that no association could be demonstrated between the PFGE types and the geographic origin of the strains.

Two strains from wastewater (Number 38, 39) in Hanoi had the same PFGE patterns (100% genetic similarity), and correlate with the origin of the patient isolates at similarity level of 97.5%.

In conclusion, the cause of the cholera outbreak in Hanoi in 2007 is *Vibrio cholerae* O1, serotype Ogawa. Originally the wastewater from Hanoi city contaminated *Vibrio cholerae* and transmitted to human.

References

1. Annual report from National Institute of Hygiene and Epidemiology from year 1995-2007.
2. National Institute of Hygiene and Epidemiology, Hanoi, Vietnam. Protocol of isolation and identification of Vibrio cholerae, 1998.
3. K.L.F. Cooper, C.K.Y. Luey, M.Bird, J. Terajima, G.B. Nair, K.M. Kam,E.Arakawa, A.Safa, D.T. Cheung, C.P. Law, H.Watanabe, K.Kubota, B.Swaminathan, and E.M.Ribot. Development and Validation of aPulseNet standardized Pulsed-Field Gel Electrophoresis Protocol for Subtyping of *Vibrio cholerae*. Foodborne Pathogens and Disease, Volume 3, Number 1, 2006: 51-58.

REPORT ON RESEARCH SPONSORED BY NATIONAL JAPANESE INSTITUTE OF INFECTIOUS DISEASES

PROJECT LEADER: PROFESSOR DR HARUO WATANABE

CO-RESEARCHER: PROF DR THONG KWAI LIN
UNIVERSITY OF MALAYA
KUALA LUMPUR, MALAYSIA

RESEARCH TITLE: Determination of PFGE genotypes, Resistance Mechanism and Development of PCR serotyping of *Salmonella* serovars from Ready-To-Eat Foods in Malaysia

SUMMARY

The Standardised PulseNet PFGE Protocol has been successfully implemented in all current analysis and we participated in the Interlaboratory PulseNet Asia Evaluation Exercise. By using this standardized protocol, we subtyped *Salmonella* spp isolated from ready to eat foods in Malaysia. The results showed that there is diversity of Salmonellae and there is no one PFGE subtypes.Some of the Salmonella serotypes identified included *S.* Tshiongwe, *S.* Enteritidis, *S.* Typhimurium, *S.* Corvallis, *S.* Bardo, *S.* Kentucky, *S.* Weltevreden, *S.* Braenderup, *S.* Paratyphi B, *S.* Hvittingfoss and *S.* Albany.

The standardized protocol was also utilized to examine Salmonella isolated from imported food provided by the Public Food and Safety Lab to assist in identifying movement of strains between countries through foods. In addition, PFGE was also applied to identify a possible carrier of S.typhi in an highly endemic state in Malaysia.

Antimicrobial susceptibility of *Salmonella* strains isolated from ready to eat foods was determined. Sixty-five percent of the strains remained susceptible to all the clinically important antibiotics while only 7% was resistant to 3 or more antimicrobial agents. In another subproject, detailed analysis of resistance of S.typhimurium showed that 14 ampicillin-resistant and 25 streptomycin-resistant strains had the bla_{TEM} and *strA* genes, respectively. However, 54% of the streptomycin resistant strains had a sequence similar to the *aadA* gene, 37% of the 27 sulphonamides-resistant strains harboured *sul1* and 89% harboured *sul2*. Among the 33 tetracycline resistant strains, 70%, 15%, and 12 % had *tet*(A), *tet*(B) and *tet*(C) genes, respectively. 37(79%) of the isolates were carried class 1 integrons, which grouped in 10 different profiles. The sequencing analysis identified *sat*, *aadA*, *pse-1* and *dhfr* genes in variable regions on class 1 integrons.

To facilitate identification was *Salmonella* serogroups or serotypes, a mPCR was optimized and applied to differentiatiate *Salmonella* genus, *S.* Typhimurium, *S.* Typhimurium DT104 and *S.* Typhi.

PURPOSE

i. To develop a sequence-based serotyping of selected *Salmonella* serovars in Malaysia

ii. To determine the pulsotypes of the *Salmonella* serovars by Pulsed Field Gel Electrophoresis

iii. To determine the antimicrobial susceptibility patterns and resistance mechanism of selected multidrug resistant *Salmonella* serovars by molecular approaches

METHODS

1. Salmonella was isolated and identified from ready to eat foods according to standard biochemical methods

PFGE was performed according to standardizes PulseNet Protocol

2. Antimicrobial susceptibility tests were carried by the disc diffusion method

3. Detection of virulence and specific targets was carried out by PCR

RESULTS AND DISCUSSIONS

1 TO DEVELOP A SEQUENCE-BASED DIFFERENTIATION OF SELECTED SALMONELLA IN MALAYSIA

Serotyping of *Salmonella* by conventional method is a problem in Malaysia- due to lack of facilities. Hence, an attempt was made to initiate the application of sequence-based approach to serotype the available *Salmonella* isolates. Primers for the specific serotypes determination were obtained from the published papers. Oligonucleotides primers were synthesized and optimized in the laboratory and apply to field isolates.

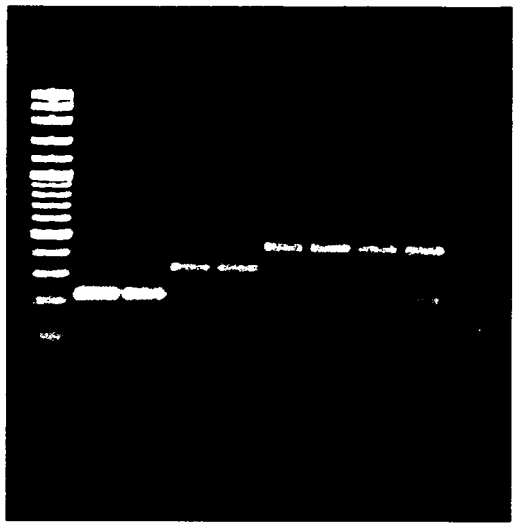
In this study, a multiplex PCR assay has been optimized. Four pairs of primers have been used: primers OMPC is used to detect a 204 bp *Salmonella* genus specific sequence (5'-ATCGCTGACTTATGCAATCG-3'), primer ENT was used to detect a 304 bp serotype Enteritidis specific sequence (5'-TGTGTTTTATCTGATGCAAGAGG-3', primer TYPH was used to detect a 401 bp serotype Typhimurium specific sequence (5'-TTGTTCACTTTTACCCCTGAA-3') while STM 104 was used to detect a 102 bp serotype Typhimurium DT 104 specific sequence (5'-ATGCGTTTGGTCTCACAGCC-3') (Alvarez *et al.*, 2004). In addition, primer ST322 oligonucleotides which was developed in-house was used

to detect a 322 bp specific Typhi sequence.

One hundred and four strains were examined by PCR to differentiate the *Salmonella* genus from non- *Salmonella* genus. Based on PCR analysis, 10% of the strains tested were not *Salmonella* genus and 90% were confirmed *Salmonella* genus. As the majority of the *Salmonella* strains were provided by other laboratories who carried out the initial isolation and identification, this study indicates that it is important to confirm the identity by this rapid PCR method before commencement of detail analysis of *Salmonella.*

Among all *Salmonella* strains, 44% were isolated from food while 56% were isolated from humans; 15% were *Salmonella* Typhimurium, 18% were *Salmonella* Enteritidis, 28% were *Salmonella* Typhi and 39% were other *Salmonella* genus. Among 14 *Salmonella* Typhimurium strains, only 3 of them were *Salmonella* Typhimurium DT 104.

Furthermore, among the *Salmonella* strains isolated from food source, 24% were *Salmonella* Typhimurium, 29% were *Salmonella* Enteritidis and 47% were other *Salmonella* genus. Among the human sources, 8% were *Salmonella* Typhimurium, 9% were *Salmonella* Enteritidis, 49% were *Salmonella* Typhi and the rest were of other *Salmonella* genus (could not be differentiated by the limited oligonucleotides used)



Representative gel for a multiplex PCR assay. Lane 1:100 bp DNA marker, lane 10 was the control negative. Lanes 2,3-other *Salmonella* genus, lanes 4,5 -*Salmonella* Enteritidis, lanes 6,7-*Salmonella* Typhimurium, and lanes 8,9- *Salmonella* Typhimurium DT104.

Conclusion: The mPCR for rapid identification of *Salmonella* of economic importance is very useful and can be implemented parallel to traditional serotyping. Future work is needed to evaluate more primers to identify at least the top ten serovars in Malaysia.

Objective 2 : Application of standardized PulseNet Protocol for Macrorestriction Analysis of *Salmonella*

A. PFGE was utilized to study the diversity of *Salmonella* isolated from street foods and imported foods

Representative XbaI pulsotypes

Approximately 270 street foods were sampled. Isolation and confirmation of Salmonellae were carried according standard microbiological and biochemical tests. Only 36 *Salmonella* strains were isolated (13%) and PFGE of *Xba*I digested DNAs showed diversity of pulsotypes. Based on limited database available, the `serotype' of *Salmonella* was predicted and this was confirmed by traditional serotyping. The common serotypes associated with street foods sampled during the study period included *S.* Tshiongwe, *S.* Enteritidis, *S.* Typhimurium, *S.* Corvallis, *S.* Bardo, *S.* Kentucky, *S.* Weltevreden, *S.* Braenderup, *S.* Paratyphi B, *S.* Hvittingfoss and *S.* Albany.

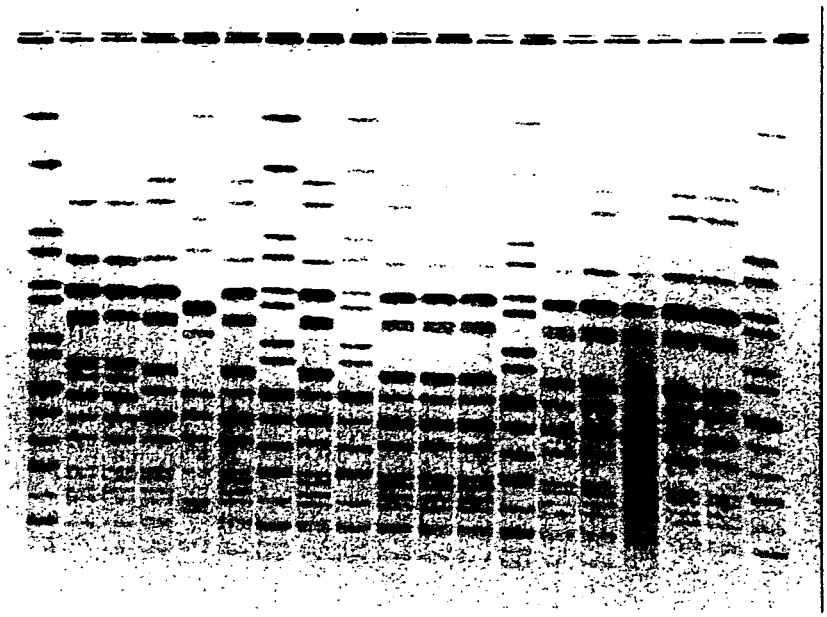
Standardised CDC PulseNet Protocol was successfully applied. Each *Salmonella* serotype was grouped into its own cluster.

B. **Continued Participation in the Interlaboratory PulseNet Asia Pacific Evaluation**.

In addition, we participated in the Interlaboratory PulseNet Asia Pacific Laboratories testing and evaluation headed by Dr Kam from the Hong Kong. We also continually provide and teach officers and researchers in Malaysia to use PFGE for subtyping bacterial pathogens and disseminate information on PulseNet activities. Continued in-house training provided to officers from public health laboratory.

c. **PFGE of S. Typhi and identification of potential typhoid carrier in a highly endemic state in Malaysia**

Kelantan is a highly endemic state for Typhoid Fever in Malaysia. *S.* Typhi isolated from patients and stools from foodhandlers were analysed. PFGE was used to analyse 92 *S.*Typhi strains derived from three recent typhoid fever outbreaks in a hype-rendemic State of Kelantan, Malaysia. Genomic DNA of strains from three outbreaks, 2005 (n=34), 2006 (n=45) and 2007 (n=13) were prepared according to the PulseNet Protocol and digested with primary enzyme, *Xba*I. Twenty-one different pulsotypes were observed among the 92 strains. Three predominant profiles, STXba06, STXba07 and STXba12 were found. The 2005 and 2006 typhoid fever outbreaks were associated with pulsotypes STXba06 and STXba07 (which differed by one *Xba*I fragment only). The recent outbreak in 2007 was associated with one predominant profile, STXba12 which was not found in previous years. Two different human carriers were identified and each of their pulsotypes corresponded to STXba07 and STXba12, respectively. This is the first report of the association of the *S.*Typhi from human carriers being linked to outbreak strains. Overall, outbreak strains were genetically homogenous while those from sporadic cases in the same locality were more diverse.



Representative PFGE profiles of S.Typhi. PFGE in lane 2 (from a carrier has same profiles as the 2007 outbreak strains in lanes6.

Objective 3: To determine the antimicrobial susceptibility patterns and resistance mechanism of selected multidrug resistant *Salmonella* serovars by molecular approaches

3.1 All *Salmonella* genus tested were susceptible to gentamicin. 12% of the strains were resistant to chloramphenicol, 11% were resistant to streptomycin, 16% were resistant to sulphamethoxazole/trimethoprim, 18% were resistant to trimethoprim, 20% were resistant to nalidixic acid, 21% were resistant to sulphonamides and ampicillin, and 5% were resistant to kanamycin. Morever, 65% of the strains were susceptible to all antimicrobial agents. 11% were resistant to one antimicrobial agent, 3% were resistant to two antimicrobial agents, 2% were resistant to three antimicrobial agents, 4% were resistant to four and five antimicrobial agents, 10% were resistant to six antimicrobial agents, and 1% was resistant to seven antimicrobial agents. Among 41 strains isolated from food source, 64% of them were susceptible to all antimicrobial agents while 20% were resistant to one antimicrobial agent, 7% were resistant to two antimicrobial

agents, 2% were resistant to either four or seven antimicrobial agents, and 5% were resistant to five antimicrobial agents. Meanwhile, among 53 strains isolated from human source, 65% of them were susceptible to all antimicrobial agents. 4% were resistant to one, three and five antimicrobial agents respectively, 6% were resistant to four antimicrobial agents, and 17% were resistant six antimicrobial agents.

3.2 Genetic Determinant of multidrug resistance in S.Typhimurium

Genetic determination of drug resistance of selected isolates of zoonotic and clinical *S.* Typhimurium was determined. About 70% of the *S.* Typhimurium isolates were resistant to two or more antimicrobial agents: tetracycline (70%), sulphonamides (57%), streptomycin (53%), ampicillin (30%), nalidixic acid (28%), kanamycin (23%), chloramphenicol (21%), and trimethoprim (19 %). Additional resistance towards cephalosporins was detected: cephalothin (28%), cephradine (21%), amoxicillin clavulanate acid (17%) and cephalexin (17%). All the 14 ampicillin-resistant and 25 streptomycin-resistant strains had the bla_{TEM} and *strA* genes, respectively. However, 54% of the streptomycin resistant strains had a sequence similar to the *aadA* gene, 37% of the 27 sulphonamides-resistant strains harboured *sul1* and 89% harboured *sul2*. Among the 33 tetracycline resistant strains, 70%, 15%, and 12 % had *tet*(A), *tet*(B) and *tet*(C) genes, respectively. 37(79%) of the isolates were carried class 1 integrons, which grouped in 10 different profiles. The sequencing analysis identified *sat*, *aadA*, *pse-1* and *dhfr* genes in variable regions on class 1 integrons.

Research output

Work presented at the 2008 Annual PulseNet Asia Pacific Meetingi Kolkata, India February, 2008

Four manuscripts in preparation for suhmission to appropriate journals.

Comparison of bacterial PFGE patterns between countries

Name of researcher: Brent Gilpin
Affiliation: Institute of Environmental Science & Research Limited, New Zealand

Summary:
In this project we established standardized regional databases for PulseNet Asia Pacific.. Databases for *Campylobacter, E. coli, Salmonella, Vibrio cholerae, Vibrio parahaemolyticus, Listeria* and *Shigella* are now available on the PulseNet Asia Pacific forum, alongwith detailed instructions. How rapidly these grow will depend on the goodwill of participants, and will provide evidence of both benefits and will for more automated regional databases.

The PFGE comparisons between NZ and USA *Campylobacter* isolates highlighted the high frequency of matches between isolates from each country. With the primary enzyme (*Sma*I), 43% of the USA patterns were indistinguishable from those in the NZ database. With the second enzyme (*Kpn*I) this dropped to 23% of patterns. Statistical analysis tools put forward and used in this study provide an initial guideline for the comparison of PFGE patterns. More robust statistical analysis would require generation of a unified database with combined frequency distributions. For individual databases, the inclusion of zero values for known patterns not in a database would be beneficial.

The importance of two enzymes is confirmed yet again. Several patterns were indistinguishable with one enzyme, but clearly different with the addition of a second. The statistical significance was also markedly increased with two enzymes from as little as 1in 10 with one enzyme, to 1 in 3,528 for two enzymes. Even with two enzymes the frequency of each pattern can make a significant difference, with **two unique patterns having a 1 in 221,557 chance of a match compared to 1 in 205 for the most common two enzyme match.**

Purpose:

The objective of this research project was to establish the framework for the comparison of PFGE databases between countries and begin the process of determining whether bacterial pathogens are actually separated or restricted by geographical boundaries (endemic) or whether the same genotypes of bacteria are distributed worldwide.

Methods:

This project had two components.

Part A: Standardised databases for *Campylobacter, E. coli, Salmonella, Vibrio*

cholerae, Vibrio parahaemolyticus, Listeria and *Shigella* have been established and made available to PulseNet Asia Pacific participants via the Asia Pacific secure forum. Instructions for the use of XML to prepare database submissions, and share data between countries have been prepared and also posted on the forum. Participants were introduced to the databases at the Asia Pacific meeting in Kolkata and submissions encouraged.

Part B: Database comparisons between PFGE databases in NZ and a subset of PulseNet USA databases comprised of outbreak and most common strains was undertaken. Bundles of PulseNet USA data were obtained and combined into a single bundle. This bundle was compared with NZ data using DICE coefficient and UPMGA with tolerance and optimization of 1% each. In this report only *Campylobacter* comparisons are given as an example.

Statistical analysis of genotypes

A range of tools were evaluated for comparison of genotypes. An excel spreadsheet was developed to calculate the Diversity Index (DI) of each database of isolates using Simpson Index (Simpson 1949), with confidence intervals around the DI estimated according to the approach described by (Grundmann et al. 2001).

	A	B	C	D
1	Ref number	Type	Counts of each type	Frequency
2	1	Sm0001	162	8.7%
3	2	Sm0037	104	5.6%
4	3	Sm0009	93	5.0%
5	4	Sm0050	75	4.0%
6	5	Sm0024	74	4.0%
7	6	Sm0021	64	3.4%
8	7	Sm0038	54	2.9%
9	8	Sm0081	44	2.4%
10	9	Sm0018	36	1.9%
11	10	Sm0002	33	1.8%
12	11	Sm0036	29	1.5%
13	12	Sm0156	26	1.4%
14	13	Sm0098	24	1.3%
15	14	Sm0013	24	1.3%
16	15	Sm0033	23	1.2%
17	16	Sm0040	23	1.2%
18	17	Sm0172	22	1.2%
19	18	Sm0030	22	1.2%
20	19	Sm0039	21	1.1%
21	20	Sm0010	20	1.1%
22	21	Sm0250	20	1.1%
23	22	Sm0053	20	1.1%
24	23	Sm0245	19	1.0%

INSTRUCTIONS- Before entering new data press the "clear data button",
This will remove data from the sheet. Enter the count data in Column C - Counts of each type
and if you wish you can enter name data in column B. The results are presented in red bello
Upto 500 different types can be entered into this sheet, please tell me if this is not enough.

Number of types	287	This is a counts types in the spreadsheet
Total population, N	1871	i.e total number of counts

Variance	1.41E-06
Confidence Interval	0.23%

	Score	Min	Max
Diversity Index	97.73%	97.50%	97.97%

clear data — Pressing this button clears all the data in the spreadsheet
Press this button to ensure there is not data in the spreadsheet prior to entering

Individual pattern frequencies in each database were calculated, and upper 95% confidence interval calculated using:

$$p + 1.96\sqrt{P(1-P)/N}$$

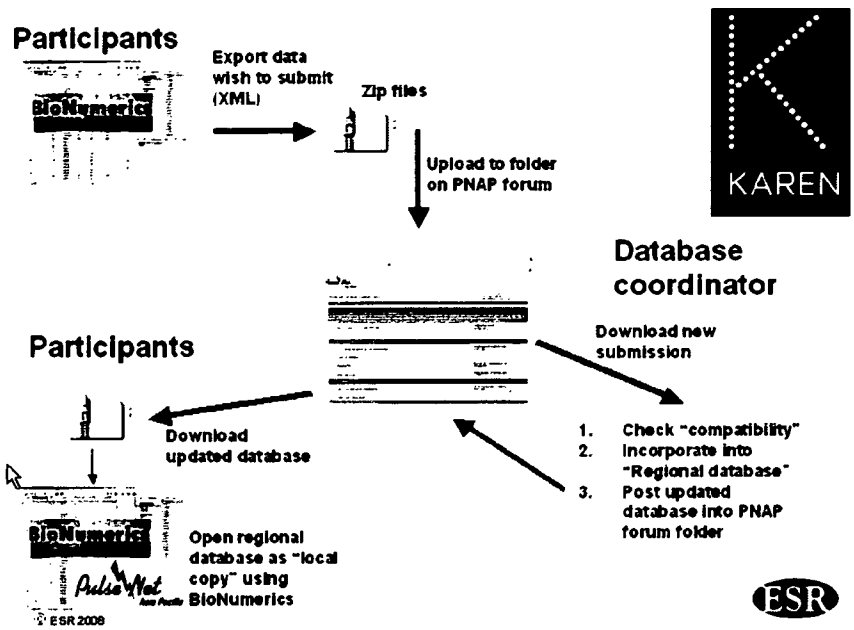
where p=frequency of particular PFGE type, and N=number of isolates in database.

Where two enzymes were available, an upper confidence interval was calculated for each, and frequencies multiplied together to yield a combined frequency. One divided by this frequency generated an estimate of how common the pattern combination is.

Results:

Standardised databases were established, and the procedure outlined below developed, tested and detailed instructions were written. Databases for *Campylobacter, E. coli,*

Salmonella, Vibrio cholerae, Vibrio parahaemolyticus, Listeria and *Shigella* are now available.



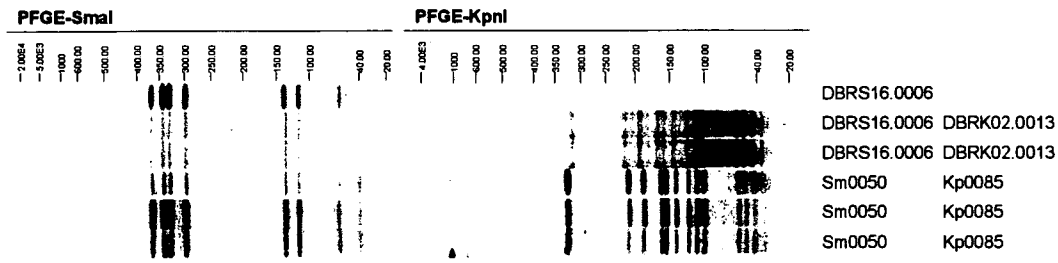
Campylobacter comparisons

Details of the isolates compared are given in table 1

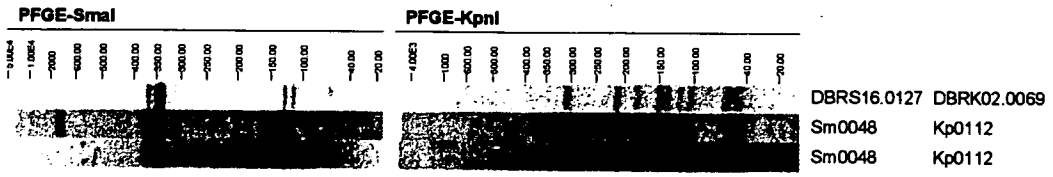
	New Zealand		USA	
	*Sma*I	*Kpn*I	*Sma*I	*Kpn*I
Isolates	1871	1097	22	22
Types	287	364	50	33
DI	97.73	99.02	94.69	96.4
95% CI	97.5-97.97	98.89-99.15	92.54-96.84	99.3-93.47

With the primary enzyme (*Sma*I), 43% of the USA patterns were indistinguishable from those in the NZ database. With the second enzyme (*Kpn*I) this dropped to 23% of patterns. A selection of comparisons are shown below.

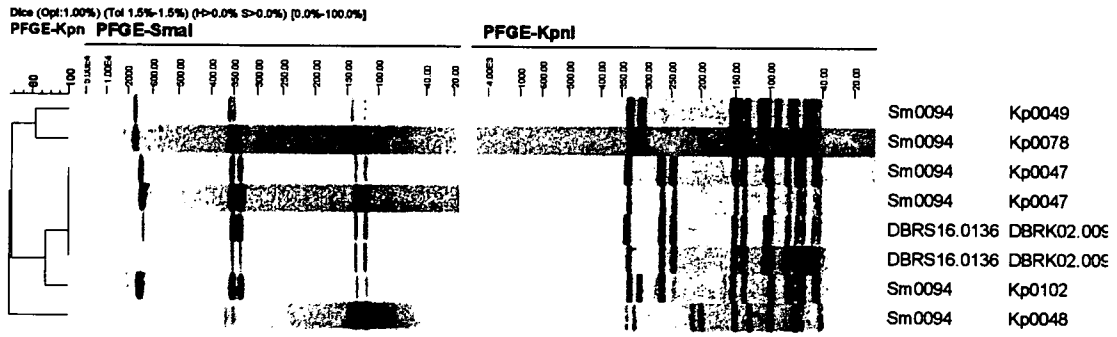
For the first panel isolates, the *Sma*I patterns are clearly indistinguishable. The US *Kpn*I patterns have I believe extra bands that are artifacts.



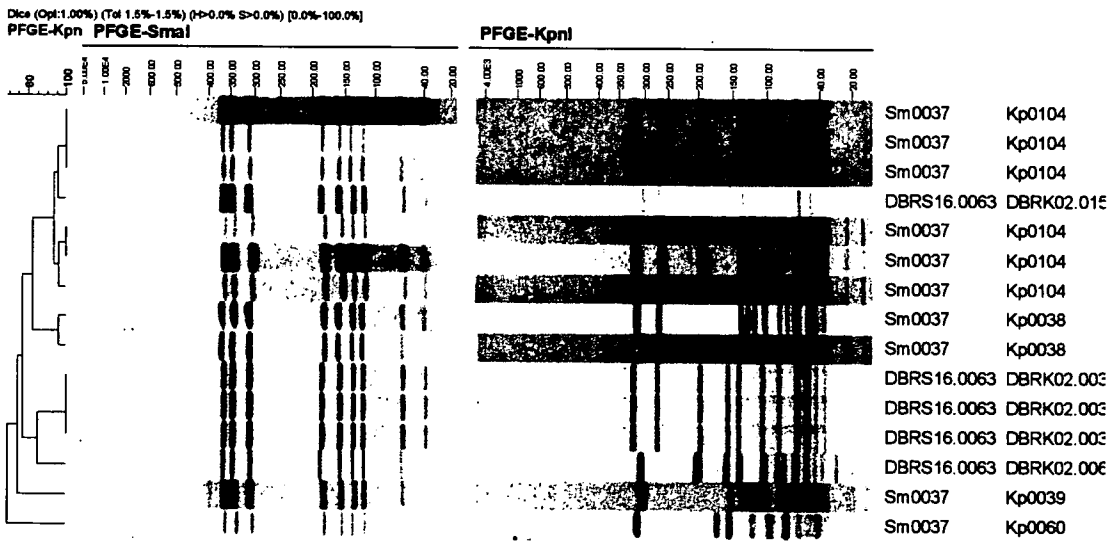
In panel 3, with both enzymes these isolates are indistinguishable. Eight other isolates in NZ database have the same *Sma*I pattern, but not Kp0112 pattern.

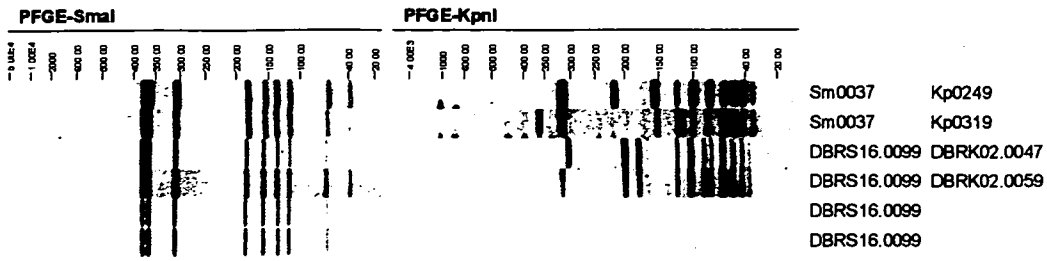


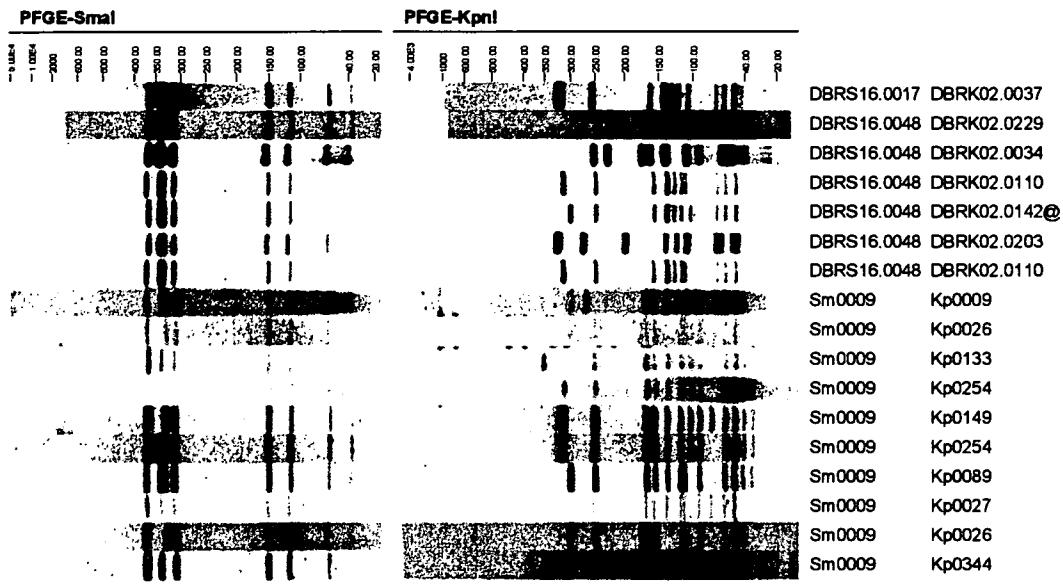
Panel 3 is a good example of indistinguishable SmaI patterns, but four different KpnI patterns, of which only one matches the USA isolate.



The three panels below have very similar *Sma*I patterns are clearly distinguished by the *Kpn*I analysis, illustrating the importance of second enzyme.







Example statistical analysis

For the first comparison given above, of pattern Sm0050 and Kp0085 how likely is a random match? Pattern Sm0050 is fairly common in the database with 75 isolates, yielding a frequency of 0.05, or 1 in 20 chance of random match. Pattern Kp0085 only had 5 representatives for frequency of 0.009, or 1 in 11 chance of random match. Combined match between two enzyme yielded frequency of 0.0004, or 1 in 2259 of random match.

For the most common SmaI pattern in the database (Sm0001), 1 in 10 chance of a match based on current database size, compared for unique pattern 1 in 632. Similarly for the most common KpnI pattern (Kp0037), 1 in 20 chance of match, compared to 1 in 350 for unique SmaI pattern. When combined to two enzyme, unique patterns have 1 in 221,557 of a chance match compared to 1 in 205 for the most common two enzyme match. The potential weight given to these matches could be quite different.

Discussion:

The establishment of standardized databases provides the basis for regional databases for PulseNet Asia Pacific. How rapidly these grow will depend on the goodwill of participants, and will provide evidence of both benefits and will for more automated regional databases.

The genome comparisons highlighted the high frequency of matches between isolates from each country. Statistical analysis tools put forward and used in this study provide an initial guideline for the comparison of PFGE patterns. More robust statistical analysis would require generation of a unified database with combined frequency distributions. For individual databases, the inclusion of zero values for known patterns not in a database would be beneficial.

The importance of two enzymes is confirmed yet again. Several patterns were indistinguishable with one enzyme, but clearly different with the addition of a second. The statistical significance was also markedly increased with two enzymes from as little as 1in 10 with one enzyme, to 1 in 3,528 for two enzymes.

Reference list:

1. Grundmann, H., S. Hori, et al. (2001). "Determining Confidence Intervals When Mesuring Genetic Diversity and the Discriminatory Abilities of Typing Methods for Microorganisms." Journal of Clinical Microbiology 39(11): 4190-4192.
2. Simpson, E. H. (1949). "Measurement of diversity." Nature 163: 688.

Publication list for this work:

1. On SLW, McCarthy N, Miller WG, and Gilpin BJ. (2008) Molecular epidemiology of *Campylobacter* species. In: *Campylobacter* 3rd Edition, Nachamkin I, Szymanski C, Blaser M. (eds), ASM Press, Washington DC.

Title PFGE Standardization and molecular epidemiological study of *Vibrio vulnificus*

Name of researcher ; Bokkwon Lee

Affiliation ; Div. of Enteric Bacterial Infections.

Korean National Institute of Health, Seoul, Republic of Korea

Summary

Vibrio vunificus (*V. vulnificus*) is a gram negative, estuarine bacterium commonly found in coastal waters, and in association with shellfish and fish. This bacterium is known to cause septicemia and severe wound infections in patients with chronic liver diseases or immuno-compromised condition. We carried out the molecular characterization of *V. vulnificus* isolates from human *Vibrio* septicemia cases and from marine environmental specimens in Korea, using ribotyping and pulsed-field gel electrophoresis (PFGE). The ribotyping showed too high discriminating power as *Not*I - digested PFGE patterns. But in many clinical isolates, their *SfiI* digested PFGE patterns were closely related and showed regional relatedness. Among these, some isolates showed same PFGE pattern with those of environmental strains isolated nearby region. Therefore *SfiI* digested PFGE can be a effective molecular epidemiological tool for *V. vulnificus* strains

Purpose:

- PFGE standardization of *Vibrio vulnificus* for PulseNet Korea and Asia.
- Comparison of PFGE patterns of clinical and environmental isolates of *Vibrio vulnificus* obtained in 2000-2005.
- Combinatorial subtype analysis of *V. vulnificus* with PFGE, RAPD, and ribotyping.

Methods:

1) Bacterial Isolates

The Thiosulfate Citrate Bile Salts Sucrose (TCBS) agar, as a selective medium, was used for isolation of *V. vulnificus*. For primary identification, the isolates were characterized by standard physiological and biochemical tests with an API 20E kit (Bio Meriux SA, France). PCR amplification of the *vvhA* gene, specific to *V. vulnificus*, was also used to identify the isolates.

2) **PFGE**

Genetic relatedness between isolates collected *V. vulnificus* sepsis was investigated using pulsed field gel electrophoresis (PFGE) as described by Gautom with modification. Lysed plugs were digested with *Sfi*I (New England Biolabs, Boston, MA, USA), and PFGE was performed in 1% agarose gels in 0.5 × Tris–borate–EDTA buffer at 14 °C using a CHEF mapper apparatus (Bio-Rad, Richmond, CA, USA) at 6 V/cm with linearly ramped switching times of 3.5–50 s for 22 hrs (used *Not*I) or block 1, 2–10 s for 13 h and block 2, 20–25 s for 6 h (used *Sfi*I). PFGE banding pattern analysis was performed using BioNumerics software (version 4.6; Applied Math, Belgium). Analysis of banding patterns was performed using the Dice coefficient with a 1.0% tolerance for the band migration distance. Clustering of the patterns was performed using the un-weighted pair-group method with arithmetic averages.

3) Ribotyping

The automatic RiboPrinter (Dupont Qualicon, Inc., Wilmington, DE) was used for ribotyping as described by Bruce. All strains were inoculated on Tryptic soy agar (DIFCO, Maryland, USA). After overnight growth, colonies were picked from individual agar plates, and resuspended in 200 μl of riboprinter buffer. Thirty microliters of cell suspension was used for Riboprinter analysis after heating was done according to the manufacturer's protocol. Suspended in lysis buffer A and B, transferred to a sample carrier, and loaded into the RiboPrinter. DNA was digested with EcoRI, after which the restriction fragments were separated by electrophoresis, transferred to nylon membranes, and hybridized with a chemiluminescent-labeled DNA probe containing the *E.coli* rrnB rRNA operon. The chemiluminescent pattern was then electronically imaged, processed, and compared to other patterns in the Ribo Printer database. Strains were identified to have the same patterns was ≥ 0.85, and assigned to a specific ribogroup. The Riboprinter automatically assigned identification at a specific genus, species, and strain level.

Results:

1) Ribotyping

We tested ribotyping as less discriminative molecular typing tool. Total 117 isolates – 32 environmental and 85 clinical isolates - were tested and all of the tested isolates are classified into 106 ribotypes. 5 clinical isolates are included in 16-S-6 type, 4 clinical isolates are included in 21-S-3 type, 5 clinical isolates and 1 environmental isolate are included in 19-S-5 type. No more indistinguishable ribotype was found in other isolates.

Table 1. Representative results of PFGE, RAPD, and ribotyping

Strain No.	Molecular type		
	PFGE	RAPD	Ribotype
Clinical			
05-12004	vvn008	D 21	23-S-5
05-12520	vvn013	D 24	23-S-6
05-12561	vvn015	D 26	23-S-7
05-14300	vvn019	D 11	[redacted]
05-14946	vvn025	D 28	23-S-5
05-17101	ND	F 5	15-S-5
05-17102	vvn040	D 3	24-S-3
05-17789	vvn047	D 30	30-S-8
Environmental			
05-09128	vvn001	B 1	24-S-4
05-09129	vvn001	A 3	24-S-4
05-09404	vvn002	D 14	24-S-6
05-09485	vvn004	D 4	24-S-7
05-12123	vvn009	E 3	25-S-7
05-12182	vvn010	C 4	24-S-8
05-12418	vvn012	A 5	25-S-1
05-12858	vvn017	D 9	25-S-2
05-13102	vvn018	B 4	25-S-3
05-13881	vvn018	D 6	[redacted]
05-14332	vvn021	C 1	26-S-1
05-14938	vvn023	C 3	26-S-3
05-15548	vvn027	D 8	26-S-5
05-15668	vvn029	F 9	26-S-6
05-15952	vvn035	D 1	26-S-2
05-16156	vvn038	F 8	21-S-1
05-16963	vvn034	A 1	26-S-7
05-16964	vvn024	B 3	25-S-5
05-17472	vvn043	D 16	[redacted]
05-17473	vvn044	D 2	31-S-4
05-17714	vvn045	F 4	30-S-5
05-17715	vvn046	D 10	30-S-6
05-ente-275	ND	D 18	27-S-5
05-ente-277	ND	F 6	30-S-7
05-ente-280	ND	D 5	27-S-6
05-ente-281	ND	D 23	23-S-4