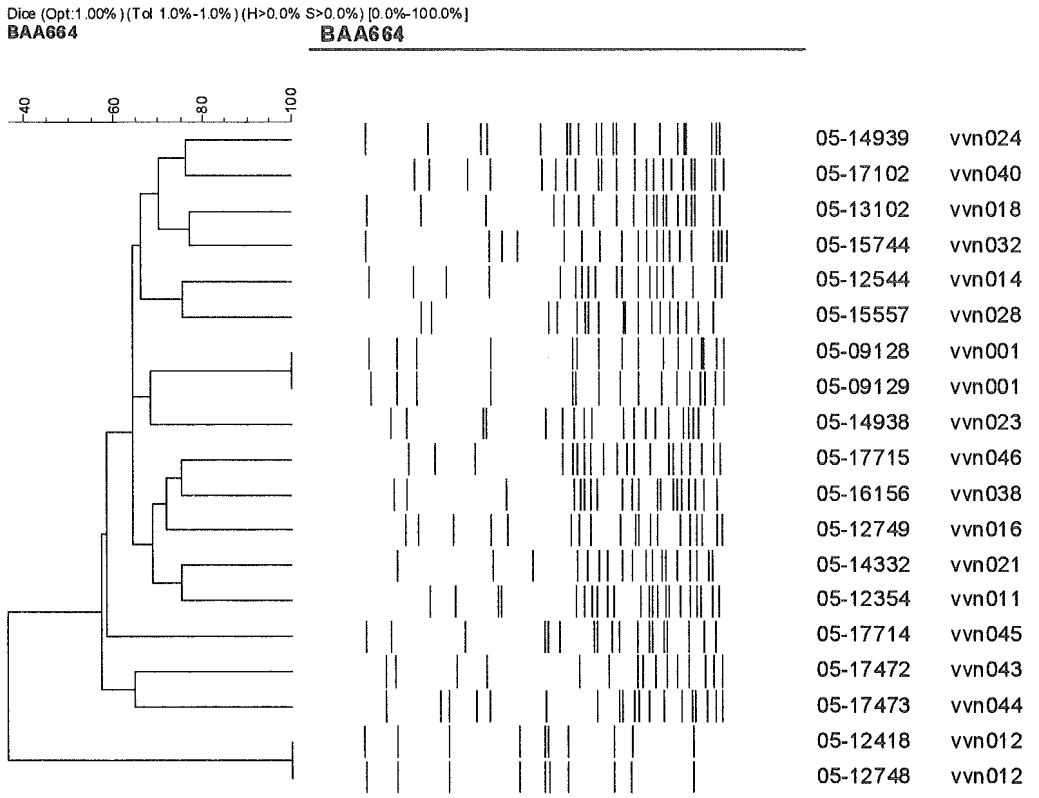


Figure 12. Dendrogram of *Not* I digested PFGE patterns of *V. vulnificus* isolates from Jeonla region in 2005.

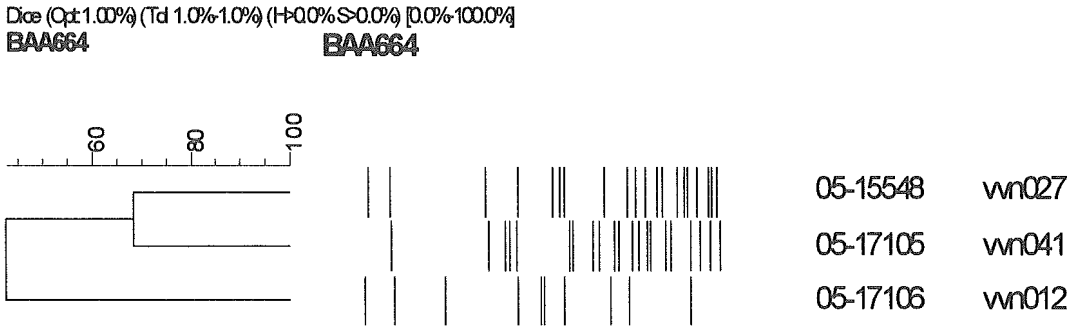


Figure 13. Dendrogram of *Not* I digested PFGE patterns of *V. vulnificus* isolates from JeJu region in 2005.

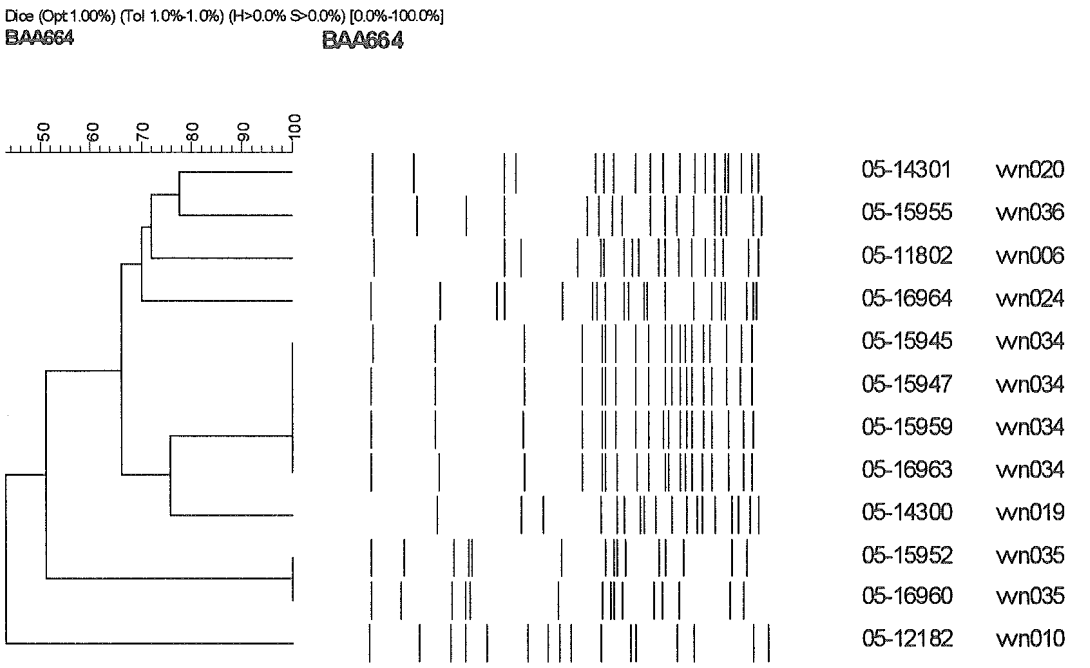


Figure 14. Dendrogram of *Not* I digested PFGE patterns of *V. vulnificus* isolates from Chungcheng and Gangwon region in 2005.

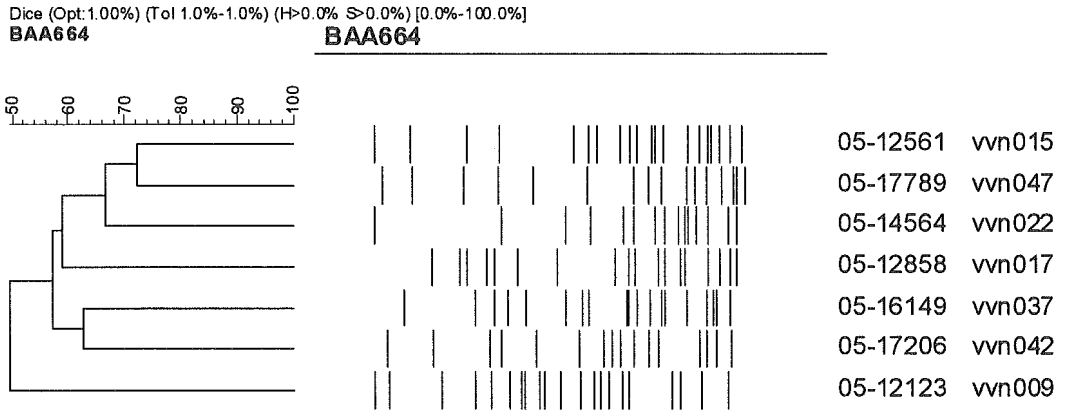


Figure 15. Dendrogram of *Not* I digested PFGE patterns of *V. vulnificus* isolates from Gyeongi and Incheon region in 2005.

3. RAPD results.

RAPD produced 1 to 12 bands in each lane (Figure 16). Five clusters could be produced by RAPD analysis (Figure 17). RAPD showed less discriminatory power than *Not* I-PFGE, but we could have more meaningful results than PFGE analysis. All clinical isolates were clustered into D type except one isolate though RAPD patterns of environmental isolates were clustered A to E type (Figure 17, Table 2). RAPD experiment always has weak reproducibility, but, in this clustering point of view, RAPD could distinguish between clinical (pathogenic) *V. vulnificus* and non-pathogenic.

We summarize our results in Table 3.

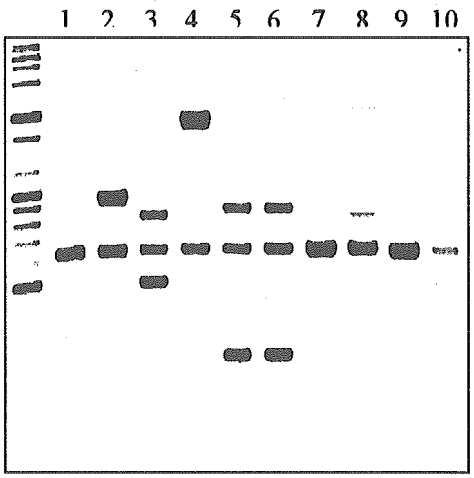


Figure 16. Representative RAPD profiles of clinical *V. vulnificus* isolates.

Lanes 1, 100-bp plus ladder; lane 2, strain 05-12354; lane 3, strain 05-12520; lane 4, strain 05-12561; lane 5; strain 05-ente-CI-1; lane 6, strain 05-ente-CI-2; lane 7, strain 05-14300; lane 8, strain 05-14301; lane 9, strain 05-17101; lane 10, 05-17102.

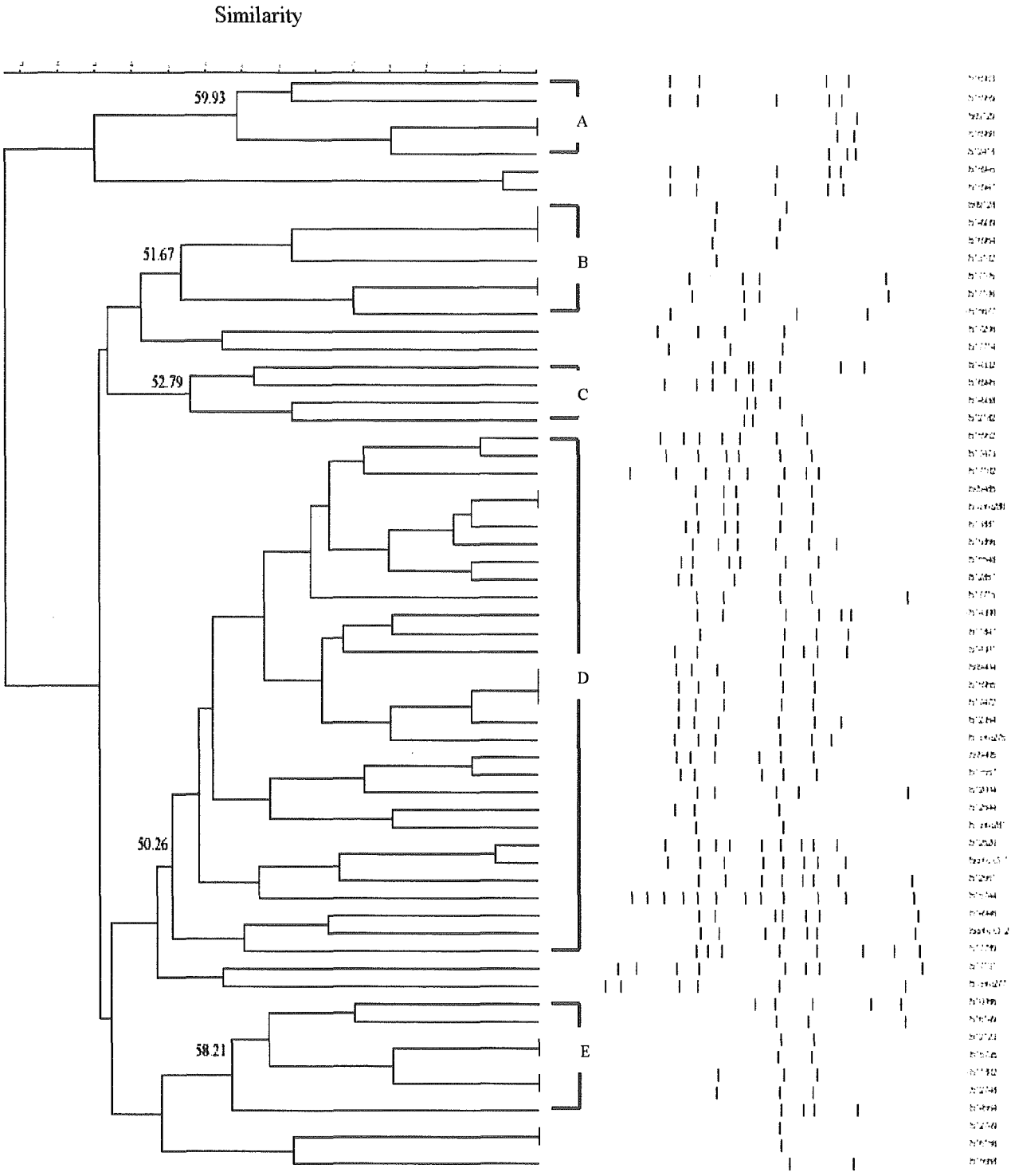


Figure 17. Dendrogram of RAPD patterns of clinical and environmental *V. vulnificus* isolates in 2005

Table 2. Percentages of *V. vulnificus* strains as analyzed by RAPD-PCR

| Type of isolates | RAPD type | | | | | |
|---|---|---|---|---|---|---|
| | A | B | C | D | E | other |
| Total | 5 (8.06%) | 7 (11.3%) | 4 (6.5%) | 30 (48.4%) | 7 (11.3%) | 9 (14.5%) |
| Clinical | - | - | - | 12 (92.3%) | - | 1 (7.3%) |
| Environmental | 5 (10.2%) | 7 (14.3%) | 4 (8.2%) | 18 (36.7%) | 7 (14.3%) | 8 (16.3%) |

Table 3. Molecular type of *V. vulnificus* as analyzed by PFGE and RAPD-PCR

| Strain No. | Molecular type | |
|---|---|---|
| | PFGE | RAPD |
| **Clinical** | | |
| 05-12004 | vvn008 | D 21 |
| 05-12354 | vvn011 | D 17 |
| 05-12520 | vvn013 | D 24 |
| 05-12561 | vvn015 | D 26 |
| 05-ente-CI-1 | vvn048 | D 25 |
| 05-ente-CI-2 | vvn049 | D 29 |
| 05-14300 | vvn019 | D 11 |
| 05-14301 | vvn020 | D 13 |
| 05-14946 | vvn025 | D 28 |
| 05-17101 | ND | F 5 |
| 05-17102 | vvn040 | D 3 |
| 05-15744 | vvn032 | D 27 |
| 05-17789 | vvn047 | D 30 |
| **Environmental** | | |
| 05-09128 | vvn001 | B 1 |
| 05-09129 | vvn001 | A 3 |
| 05-09404 | vvn002 | D 14 |
| 05-09405 | vvn003 | D 19 |
| 05-09485 | vvn004 | D 4 |
| 05-10066 | vvn005 | E 1 |
| 05-11802 | vvn006 | E 5 |
| 05-11847 | vvn007 | D 12 |
| 05-12123 | vvn009 | E 3 |
| 05-12182 | vvn010 | C 4 |
| 05-12418 | vvn012 | A 5 |
| 05-12748 | vvn012 | E 6 |
| 05-12544 | vvn014 | D 22 |
| 05-12749 | vvn016 | F 7 |
| 05-12858 | vvn017 | D 9 |
| 05-13102 | vvn018 | B 4 |
| 05-13881 | vvn018 | D 6 |
| 05-14564 | vvn022 | E 7 |
| 05-14332 | vvn021 | C 1 |
| 05-14938 | vvn023 | C 3 |
| 05-14939 | vvn024 | B 2 |
| 05-15557 | vvn028 | D 20 |
| 05-15548 | vvn027 | D 8 |
| 05-15668 | vvn029 | F 9 |
| 05-15677 | vvn030 | B 7 |
| 05-15735 | vvn031 | E 4 |
| 05-15866 | vvn033 | D 7 |
| 05-15945 | vvn034 | F 1 |
| 05-15947 | vvn034 | F 2 |
| 05-15952 | vvn035 | D 1 |
| 05-15959 | vvn034 | A 2 |
| 05-16149 | vvn037 | E2 |
| 05-16156 | vvn038 | F 8 |
| 05-16945 | vvn039 | C 2 |
| 05-16960 | vvn035 | A 4 |
| 05-16963 | vvn034 | A 1 |
| 05-16964 | vvn024 | B 3 |
| 05-17105 | vvn041 | B 5 |
| 05-17106 | vvn012 | B 6 |
| 05-17472 | vvn043 | D 16 |
| 05-17473 | vvn044 | D 2 |
| 05-17714 | vvn045 | F 4 |
| 05-17715 | vvn046 | D 10 |
| 05-ente-275 | ND | D 18 |
| 05-ente-277 | ND | F 6 |
| 05-ente-280 | ND | D 5 |
| 05-ente-281 | ND | D 23 |
| 05-15955 | vvn036 | D 15 |
| 05-17206 | vvn043 | F 3 |

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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 12 patients with culture-confirmed quinolone-resistant nontyphoidal Salmonella were identified. Only 11 patients had available information from chart review. All patients came from various cities in Metro Manila. Mean age was 17 years old (range: 0.03-76.33 years). Seven of the patients were children, 5 of whom were less than 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. 3 patients had been administered fluoroquinolones prior to availability of culture and sensitivity results. All patients eventually recovered from their illness.

Seven (7) serotypes were identified with Salmonella *choleraesuis var Kunzendorf* accounting for 27% 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. Acquisition of equipment was achieved in February 2006. ***Initial PFGE procedures will follow by the second year of the project following acquisition of technology.***

**Results**

1) Epidemiologic Investigation. A total of 12 patients with culture-confirmed quinolone-resistant nontyphoidal Salmonella were identified. Only 11 patients had available information from chart review Table 1). Patients came from various cities in Metro Manila but 2 patients with ciprofloxacin-resistant Salmonella came from the district of Sampaloc. There were 7 males and 4 females with a mean age of 17 years old (range: 0.03-76.33 years). Seven of the patients were children, 5 of whom were less than 1 year old, 1 was 1.6 years old and one 8 years 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. 3 patients had been administered fluoroquinolones prior to availability of culture and sensitivity results. The eight other patients were given antimicrobials of other classes, most of which were cephalosporins. 9 patients were febrile, 3 had diarrhea and 3 had vomiting. All patients eventually recovered from their illness.
2) Laboratory Investigation. Seven (7) serotypes were identified with Salmonella choleraesuis var Kunzendorf accounting for 27% 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 11 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, Metro Manila, Philippines

| Characteristics | N=11 |
|---|---|
| 1. Age (years) | |
| Mean | 17.24 |
| Range | 0.03-76.33 |
| 2. Female sex – no. (%) | 4(36.37) |
| 3. Treatment with antimicrobial agent | |
| a. Fluoroquinolones | 3 |
| b. Others | 8 |
| 4. Concomittant medical problems | |
| a. HIV and pulmonary | 1 |
| b. cardiac and pulmonary | 1 |
| c. genitourinary | 1 |

Table 2. Serotypes of nontyphoidal Salmonella isolates.

| Serotype | No. (%) |
|---|---|
| Salmonella choleraesuis var. Kunzendorf | 3 (27.3) |
| Salmonella Schwarzengrund | 2 (18.2) |
| Salmonella Hissar | 2 (18.2) |
| Salmonella Lomita | 1 (9.1) |
| Salmonella Stanley | 1 (9.1) |
| Salmonella Oritamerin | 1 (9.1) |
| Salmonella typhimurium | 1 (9.1) |
| | **11 (100.00)** |

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

| ID NO. | ORGANISM | AMP | CHL | CIP | SXT | NAL |
|---|---|---|---|---|---|---|
| 200001 | Salmonella Lomita | >256 | 192 | <1 | >32 | ≤8 |
| 300002 | Salmonella Stanley | ≤8 | ≤8 | <1 | <2 | ≤8 |
| 400003 | Salmonella Oritamerin | >256 | >256 | <1 | >32 | ≤8 |
| 400004 | Samonella Hissar | >256 | >256 | <1 | >32 | ≤8 |
| 400005 | Salmonella Typhimurium | >256 | >256 | <1 | >32 | >256 |
| 400006 | Salmonella Hissar | >256 | ≤8 | <1 | >32 | >256 |
| 400007 | Salmonella Choleraesuis var. kunzendorf | ≤8 | 192 | <1 | >32 | >256 |
| 400008 | Salmonella Choleraesuis var. kunzendorf | >256 | ≤8 | <1 | >32 | >256 |
| 400009 | Salmonella Choleraesuis var. kunzendorf | >256 | ≤8 | <1 | >32 | >256 |
| 500010 | Salmonella Schwarzengrund | >256 | ≤8 | >32 | <2 | >256 |
| 500011 | Salmonella Schwarzengrund | >256 | >256 | >32 | >32 | >256 |

## Title

**Surveillance and molecular tracking of *Vibrio cholerae* O1/O139 and *Vibrio parahaemolyticus* in Bangladesh and in the Asia Pacific region**

## Name of the researcher

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## Summary

A standardized PFGE protocol for *V. cholerae* strains isolated form different countries of Asia Pacific region was developed and validated. Results from all laboratories were uniform and thus the validated PulseNet PFGE protocol for PFGE can now be used for analysis of *V. cholerae* strains from all over the world and from the Asia Pacific.

Three most common serotypes of *V. parahaemolyticus* from ICDDR,B, Bangladesh were selected for the development and validation of PFGE protocol for *V. parahaemolyticus*. This protocol will then be used to determine if the current pandemic strain of *V. parahaemolyticus* isolated from the Asia Pacific region is clonal.

## Purpose

To maintain surveillance on *V. cholerae* O1/O139 and *V. parahaemolyticus* among hospitalized patients admitted to the ICDDR,B Hospital in Dhaka

To perform phenotypic, serological, antimicrobial resistance and molecular analysis to enable
cataloguing of different genotypes of toxigenic and non-toxigenic *V. cholerae* O1/O139 and *V. parahemolyticus* isolated during the above surveillance and perform the same laboratory analysis for strains of the above pathogens which we receive from different parts of the Asia Pacific region.

To participate in the establishment of the the Pulse Field Gel Electrophoresis molecular typing protocols being developed by the PulseNet Asia Pacific group and to compare and exchanges profiles of strains from Bangladesh and and from other countries to identify outbreak strains and new emerging clones.

The long-term objective of this project is to understand the various types of *V. cholerae* and *V. parahaemolyticus* in circulation and to monitor the emergence of new clones of the above vibrios in the Asia Pacific region and in other parts of the world.

## Methods

Initially a panel of 40 *Vibrio cholerae* strains of different biotypes and serogroups (O1 and O139) from Japan, Hong Kong and Bangladesh were selected and used for development and validation of a standardized *V. cholerae* PulseNet PFGE protocol. For the final validation, however, seven isolates were chosen from the initial panel of 40 strains. All the *V. cholerae* strains included in this study were initially grown on TTGA agar plate from stool sample and then subjected to enrichment. The bacterial strain were then subjected to serological confirmation by using monoclonal antibodies and biochemical tests (chicken cell agglutination, sensitivity to polymixn B and phage IV and 5, Vogues-Proskauer test). Further confirmation of the strains was carried out by PCR using species-specific *ompW* and serogroup- specific *rfbO1* and *rfbo139* primers. Toxigenic trait of these strains was then tested by *ctxAB* PCR. For PFGE analysis two rare-cutting restriction enzymes, namely, *Not*I and *Sfi*I were selected for the validation process of the PulseNet PFGE protocol and used in our laboratory using the supplied standardized protocol. In ICDDR,B all the 40 *V. cholerae* strains were restricted only with *Not*I enzyme and the seven finally selected isolates were restricted with both the restriction enzymes, *Not*I and *Sfi*I. A CHEF DR-II apparatus was used to electrophorese the resulting restricted DNA fragments for 19 hours. At the end of electrophoresis products were stained with ethidium bromide and destained with water. Finally, gel embedded products were visualized under UV light and photographed on a gel documentation system.

A total 6 strains of *V. parahaemolyticus*, two each of the most common serotypes were grown on TCBS from stool culture. The resulting strains were then further grown on LA supplemented with 3% sodium chloride. Initially all the strains were subjected to serological confirmation by using polyconal antibodies. Further confirmation of the resulting strains was carried out by PCR using species-specific *toxR*. Toxigenic ability of these strains was then tested by *tdh* and *trh*. Pandemic potential of these strains were confirmed by GS and ORF8 PCR.

## Results

### Epidemiological investigation

*Vibrio cholerae* causes cholera which is characterized by severe watery diarrhea and vomiting. Till date 206 serogoups of *V. cholerae* have been recognized and among the serogroups only O1 and O139 have been found to associated with pandemic and/or epidemic outbreak. The O1 serogroup of *V. cholerae* is classified into two biotypes namely, classical and El Tor. The El Tor biotype was responsible for the fifth an sixth pandemics and although there is no hard evidence, presumably for the earlier pandemics also. In 1961 El Tor vibrios caused the seventh pandemic and gradually replaced the classical strains and continue to persist till date. Cholera is an easily treatable disease but when left untreated cholera can kill quickly following the onset of symptoms and without treatment the case fatality rate for severe cholera is about 50%. Between 1817 and 1917 this disease caused deaths in India alone are estimated at over 38 million and early of this century (in 2001) 58 countries reported 184 311 cases with 2 728 deaths. In 1992, the new serogroup O139 first reported in India and till date continue to spread in other countries. Recently, new variants of *V. cholerae* O1 strains associated with acute secretory diarrhea were reported. These strains have been classified as hybrids as they were found to have traits of both the biotypes,

classical and El Tor. In early 2004, such hybrids of *V. cholerae* were responsible for a huge outbreak of cholera in Mozambique in the African continent.

*Vibrio parahaemolyticus* is currently recognized as a major, worldwide cause of gastroenteritis, particularly in the Far East where seafood consumption is high. In February 1996, a sudden increase in the incidence of the *Vibrio parahaemolyticus* was observed among hospitalized patients in Calcutta, a city in the northeastern part of India. Analysis of the strains revealed that the unique serotype O3:K6, previously isolated during the surveillance in Calcutta, accounted for 50 to 80% of the infections. O3:K6 isolates similar to those isolated in Calcutta were reported from food borne outbreaks and from sporadic cases in Bangladesh, Chile, Japan, Korea, Laos, Mozambique, Russia, Taiwan, Thailand and United States. Other serotypes like the O4:K68, O1:K25 and O1:KUT (untypable) that had molecular characteristics identical to that of the pandemic O3:K6 clone were subsequently documented. These new serotypes appear to have diverged from the pandemic O3:K6 clone by alteration of the O:K antigens and were postulated to be clonal derivatives of the O3:K6 serotype. Twenty different serotypes are currently included in the pandemic group list and all have show to be virtually similar to the O3:K6 serotype by a variety of molecular typing techniques. Clearly, the pandemic spread such as one never seen before, of a specific clone of *Vibrio parahaemolyticus* is apparent and the new clone has now spread into four continents (Asia, America, Africa and Europe)

*V. cholerae* and *V. parahaemolyticus* caused diseases in almost all parts of the world and thus establishing a collaborative network between Asia Pacific Laboratories might me useful to understand the genetic relationships of individuals of theses species. To do so strains isolated from Asia Pacific regions will be typed based on PFGE analysis and results will be uploaded in a unified database. To develop such database a standardized protocol of PFGE is essential, as this will ensure the uniformity of the test in different laboratories in different countries. Access to this database will allow the collaborators to understand the various strains in circulation and the emergence of new clones, which will in turn alert the regional laboratories in advance.

**PFGE analysis:**

The PFGE images generated in ICDDR,B were sent to PHLC, Hong Kong for analysis and inter-laboratory comparison. The results obtained in ICDDR,B matched well with the results obtained by the other two members of PulseNet Asia Pacific (PHLC, Hong Kong; NIID, Japan), which ensured the reproducibility and robustness of the protocol (Cooper et al., Manuscript communicated).

Six strains of epidemiologically unrelated three most common serotypes of *V. parahaemolyticus* strains were sent to PHLC, Hong Kong and one set of strains have been preserved for future reference.

**Discussion**

In ICDDR,B, *V. cholerae* strains isolated from different parts of the world were examined by PFGE using previously used and the newly standardized PulseNet protocol. Significant qualitative differences were noticed between the previously practiced and new PulseNet PFGE protocol during digital analysis. Identification of

closely migrating bands is a commonly observed error generated by general PFGE protocol used in different laboratories. In our laboratory, we observed such errors while examining *V. cholerae* strains isolated from a recent outbreak in Biera, Mozambique. Two closely migrating high molecular weight restriction fragments placed the 24 hours long local laboratory protocol in question. The PulseNet standardized PFGE protocol, however ensured successful identification of such closely migrating bands during digital analysis. Use of wider combs in the PulseNet standardized protocol gave extra sharpness to the separated DNA fragments, which in turn made individual band identification easier by increasing the inter fragment physical gap. In the ICDDR,B laboratory we have also compared the PFGE patterns generated by *Xba*I and the two enzymes: *Not*I and *Sfi*I used in the validation process of PulseNet PFGE protocol. Comparison revealed that the rare-cutting *Not*I and *Sfi*I are better choices than *Xba*I. In our laboratory we observed the tendency of the restricted DNA fragments, especially the low molecular bands to become diffused and fuzzy due to long running time. Developing a short running time with maximum separating ability of PulseNet protocol has also minimized this problem. In the validation process, we observed that the variation in equipment could cause a little variation in the final outcome.

All the *V. parahaemolyticus* strains are awaiting redistribution to all participating laboratories. PFGE run will be conducted following the standardized *V. parahaemolyticus* PFGE protocol by PulseNet.

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**1. Title of the Project**

Phenotypic and Genotypic Characterization of Common Enteric Pathogens Isolated from Diarrheal Patients: a National Study

**2. Name of the Investigator**

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**3. Summary**

We have investigated several outbreaks and sporadic cholera in many States of India during 2004-05. An outbreak of diarrhea occurred in Delhi during May 2004, in which 28 (64%) cases were positive for *V. cholerae* O1 serotype Inaba. Following this outbreak, in a span of 18 months, cholera was recorded in 14 other States with 6 outbreaks. *V. cholerae* O1 Inaba serotype was exclusively identified in 9 States. In the antimicrobial susceptibility assay, large numbers of Inaba isolates were resistant to chloramphenicol, streptomycin and majority (91%) of the Inaba isolates showed reduced susceptibility for ciprofloxacin. The tested isolates harbored *ctxA* and the El Tor allele of *tcpA* in the PCR assay. Ribotyping analysis showed that most of the recent Inaba isolates belongs to a new ribotype (RIV) and isolates of Ogawa during the same period and old Inaba were identified as ribotype RIII. In the pulsed-field gel electrophoresis (PFGE), majority of the Inaba isolates belongs to 'H' or 'H1' pulsotype. Identification of 6 new pulsotypes demonstrates that the genome of recent *V. cholerae* O1 is under flux. Since the *V. cholerae* serogroup O139 is no longer existing in India, we assume that the Inaba serotype has acquired the potential to affect the population at a large.

*Vibrio fluvialis* causes sporadic diarrhea, but its incidence was not frequently reported among diarrheal patients due to the complications in the conventional identification methods. In order to identify this pathogen, we formulated a new PCR based assay and validated its sensitivity and specificity in comparison with 16S rDNA sequence and API 20E identification systems. This PCR assay may be useful for confirmation of *V. fluvialis*.

**4. Purpose**

During 2004-05, we have identified the reemergence and progression of *V. cholerae* O1 serotype Inaba with outbreaks and sporadic cases in many parts of India. Considering the changes in the phenotypic and genotypic characteristics of *V. cholerae* O1, we have analyzed the Inaba isolates collected from different cholera outbreaks as well as strains received at the National Institute of Cholera and Enteric Diseases, Kolkata, India.

*Vibrio fluvialis*, has been reported to cause sporadic infections and outbreaks of diarrhoea in humans and has also been isolated from the marine and estuarine environments. However, public health significance of this pathogen is not studied in detail due to the lack of simple and reliable diagnostic tests. Information regarding virulence genes, and standard genetic markers for the identification of this organism, are not fully exploited. Despite the use of an array of biochemical tests including commercial identification systems, proper identification of *V. fluvialis* still remains a problem due to its phenotypic similarity with *Aeromonas* species. In this study, we made an attempt to establish a PCR based identification method for *V. fluvialis*.

**5. Methods**

***5.1 Molecular characterization of recent V. cholerae O1 isolates from India***

Four hundred and two *V. cholerae* O1 isolates consisting of 174 Ogawa and 228 Inaba serotypes isolated either from sporadic cases or outbreaks of cholera from 15 States were included in this study (Table 1). Representative *V. cholerae* O1 Ogawa/Inaba isolates covering all the areas were selected randomly for molecular typing.

**PCR assay**

The presence of A-subunit cholera toxin gene (*ctxA*) and *tcpA* variants (major structural subunit gene of the toxin coregulated pilus) of the classical and El Tor biotypes was determined by a multiplex PCR assay (1, 2) using 10X amplification buffer (500 mM KCl, 100 mM Tris HCl, 15 mM $MgCl_2$), [pH 8.3]), 2.5 mM each of dNTP mixture, 10 pmole each of the primer, 1.25 unit of rTaq DNA polymerase (Takara Shuzo, Otsu, Japan) and 5 µl of template. The reaction volume was adjusted to 25 µl using sterile triple distilled water. Multiplex PCR was performed in an automated thermocycler (Gene Amp PCR system 9700, Applied Biosystems)