Introduction:

Vibrio paraheamolyticus is a halophilic gram-negative bacterium, is widely reported as an agent foodborne associated illness in the United States, Taiwan, Japan, and Southeast Asian coutries (1, 2, 3, 4). Most of the reported cases of acute gastroenteristis in outbreaks due to *Vibrio paraheamolyticus* were related to ingestion of primarily raw or improper uncooked seafood (2, 5). In Vietnam, the source of *Vibrio paraheamolyticus* infection was also contaminated food, seafood or seafood originated products (7).

Serologic typing of O and K antigen is commonly used in identification and differentiation of *V. paraheamolyticus*. However, a number of molecular typing methods have been applied for investigating of outbreak and studying epidemiology of *V. paraheamolyticus*. The procedure of pulse-field gel electrophoresis (PFGE) for *V. paraheamolyticus* was first developed in 1986 (8) and has been become one of the most useful tool to assist epidemiologists in investigating outbreak by detecting food-borne disease case clusters and facilitating early identification of common source outbreaks (9). PFGE together with serotyping, were imployed in the study.

Materials and methods:

Specimen collection: The study was conducted between 1997 and 2001 in Khanh Hoa province (Nha Trang) on the coast of central Vietnam. All residents admitted to healthcare centers (commune health centers, policlinics, or hospital), in a case of diarrheal episode were provided with a set of questions regarding the socioeconomic status of his/her household and demographic characteristics. The clinical history and physical findings of the patient were captured through a detailed case-report form. A stool specimen obtained from each patiens was transported in Cary Blair medium (0.16% agar) to the lab at the Institute Pasteur in Nha Trang for bacteriological examination.

Cultivation and isolation:

All stool specimens were examined for *V. paraheamolyticus* by standard methods of common bacterial gastroenteritis pathogens (10). Rectal swabs were suspended in 3ml normal saline solution and inoculated directly onto selective medium (TCBS agar). A portion of each faecal suspension was enriched in 5ml alkaline pepton water containing 0.5% NaCl and incubated overnight at 37°C. Bacterial suspension was then applied again to TCBS medium agar. Presumtive colonies of *V. paraheamolyticus* was determined by the appearance of typical blue –green colonies on TCBS agar. Suspected colonies were selected for further biochemical testing with the identification of oxidase-positive, glucose-nonfermenting, lysine decacboxylase-, ornithine decacboxylase- and urease-positive.

Serotyping:

Confirmed isolations of *V. paraheamolyticus* were serotyped with the use of commercial set of O and K antisera by the appearance of agglutination (Denka Seiken Co., Tokyo, Japan), according to the manufacture's instructions.

PFGE:

NotI – PFGE was performed on all 28 *V. paraheamolyticus* isolates according to the PulseNet Protocol (Center for Disease Control and Prevention, 2004). According to the proposed method of Tenover et al. (11), different designations (e.g., A, B, etc.) were given to the PFGE patterns if they differed by more than 6 bands. The PFGE patterns that differed from a designated type by one to 6 bands were classified as subgroups within the designated pattern and subgroup designations (e.g., A1, A2, etc.) were given accordingly. *V. paraheamolyticus* cultures were grown for 14-18h on Trypticase Soy Agar plates (Difco Laboratories, Detroit, MI, USA) with 5% defibrinated sheep blood (TSA-SB) at 37°C. The cells were havested and, washed and then resuspended in Cell Suspension Buffer (CSB-100 mM Tris: 100mM EDTA, pH 8.0). A portion of this suspension was mixed with an equal volume of 1.5% SeaKem Gold agarose, distributed into molds, and allowed to solidify at 4°C for 30 min. The bacterial cells in the agarose plugs were treated with Cell Lysis Buffer (CLB-50 mM Tris: 50 mM EDTA [pH 8.0], 1.0% N-lauroylsarcosine, 0.1mg of proteinase K per ml) and incubated at 54°C for 1 hour. After lysis, the plug was washed twice with 10 -

15ml preheated sterile Ultrapure water and washed four times with preheated sterile TE buffer (10 mM Tris: 1mM EDTA, pH 8.0).

For restriction endonuclease digestion, one section of the plug (2mm in width) was equilibrated with 20 µl of enzyme buffer supplied by Roche Molecular Biochemicals. 10 U restriction enzyme *NotI* was added to the mixture. The mixture of restriction enzyme, enzyme buffer and plug was incubated at 37^0C for 4h. Restriction fragments were separated by PFGE in 1% agarose gel (Bio-Rad, Hercules, CA, USA) in 0.5X TBE buffer (100 mM Tris: 45 mM Boric acid: 100 mM EDTA, pH 8.0) using the Bio-Rad CHEF-MAPPER apparatus (Bio-Rad Laboratories, Richmond, CA, USA). The initial pulse time of 2 sec was increased linearly to 10 sec over 13 hours at 6 V and 14^0C. After electrophoresis, the gel was stained in a solution of ethidium bromide (0.5µg/mL) for 30 minute. Gels were then washed in distilled water for every 20 mins before being photographed. DNA bands were visualized with short wave UV light (254 nm) and photographed.

The images of DNA band patterns were analyzed for cluster analysis using GelcomparII sofware version 4.0 (Applied Maths) based on the Dice similarity coefficient and unweighted pair-group method with arithmetic averages.

Results and Discussion:

PFGE was performed on 28 *V. paraheamolyticus* isolates . Serotyping of all isolates is belong to O3 : K6 (strains in 1996, 1997), O4: K68 (strains in 1998) and O1 : K25 (strains in 1998 and 1999) (12).

Fig. 1

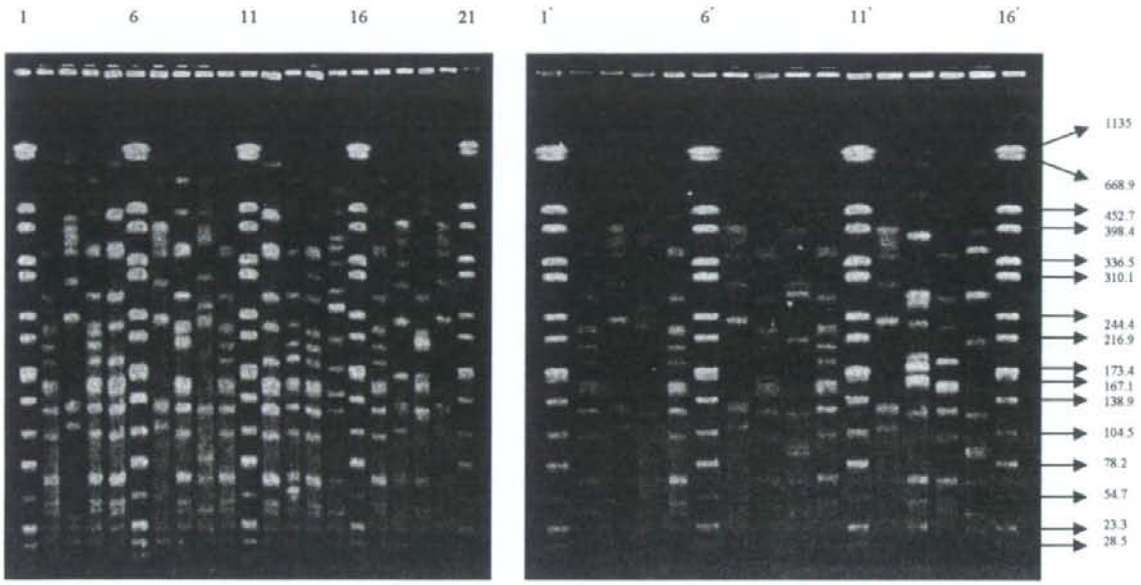
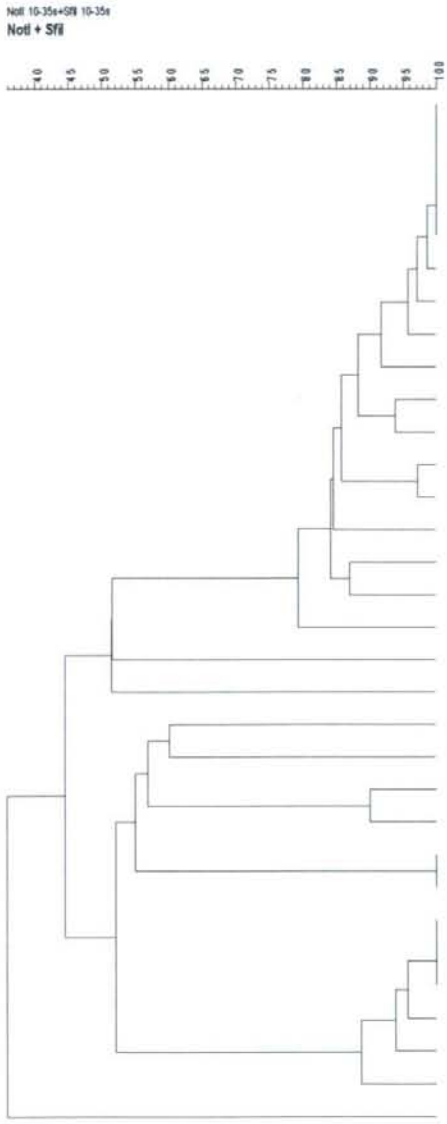


Fig.1. *Not*I-PFGE of selected *V.parahaemolyticus* isolates. Lanes 1, 6, 11, 16, 21 and 1', 6', 11', 21', molecular mass marker of Salmonella serotype Branderup H 9812 with different serotype.

Fig. 2. Dendrogramme and PFGE pattern related following the year of isolates.



Key	year	SourceCounty	PatientName	PatientAg
15816	1999	Khoa Nhi	Nguyen Tan Bao	4
1993	1997	CC luu	Phung Tu Oai	36
4097	1997	CC luu	Nguyen Tuan Vu	8
7843	1997	Dept of Infections	Thai Van Thien	30
791	1996	Khoa Lay	Le Thi Thuy Diem	24
14096	1999	CC luu	Cao Thi Lieu	36
2500	1997	PK 3	Huynh Thi Hoang	45
9192	1998	CC luu	Le Vu Truong	26
14151	1999	Khoa Lay N.Hoa	Doan Thi Trung	34
5417	1997	Khoa Nhi	Ho Hai Quynh Chau	9
775	1997	Khoa Lay	Nguyen Thi Thao Trang	24
15813	1999	Khoa Nhi	Nguyen Van Dinh Thinh	4
15885	1999	Khoa Lay	Nguyen Trong Bao Tram	12
5418	1997	Khoa Nhi	Luu Tran Tuong Vi	16
7264	1998	PK 2	Pham Kim Loan	7
9696	1999	Khoa Lay	Nguyen Thi Thanh Loan	28
1976	1997	Khoa Lay	Nguyen Thi Minh Phuong	33
6699	1997	Khoa Lay	Tran Thi Tuyet	40
1978	1997	CC luu	Tran Van Nghia	21
2559	1997	PK4	Tran Thi Van Khanh	52
7612	1997	CC luu	Nguyen Huu Nhat Anh	7
12035	1999	Ninh Diem	Nguyen Van Hien	25
3005	1997	Khoa Lay	Le Xuan Cuong	30
15884	1999	Khoa Lay	Le Thi Cam Tan	28
6180	1998	CC luu	Truong Ai Loan	55
11653	1999	Ninh Loc	Nguyen Thi Luong	67
1963	1997	CC luu	Le Thi Kim My	16
48608	2001	PK 1	Le Thi Ngan	43
3068	1996	Khoa Lay	Truong Thi Mai	26
2854	1997	CC luu	Tran Van Thu	34
2478	1997	PK 3	Le Song Hao	39
H9812				

Of the 31 isolates of *V. paraheamolyticus* , 16/31 (52%) shared a coefficient of similarity at ≥ 85% .

5/31 (16%) had the same PFGE pattern (similarity at 100%). These isolates were obtained 3 isolates in 1997, 2 isolates of the year 1996 and 1 isolate in 1999.

3/31 (similarity at 100%) obtained isolates of 1999, 2001 and 1997.

Almost 50% of strains isolated were belong to pandemic strains that emerged around 1996 and spread to many countries (12).

Obviously a transition of major serovars occurs among the pandemic strains represented by emergence of O3:K6 in 1997, O4:K8 in 1998 and O1:K25 in 1998 and 1999.

Acknowledgement

Thanks for grant support of the National Institute of Infectious Diseases (NIID), Tokyo, Japan. Thanks for Association of public health Laboratories (APHL) and Centers for Disease control and prevention (CDC), Atlanta, George, USA for technical training.

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Title: Distribution of Genotypes and Antimicrobial Resistance of *Salmonella* Typhi and Paratyphi A : An intercountry collaborative study (Malaysia, Taiwan and Vietnam)

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National Institute of Infectious Diseases
Tokyo, Japan

CO-RESEARCHERS: Prof. Dr Thong Kwai Lin
University of Malaya, Kuala Lumpur
Malaysia.

STUDY FACILITY: BioMedical Science and Molecular Typing Laboratory, Institute of Postgraduate Studies, University of Malaya, Kuala Lumpur, Malaysia

Summary:

The project will aim at inter-country collaborative study on infectious bacterial pathogens. The scope of study includes epidemiological investigation of diseases by comparison of genotypes (PFGE and MLVA) and antimicrobial resistance profiles of bacterial strains and evaluation of new genotyping methods. In the first and second year, the study will work on the investigation of distribution of (PFGE and MLVA genotypes) and antimicrobial resistance profiles of *Salmonella enterica* ser. Typhi and Paratyphi A isolates in Malaysia, Taiwan and Vietnam. In the third year of the study, the investigators will work on the evaluation of usefulness of MLVA method for *Shigella flexneri* of various serotypes.

Purpose:

1. To promote inter-country collaboration on study of epidemiology of bacterial infectious diseases.
2. To obtain epidemiological trend on antimicrobial resistance and strain distribution of *Salmonella* Typhi, *S.* Paratyphi A, Shgiella *flexneri* in the participant countries.
3. To evaluate the usefulness of MLVA methods on various serotypes.

Methods:

1. Collection of bacterial isolates. Each participant country provides 50-100 isolates of *Salmonella* Typhi and *S.* Paratyphi A. For each isolate, the isolate information and results of testing will be saved in Excel file. The Excel file will record code of isolate, isolation country, isolation province/state/county, isolation date, and traveling history, results of antimicrobial susceptibility

testing, code of PFGE pattern, code of MLVA, VNTR profile.

2. Antimicrobial susceptibility testing. The antimcirobial susceptibility of isolates will be determined by disk diffusion method or microbroth dilution method followed Clinical and Laboratory Standards Institute (CLSI) guideline. For disk diffusion method, E. coli ATCC25922 will be used as the QC strain.
3. PFGE analysis. PFGE will be carried out using the standardized PulseNet PFGE protocol. *S.* Typhi and Paratyphi will be analyzed with XbaI;
4. MLVA analysis. All the *S.* Typhi and *S.* Paratyphi isolates will be analyzed by the MLVA method (with 35 VNTR loci) developed in Dr. Chien-Shun Chiou's lab. *Shigella flexneri* will be analyzed by the MLVA method (36 VNTR loci) developed in Dr. Chien-Shun Chiou's lab.

Results and Discussion

1) Antimicrobial susceptibility profiles

The majority of the S.Typhi strains tested were drug susceptible except for 5 strains.

2) PFGE analysis

To establish the relationship between the isolates of the strains were analyzed by pulsed field gel electrophoresis of *Xba*I-digested fragments. Sixty two profiles were obtained from 197 S. typhi. Strains from the outbreak cases were homogenous while those from sporadic cases were more heterogenous. There was one predominant genotype that persisted in the highly endemic state of Kelantan.

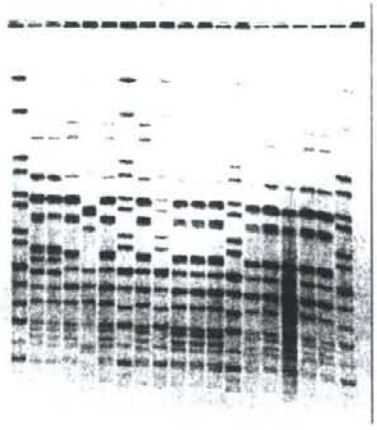


Fig 1. Representative PFGE profiles of S. Typhi

3. MLVA analysis

We are still setting up facilities for MLVA work together with Dr Chiou from Taiwan. Dr. Chiou has provided the newly developed MLVA protocol. As we don't have the full facilities to conduct MLVA, we have sent extracted DNA of S.Typhi, S. Paratyphi A and Shigella sonnei, Shigella boydii, Shigella flexneri to Dr Chiou. We are in the process of acquiring BioNumerics to analyse the data.

While the facilities for proper MLVA work is being set up, we attempted to initiate VNTR analysis using published TRs (Liu et al, 2005). VNTR was evaluated for its usefulness in the molecular typing of *Salmonella enterica* serovar Typhi isolates from outbreak and sporadic cases of typhoid fever in Malaysia. Multiplex PCR assay displayed diversity based on four VNTR loci studied, with total allele numbers ranging from 2 to 19 and Nei's diversity (*D*) values ranging from 0.04 to 0.93. Sequence analysis of individual alleles confirmed the presence of VNTRs between isolates and identified 42 VNTR profiles. Our results revealed that the copy number of repeats in CT18 strain of *Salmonella* Typhi was highly correlated with the *D* value (R^2 = 0.57) or the number of alleles (R^2 = 0.87) observed across diverse isolates.

(Appendix 1, Table 1) Hence, VNTR analysis appears capable of both identifying *S.* typhi isolates from well-characterized outbreaks and successfully classifying more distant relationships. The study demonstrates that VNTR provides a rapid, highly discriminatory, and reproducible typing method for epidemiological surveillance and outbreak investigation of *S.* Typhi strains.

Publication

Chiou, CS, Watanabe, H., Wang,YW, Terajima J., Thong,KL, Phung,DC, and Tung, SK. 2009. Utility of Multilocus variable number tandem repeat analysis as a molecular tool for phylogentic analysis of Shigella sonnei. J. Clin. Microbiol. 47: 1149-1154

Manuscript in Preparation

Thong KL., S. Rezanejad, Teh CSJ, Chiou, CS, H. Watanabe Molecular subtyping of *Salmonella enterica* serovar Typhi by Variable-Number Tandem Repeats.

Thong, KL. et. al. Molecular restriction of S.Typhi in a highly endemic state in Malaysia.

Appendix 1

Table 1 VNTR profiles of S.Typhi

VNTR Profile (TR1/TR2/TR3)	Strain No/year	VNTR Types	TR1 Types	TR2 Types	TR3 Types
7 *x* / 20.9 *x* / 1.3 *x*	TP43/98	MY1	A1	B1	C1
7 *x* / 18.9 *x* / 1.3 *x*	TP40/98	MY2	A1	B2	C1
7 *x* / 18.9 *x* / 2.3 *x*	TP41/98	MY3	A1	B2	C2
6 *x* / 18.9 *x* / 1.3 *x*	TP34/98, TP46/98	MY4	A2	B2	C1
15 *x* / 5.9 *x* / 2.3 *x*	TP128/99	MY5	A3	B3	C2
16 *x* / 25.9 *x* / 2.3 *x*	TP130/99	MY6	A4	B4	C2
4 *x* / 9.9 *x* / 2.3 *x*	TP160/99	MY7	A5	B5	C2
4 *x* / 10.9 *x* / 2.3 *x*	TP172/99	MY8	A5	B6	C2
5 *x* /28.9 *x* / 2.3 *x*	TP247/99	MY9	A6	B7	C2
11 *x* / 16.9 *x* / 3.3 *x*	TP1/00	MY10	A7	B8	C3
9 *x* / 19.9 *x* / 3.3 *x*	TP2/00	MY11	A8	B9	C3
8 *x* / 5.9 *x* / 1.3 *x*	TP5/00	MY12	A9	B3	C1
5 *x* / 16.9 *x* / 2.3 *x*	TP118/00	MY13	A6	B8	C2
5 *x* / 19.9 *x* / 2.3 *x*	TP168/00	MY14	A6	B9	C2
10 *x* / 14.9 *x* / 1.3 *x*	TP2/01	MY15	A10	B10	C1
9 *x* / 13.9 *x* / 1.3 *x*	TP3/01	MY16	A8	B11	C1
12 *x* / 17.9 *x* / 2.3 *x*	TP5/01, ST142/05	MY17	A11	B12	C2
12 *x* / 9.9 *x* / 2.3 *x*	TP38/01	MY18	A11	B5	C2
5 *x* / 13.9 *x* / 2.3 *x*	TP58/01	MY19	A6	B11	C2
10 *x* / 18.9 *x* / 1.3 *x*	TP20/02	MY20	A10	B2	C1
5 *x* / 0 *x* / 1.3 *x*	TP24/02	MY21	A6	B13	C1
10 *x* / 10.9 *x* / 2.3 *x*	TP35/02, TP90/04	MY22	A10	B6	C2
12 *x* / 16.9 *x* / 2.3 *x*	TP41/02	MY23	A11	B8	C2
12 *x* / 21.9 *x* / 2.3 *x*	TP43/02	MY24	A11	B14	C2
5 *x* / 26.9 *x* / 2.3 *x*	TP195/03	MY25	A6	B15	C2

5 x / 32.9 x / 2.3 x	TP282/03	MY26	A6	B16	C2
12 x / 24.9 x / 2.3 x	TP322/03	MY27	A11	B17	C2
11 x / 17.9 x / 2.3 x	TP323/03, ST117/05	MY28	A7	B12	C2
12 x / 15.9 x / 2.3 x	TP329/03	MY29	A11	B18	C2
10 x / 12.9 x / 2.3 x	TP78/04, ST37/06	MY30	A10	B19	C2
10 x / 9.9 x / 3.3 x	TP80/04	MY31	A10	B5	C3
12 x / 14.9 x / 2.3 x	TP85/04	MY32	A11	B10	C2
6 x / 9.9 x / 2.3 x	TP108/04	MY33	A2	B5	C2
12 x / 18.9 x / 2.3 x	ST156/05	MY34	A11	B2	C2
- / 0 x / 2.3 x	ST189/05	MY35	A12	B13	C2
- / 17.9 x / 2.3 x	ST208/05	MY36	A13	B12	C2
10 x / 10.9 x / 1.3 x	ST2/06	MY37	A10	B6	C1
18 x / 0 x / 2.3 x	ST3/06	MY38	A14	B13	C2
12 x / 13.9 x / 2.3 x	ST13/06	MY39	A11	B11	C2
13 x / 15.9 x / 2.3 x	ST16/06	MY40	A15	B18	C2
10 x / 12.9 x / 1.3 x	STS314/07, STB4738/07, STB4571/07, STS334/07	MY41	A10	B19	C1
12 x / 0 x / 2.3 x	STPH014/05	MY42	A11	B13	C2

Dr Thong Kwai Lin

Malaysia.

Validation of new *Listeria* PFGE protocol and analysis of recalcitrant isolates

Name of researcher: Brent Gilpin

Affiliation: Institute of Environmental Science & Research Limited, New Zealand

Summary:
PulseNet USA have recently modified the *Listeria* PFGE protocol to include more lysozyme, more proteinase K and an increased temperature of lysis. We evaluated this protocol on isolates previously analysed and found that the new protocol produced PFGE images of at least as good a quality as the previous method. We also tested isolates that had previously proven to be recalcitrant to PFGE analysis and found that this new protocol produced high quality images with these isolates.

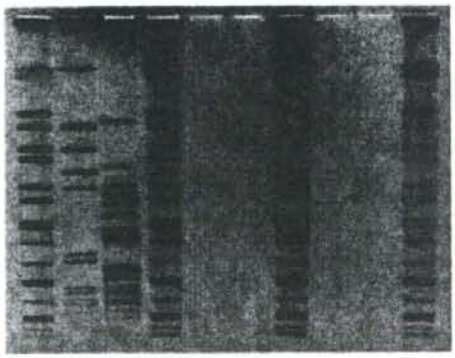
Surprisingly some of these isolates that previously didn't produce PFGE images, had a PFGE profile indistinguishable to that previously obtained from other isolates using the standard protocol. This genotype is common worldwide. Analysis of isolates from these two groups is ongoing and involves examination for the presence of plasmids, virulence elements, antibiotic resistance, and cell wall analysis. To date no differences have been identified.

Purpose:

Validate new protocol, analyse isolates that previously couldn't be typed by PFGE, and identify isolates for further characterization by other methods.

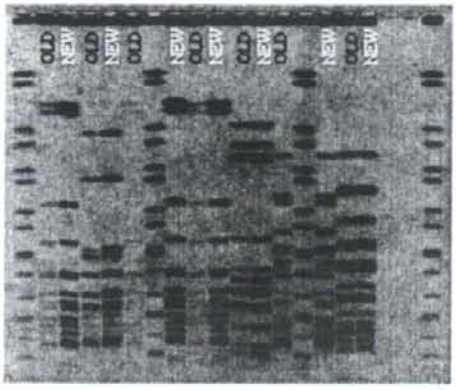
Results

Analysis of a number of isolates using the original protocol yielded no bands as illustrated in lanes 5, 6, 7 and 8 of the figure below.



These isolates when repeated with the new protocol yielded PFGE patterns that could be analysed.

The second gel figure illustrates a set of isolates analysed with the old protocol and with the new. As can be seen, the new protocol produced much stronger banding patterns.



Interestingly several isolate patterns which could only be generated with the new protocol were indistinguishable to patterns which previously could be generated routinely with the old protocol.

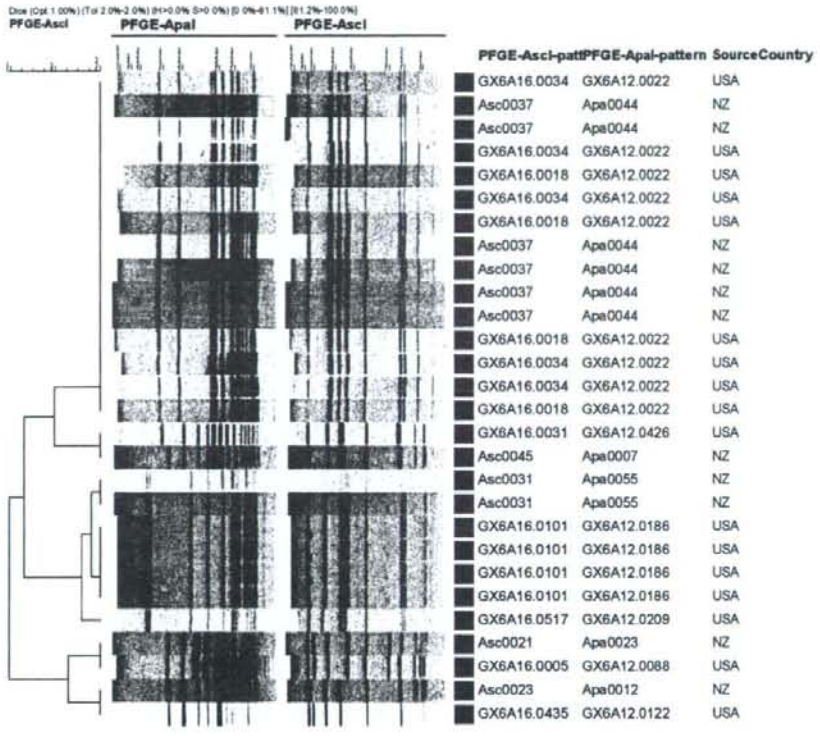
This pattern, (lanes 2 and 3) of the figure above is actually the most frequent pattern in the New Zealand database and is also common in the USA database

Characterisation of isolates

Analysis of isolates from these two groups is ongoing and involves examination for the presence of plasmids, virulence elements, antibiotic resistance, and cell wall analysis. To date no differences have been identified between the two groups of isolates.

Comparison of isolates

344 isolates from New Zealand and 113 isolates from USA were compared using BioNumerics 5.1. Six pattern were very similar, if not indistinguishable when analysed using two enzymes. A selection of these are illustrated in the figure below.



Discussion:

The new *Listeria* protocol is robust and produces PFGE images with all isolates tested including isolates which previously couldn't be analysed. The PulseNet USA modifications to the *Listeria* PFGE protocol included more lysozyme, more proteinase K and an increased temperature of lysis. The new protocol produced PFGE images of at least as good a quality as the previous method. We also tested isolates which had previously proven to be recalcitrant to PFGE analysis and found that this new protocol produced high quality images with these isolates.

Surprisingly some of these isolates that previously didn't produce PFGE images, had a PFGE profile indistinguishable to that previously obtained from other isolates using

the standard protocol. This genotype is common worldwide. Analysis of isolates from these two groups is ongoing and involves examination for the presence of plasmids, virulence elements, antibiotic resistance, and cell wall analysis. To date no differences have been identified.

Reference list:

1. Listeria protocol: http://www.pulsenetinternational.org/protocols/protocols.asp

Publication list for this work:

1. Gilpin, Robson (in preparation) Strains of *Listeria* recalcitrant to PFGE.

Title: **Application of Multilocus variable-number tandem repeat analysis for typing of *Shigella* spp.**

Name of researcher; **Bok K won Lee**
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Summary:

Shigellosis has been prevalent in the Republic of Korea since 1998. Even with its high incidence rates over the short period, the genetic relatedness of *Shigella* isolates using Pulse-field Gel Electrophoresis (PFGE) is not completely established. In this reason, we set up the MLVA typing method to complement the PFGE, and this next generation molecular typing method identified as more strong discriminative method than PFGE. We tested 26 VNTR loci, and among these, most variable VNTR loci SS01, SS03, SS06, and SS10 were enough to used as new molecular typing tool as complementation for PFGE to investigate the genetic relatedness between *S. sonnei* isolates in Korea..

Purpose:

1. Development of a next generation molecular typing method to identify *Shigella* spp. and its application
2. Validation of the usefulness of multilocus variable-number tandem repeat analysis (MLVA) for typing of *S. sonnei* isolates in Korea
3. Database build-up with MLVA profiles of *S. sonnei*

Methods:

Bacterial Isolates

A retrospective analysis was performed for *S. sonnei* isolates, those collected by public health network of Korea Centers for diseases Control from 1998 to 2004, using the PFGE database of the Korean National Institute of Health (KNIH). A total **62** isolates were selected based on their *XbaI*-digested PFGE type and epidemiological history to compare the discriminatory powers of MLVA and PFGE.

The MacConkey agar, as selective media, used for isolation of *Shigella* spp., For primary identification, the isolates were characterized by conducting standard physiological and biochemical tests with an VITEK GNI plus card and VITEK system (Bio Meriux VITEK, MO, USA). Grouping and Typing antisera (DenkaSeiken, Tokyo, Japan) used for the serological identification of *Shigella sonnei.*

Preparation of crude bacterial DNA.

S. sonnei isolates were plated onto tryptic soy agar and incubated overnight at 37°C. A loopful of bacterial colony was suspended in 100ml of 10mM Tris in an Eppendorf tube, and boiled for 5 min. After centrifugation at 13,000rpm for 10 min, the supernatant was used.

MLVA

The information about selected VNTR loci and the sequence of primer sets for PCR amplification of the 26 VNTR loci for MLVA of *S. sonnei* was kindly provided from Dr. Chiou in Taiwan CDC (Liang et. al.), and synthesized from Applied BioSystems (Foster City, CA).

The PCR conditions were slightly modified from Liang's method. Briefly, M1 to M6 were performed with a denaturing step at 94°C for 5 min, followed by 30 cycles of amplification at 94°C for 45 s, 55°C for 50 s, and 72°C for 60 s. M7 was performed under the same conditions except that the annealing temperature was set at 64°C.

Before the fragment analysis, the amplicons were diluted in de-ionized distilled water in a 1: 10 or 1:100 ratio. For the capillary electrophoresis, 1ul of diluted solution, 1ul of GeneScan 500 LIZ standard (Applied Biosystems), and 8.9 ㎕ Hi-Di-formamide were mixed and denaturated at 95℃. Capillary electrophoresis was performed by ABI Prism 3730xl genetic analyzer.

Data were collected, and the lengths of the amplicons were determined with GeneScan data analysis software, v. 2.0 (Applied Biosystems). All amplicons of different lengths from each locus were subjected to nucleotide sequence determination to verify the repeat sequence and the number of repeat units in the amplicons.

Data analysis of MLVA

The number of repeat units for each locus was saved as "character type" data in BioNumerics software (version 5.1; Applied Maths, Kortrijk, Belgium) and then subjected to cluster analysis using the minimum spanning tree method.

Results:

1) Set up of the reaction condition for MLVA

The optimal condition for 7 multiplex PCR reactions was tested. The optimal reaction condition of PCR set M1, M2, M3, M4, M5, and M6 was same as previously published method (Liang et. Al.), but for M7 set, the annealing temperature was modified to 64°C. The optimal results were obtained by using Platinum *Taq* (Invitrogen, Carlsbad, CA).

For the fragment analysis, the optimal amplification cycle was 30 cycle and the optimal dilution scale of amplicon was 1:10 (M1, M2, M3, M4, and M5 set) and 1:100 (M6 set)

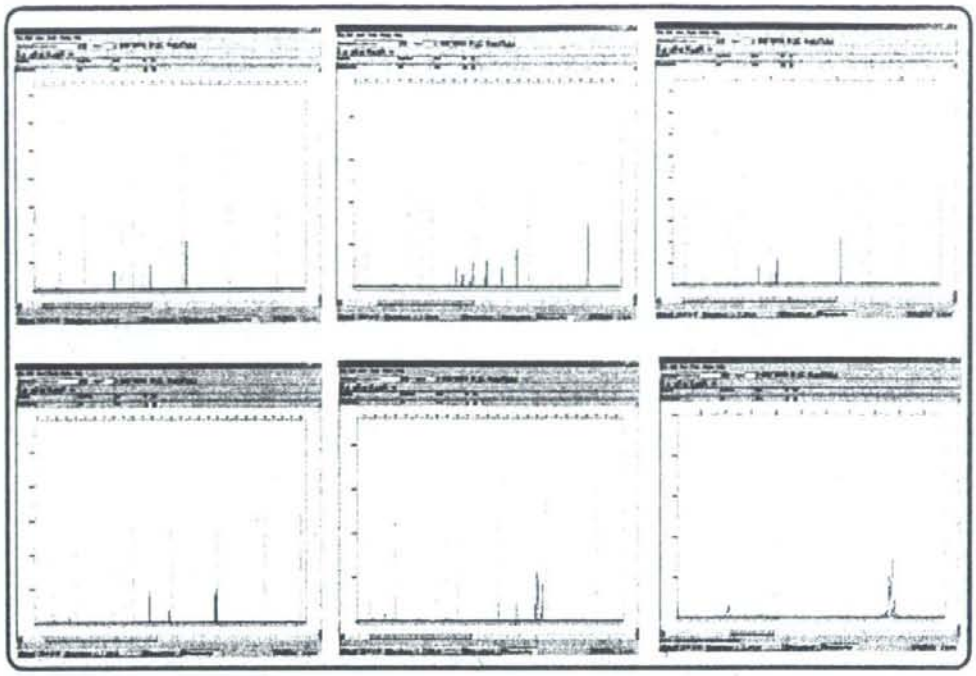

Figure 1. The results for fragment analysis of 6 multiplex PCR set : from upper left, M1, M2, M3, M4, M5 and M6 set.

2) Variability of each VNTR locus

A total 62 of *S. sonnei* isolates were tested. From the MLVA result of these 62 isolates, VNTR loci SS06 and SS01 were most variable, the variation rate were 16.1% and 14.5% respectively. The next variable VNTR loci were SS3 and SS10, and the variation rate was 9.7% both. Among VNTR 26 loci, 6 loci showed more than 6% of variation rate and 9 loci have no variation (table 1). VNTR locus SS26 was not amplified in all the 62 tested isolates.

In the previous report (Liang et. Al.), 4 VNTR loci combination (SS01, SS03, SS06, and SS09) and 8 VNTR loci combination (SS01, SS03, SS06, SS09, SS10, SS11, SS12 and SS13) MLVA have enough discriminatory power compare to PFGE, but, from this study, for the 4 VNTR loci combination for Korean *S. sonnei* isolates, combination of SS01, SS03, SS06, and SS10 will have stronger discriminatory power.