Dade turbidometer up to 0.64-0.66 .Keep in ice if sample no is more than 6.
4. Take 200µl (cell of CSB) in an Eppendorf tube and keep them in ice.
5. Prepare 1% SKG and place it in a water bath in 55°C-60°C
6. Pre heat the Eppendorf tube in 37°C for (5-10) min and mix with10µl of freshly prepared Proteinase K (800U/ml)
7. Take 200µl SKG and mix properly and pour it in the previously labeled plug mold
8. Put the plug at RT for 15min or in 4°C for 5min
9. Prepare Cell Lysis buffer (50mM Tris, pH 8, 50mM EDTA, pH 8 +1% N-Laryl Sarcosine)
10. Take 5ml of CLB and 25µl PK in a Sorval tube, mix vigorously and transfer the plug in the Sorval tube.
11. Place the Sorval tube into water bath at 54°C with constant and vigorous agitation (175-200 rpm) for 1 hour.
12. After 1 hr remove the CLB. Ensure complete removal of the CLB
13. Add 15 ml preheated (50°C) ultra pure water to the Sorval tube and put them in water bath at 50°C with constant and vigorous agitation (175-200 rpm) for15min.

14. After 15min remove the water. Ensure complete removal of the water

15. Repeat the above step.

16. Add 15 ml preheated (50°C) TE buffer(10mM Tris, pH 8, 1mM EDTA, pH 8) to the Sorval tube and put them in water bath at 50°C with constant and vigorous agitation (175-200 rpm) for15min.

17. Repeat the above step (3 times)

18. After the 4th washing transfer the plug in Eppendorf tube containing fresh TE buffer.

19. Keep the plug in 4°C.

20. Restriction Digestion of DNA in Agarose Plugs with *XbaI*

Requirements:

1. Filtered deionized water
2. 10X H buffer
3. Xba I enzyme (10 U/ml)

4.NotI enzyme (10 U/ml)

Plugs were removed from tubes containing TE with wide end of spatula and were placed in a sterile disposable petri dish and were cut at a 2 mm wide slice from test samples and transferred to the labeled 1.5 ml micro centrifuge tubes containing 200 µl diluted H buffer (1:10 dilution). The rest of plugs were replaced in original tubes that contained 1 ml TE Buffer. Two 2 mm wide slices of Salmonella ser. Braenderup standard plugs were cut and transferred to tubes of diluted H buffer. The tubes were incubated in room temperature for 10-15 min. After incubation of plug slices H buffer were removed.

Then each of the plugs was immersed in 200 µl of reaction mixer, which was prepared according to the following calculations:

For Marker

10x Xba I restriction enzyme buffer = 20 µl

Xba I restriction enzyme (10U/µl) = 4µl

Filtered deionized water = 176µl

Total = 200µl

For Test strain

10x Not I/ SfiI restriction enzyme buffer = 20 µl

Xba I restriction enzyme (10U/µl) = 4µl

BSA (20mg/ml) = 2µl

Filtered deionized water = 174µl

Total = 200µl

The NotI digested samples and the control tubes were incubated at 37°C and SfiI digested samples were incubated at 50°C water bath for 4 hrs.

Electrophoresis Conditions	
Voltage Gradient	6v/cm
Included Angle	120°
Ramping	Linear
Initial Switch Time	10s
Final Switch Time	35.03s
Run Time	18hrs
Initial Milliamps	NA

Well	Isolate	Enzyme	Year	Units	Serotype
1	H9812	XbaI	40U	Salmonella braenderup	
2	VPS04	NotI	1998	40U	O4:K68
3	VPS10	NotI	2005	40U	O4:K8
4	VPS14	NotI	2001	40U	O3:K6
5	H9812	NotI	40U	Salmonella braenderup	
6	VPS19	NotI	2004	40U	O1: KUT
7	VPS27	NotI	2005	40U	O2:K28
8	VPS31	NotI	1997	40U	O4:K12
9	VPS35	NotI	2003	40U	O6:K18
10	H9812	XbaI	40U	Salmonella braenderup	

Electrophoresis Conditions	
Voltage Gradient	6v/cm
Included Angle	120°
Ramping	Linear
Initial Switch Time	10s
Final Switch Time	35.03s
Run Time	18hrs
Initial Milliamps	NA

Well	Isolate	Enzyme	Year	Units	Serotype