APPLICATION OF RIBOSOMAL RNA GENE RESTRICTION PATTERNS ANALYSIS AND PULSED-FIELD GEL ELECTROPHORESIS IN DISTINGUISHING *SALMONELLA WELTEVREDEN* ISOLATES IN MALAYSIA

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Abstract. A representative sample of 20 isolates of *Salmonella weltevreden* strains from stool cultures of patients admitted at the University Hospital, Kuala Lumpur, Malaysia were analyzed. All the strains were susceptible to ampicillin, ceftriaxone, ciprofloxacin, chloramphenicol, tetracycline, trimethoprim, gentamicin and co-trimoxazole. Ribosomal RNA gene restriction pattern analysis of *Pst*I-digested DNA gave three ribotypes while pulsed-field gel electrophoresis (PFGE) analysis of *Xba*I-digested DNA gave ten distinct profiles. PFGE was more discriminative than ribotyping in distinguishing the strains. The majority of the strains analyzed were very closely related with similarity coefficient values ranging from 0.8 to 1.0. Both PFGE and ribotyping could distinguish one of the strains which was obtained from a patient following a bone marrow transplant for β-thalassemia major, indicating that this particular strain was unrelated to the rest of the strains from patients with acute gastroenteritis.

INTRODUCTION

Acute gastroenteritis caused by *Salmonella* spp continues to be a public health problem in many parts of the world especially in the developing countries, including Malaysia. The most common agent associated with non-typhoidal salmonellosis is *Salmonella enteritidis*. However, outbreaks of gastroenteritis caused by *Salmonella weltevreden* are rarely reported. In terms of its distribution, *S. weltevreden* was the fourth commonest serotype isolated (17/173 or 9.8%) from the University Hospital, Kuala Lumpur (Lee *et al*, 1998). In another survey, Koe *et al* (1991) in their study of 97 children with acute gastroenteritis, showed that the most common pathogen isolated was food poisoning *Salmonella* spp (25.8 %) and among these strains, 15% (3/20) were *S. weltevreden*. *S. weltevreden* was the second most common serotype isolated from clinical specimens in Malaysia (*ie* 326/1154) (IMR Annual Report, 1993). In addition, *S. weltevreden* also has one of the widest zoological distributions, which include cattle, beef, mutton, duck, prawn, dog, monkey and rats. In the past decade, molecular-based methods have been widely applied to study the genetic relatedness of pathogens to track the source of infections, and to investigate the extent of genetic variations among isolates belonging to the same clone. As there are no published reports for this species, the objective of the work was to apply the technique of ribotyping and pulsed-field gel electrophoresis to study the extent of genetic variation of clinical strains of *S. weltevreden* from sporadic cases of acute gastroenteritis in Malaysia.

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MATERIALS AND METHODS

Bacterial strains

A total of 20 clinical isolates of *S. weltevreden* from feces or rectal swabs obtained from different individuals with sporadic cases of gastroenteritis admitted to the University Hospital, Kuala Lumpur were used in this study. The organisms were isolated, maintained and identified using standard methods at the Medical Microbiology Laboratory, University Malaya Medical Center. All isolates were tested for antibiotic sensitivity by standard disc diffusion procedures.

Preparation of DNA

Chromosomal DNA for pulsed-field gel electrophoresis (PFGE) analysis and ribotyping was prepared in agarose plugs as previously described

(Thong and Pang, 1996). Very briefly, pelleted cells from an overnight culture were suspended in 0.5 ml of SB solution (10 mM Tris-HCl pH 7.5, 1 M NaCl). The suspension was then mixed with an equal volume of 1.5% low melting point agarose (Incert agarose, FMC Bioproducts, Rockland, ME, USA) in plastic moulds. The agarose blocks/plugs containing the bacterial cells were transferred to Bijou bottles containing 2 ml of lysis buffer (6 mM Tris-HCl, pH7.5, 100 mM EDTA, pH 8.0 1 M NaCl, 0.5% Brij 58, 0.2% sodium deoxycholate, 0.5% sodium lauryl sarcosine, 1 mg/ml lysozyme 1 U/ml RNase) and incubated overnight at 37ºC. The plugs were then subjected to overnight digestion with Proteinase K (1mg/ml) followed by extensive washing with TE buffer (10 mM Tris-HCl, 10 mM EDTA, pH 7.4).

Ribotyping, Southern blot hybridization and gene probe

Slices of DNA-containing agarose plugs were digested to completion with 20U of *Pst*I. The restricted DNA fragments were separated in a 0.8% agarose gel in TBE buffer at 70 V for 27 hours, stained with ethidium bromide (1 μg/ml), and then transferred to a Hybond-N nylon membrane by using the method of Southern as described in Sambrook *et al* (1989). Lambda *Hind*III was used as a DNA size standard. The 16S rRNA gene probe was generated by PCR from *S. weltevreden* genomic DNA with primers F1 (5′-GAGTTTGATCCTGGC TCAG-3′) and R13 (5′-AGAAAGGAGGTGATCC AGCC-3′) (Weisburg *et al*, 1991). The 1.5 kb PCR product was excised from the gel after electrophoresis through 1% Gibco BRL Ultrapure Low Melting Point Agarose in TAE buffer and purified with Geneclean II (BIO 101, CA, USA). Probe was labeled by the nonradioactive ECL system (Amersham International, Amersham, UK). Hybridization and signal detection were carried out strictly according to the manufacturer's protocol.

PFGE analysis

Slices of DNA-containing agarose plugs were digested overnight with 20U of *Xba*I (Promega) at 37ºC and then electrophoresed on a CHEF-DR II/III system (Bio-Rad laboratories) for 26 hours at 6V/cm, with a ramped pulsed time of 2 to 40 seconds at 12ºC. Lambda DNA concatemer PFG marker was used as a DNA size standard. The gel was stained with ethidium bromide (1 μg/ml; Sigma) for 15 minutes, destained in distilled water for 15 minutes, and photographed under UV illumination.

Data analysis

Dice coefficients of similarity were calculated to compare the macrorestriction patterns. Clustering was based on the unweighted pair group average method (UPGMA) and was performed with GelCompar; Applied Maths, Kartrijk, Belgium.

RESULTS

All the strains were susceptible to ampicillin, ceftriaxone, ciprofloxacin, chloramphenicol, tetracycline, trimethoprim, gentamicin and co-trimoxazole. All the DNA samples could be restricted by *Pst*I and ribotyping was carried out to simplify the interpretation of the complex banding patterns. Three *Pst*I ribotypes (designated A to C) were observed among the 16 *S. weltevreden* isolates (Fig 1A). The *Pst*I ribotypes consisted of five to seven fragments with sizes ranging from 6.5 kb to 23 kb. The most common pattern was ribotype A (14/16 isolates) while ribotypes B and C were each represented by a single strain. Ribotype A differed from ribotype C by a single band (F = 0.96).

Macrorestriction analysis of total genomic DNA digested with *Xba*I yielded ten different PFGE patterns (designated X1-X10). The total number of fragments ranged from 11 to 16 with sizes ranging from 48.5 kb to 533.5 kb which could be easily discerned and analyzed (Fig 1B). Pulsed-field profile (PFP) X1 and X2 were the most common patterns, each represented by six out of twenty strains (X1 and X2 differed in only one DNA band, F=0.96). PFPs X3-X10 were each represented by a single strain. Wide genetic variations were detected among these isolates as evidenced by the wide range of similarity indices from 0.44 to 1.0 (Fig 2A). A dendrogram, based on the matrix of F values, was constructed using a clustering algorithm of the unweighted pair group arithmetic means method (UPGMA). The dendrogram showed two major clusters of *S. weltevreden* (Fig 2B). Based on 85% similarity, the majority of the *S. weltevreden* (16/20) fell into one cluster. Strain SW11 was distinctly different from the rest of the strains.

The ribotypes and the PFGE patterns were stable and 100% reproducible when the analysis was repeated three times. Both ribotyping and PFGE could distinguish SW9 and SW11 *ie* SW9 (ribotype B, X6 and SW11 (ribotype C, X8). PFGE was more discriminative and was able to further

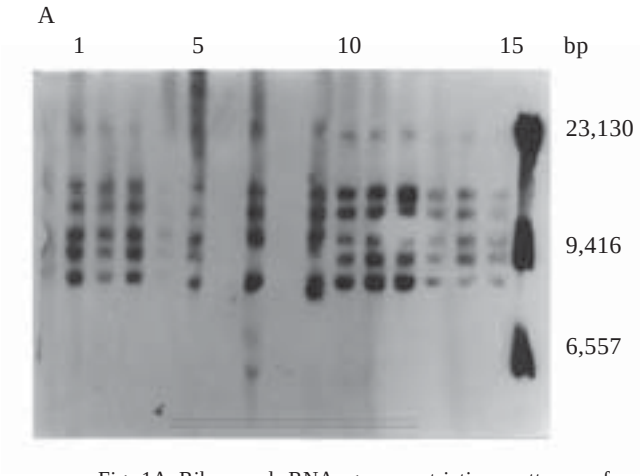

Fig 1A–Ribosomal RNA gene restriction patterns of *S. weltevreden* DNA digested with *Pst*I. Lanes 1-3, lane 5: SW1,SW3, SW4 and SW7 (ribotype A); lane 7: SW9 (ribotype B); lane 9:SW11 (ribotype C); lanes 10-15: SW12,SW14,SW16, SW20, SW22 (ribotype A); lane 16:Lambda *Hind*II DNA marker. Numbers at the side indicate marker bands.

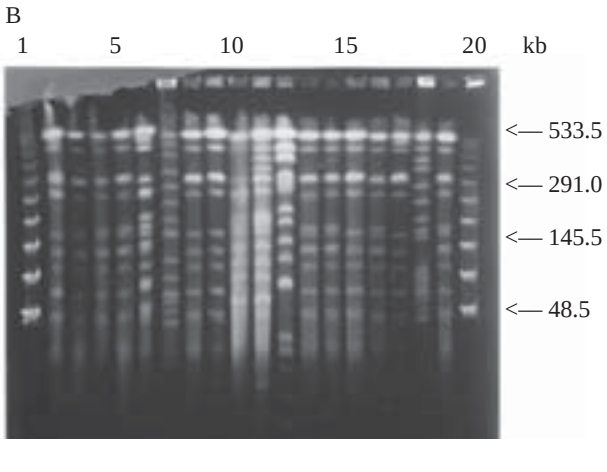

Fig 1B–*Xba*I-PFGE patterns of 18 sporadic *S. weltevreden* isolates. Lanes 1, 20: Lambda DNA concatemer PFGE marker; Lanes 2-19: SW1 (X1), SW 2 (X2), SW3(X2), SW4 (X2), SW5 (X4), SW6 (X5), SW 7(X1), SW 8(X1), SW9(X6), SW10 (X7), SW11 (X8), SW12 (X2), SW13 (X3), SW14 (X1), SW16 (X2), SW 17(X1), SW18 (X9) and SW22 (X10). Electrophoretic conditions: 2-40 seconds, 26 hours, 200 V, 12ºC.

Table 1
Date of isolation, *Pst*I ribotypes and *Xba*I-PFPs of *Salmonella weltevreden* from sporadic cases of gastroenteritis.

Strains	Date of isolation	Comments	*Pst* I-ribotype pattern	*Xba* I-PFGE pattern
SW1	6.3.97	AGE[a]	A	X1
SW2	2.9.97	AGE	A	X2
SW3	2.6.97	Fever, AGE	A	X2
SW4	1.7.97	Inflammation of the ileum	A	X2
SW5	5.6.97	AGE	A	X3
SW6	12.6.97	AGE	ND[b]	X5
SW7	12.6.97	Dysentery	A	X1
SW8	7.3.97	AGE	A	X1
SW9	8.10.97	AGE	B	X6
SW10	31.7.97	AGE	ND[b]	X7
SW11	16.5.97	β-thalassemia major post BMT	C	X8
SW12	6.7.96	Febrile fits with diarrhoea	A	X2
SW13	23.9.96	AGE	A	X3
SW14	23.1.96	AGE	A	X1
SW16	29.1.96	AGE	A	X2
SW17	4.11.97	AGE	ND[b]	X1
SW18	19.1.96	AGE	ND[b]	X9
SW20	5.9.96	AGE	A	X1
SW22	16.10.97	AGE	A	X10
SW23	1.11.97	Pregnancy with diarrhea	A	X2

[a]AGE=Acute gastroenteritis
[b]ND=Not determine

A

sw9	100																		
sw10	93.3	100																	
sw2	92.3	85.7	100																
sw3	92.3	85.7	100	100															
sw4	92.3	85.7	100	100	100														
sw12	92.3	85.7	100	100	100	100													
sw16	92.3	85.7	100	100	100	100	100												
sw23	92.3	85.7	100	100	100	100	100	100											
sw13	88.9	89.7	96.0	96.0	96.0	96.0	96.0	96.0	100										
sw1	88.0	81.5	96.7	96.7	96.7	96.7	96.7	96.7	91.7	100									
sw7	88.0	81.5	95.7	95.7	95.7	95.7	95.7	95.7	91.7	100	100								
sw8	88.0	81.5	95.7	95.7	95.7	95.7	95.7	95.7	91.7	100	100	100							
sw14	88.0	81.5	95.7	95.7	95.7	95.7	95.7	95.7	91.7	100	100	100	100						
sw17	88.0	81.5	95.7	95.7	95.7	95.7	95.7	95.7	91.7	100	100	100	100	100					
sw20	88.0	81.5	95.7	95.7	95.7	95.7	95.7	95.7	91.7	100	100	100	100	100	100				
sw22	86.7	81.3	85.7	85.7	85.7	85.7	85.7	85.7	89.7	81.5	81.5	81.5	81.5	81.5	81.5	100			
sw5	80.0	75.0	78.6	78.6	78.6	78.6	78.6	78.6	75.9	74.1	74.1	74.1	74.1	74.1	74.1	75.0	100		
sw6	73.3	75.0	64.3	64.3	64.3	64.3	64.3	64.3	69.0	66.7	66.7	66.7	66.7	66.7	66.7	62.5	68.8	100	
sw18	80.0	68.8	50.0	50.0	50.0	57.1	50.0	50.0	55.2	44.4	44.4	44.4	51.9	51.9	51.9	62.5	62.5	50.0	100
sw11	50.0	60.0	46.2	46.2	46.2	46.2	46.2	46.2	51.9	48.0	48.0	48.0	48.0	48.0	48.0	46.7	53.3	53.3	46.7	100

Fig 2A–Matrix of Dice coefficient (F) for *S. weltevreden* isolates. The original F values were multiplied by 100 to give similarity percentages.

B

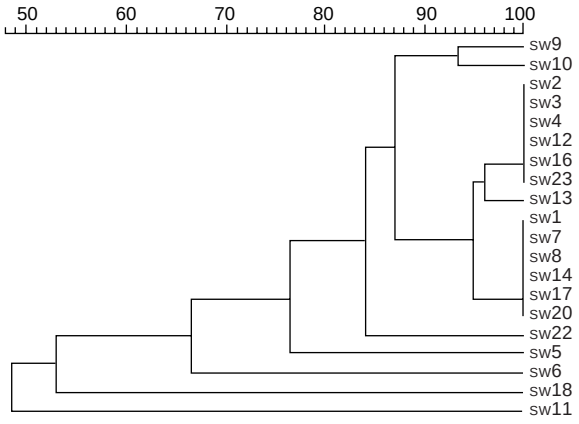

Fig 2B–Dendrogram based on similarity coefficient from *Xba*I generated PFPs of *S. weltevreden*. The dendrogram was constructed based on unweighted pair group average method (UPMGA).

subtype ribotype A into five different PFGE profiles (Table 1).

DISCUSSION

Acute gastroenteritis continues to be a public health problem in developing countries as a result of many interrelated factors including increased urbanization, inadequate supplies of clean water and inadequate sanitary measures. In relation to effective surveillance, the availability of detailed and accurate data related to the molecular epidemiology of non-typhoidal *Salmonella* spp is crucial. Both ribotyping and PFGE have been used for the study of many bacterial species including *S. typhi* (Thong *et al*, 1995a), *S. enteritidis* (Thong *et al*, 1995b) and *S. typhimurium* (Mhand *et al*, 1999). However molecular analysis of *S. weltevreden* has rarely been done. In this study, ribotyping of *Pst*I digested *S. weltevreden* showed three ribotypes among the 16 isolates analyzed. Since *Salmonella* spp contains seven *rrn* operons distributed throughout the chromosome, its genomic DNA will show a maximum of seven DNA hybridizing fragments when digested with *Pst*I. The limited number of ribotypes observed (ribotype A1-A3, F=0.96-1.0) suggests that ribotyping alone is of limited value in the epidemological analysis of *S. weltevreden* in this study.

Genomic macrorestriction fragment length analysis by PFGE is now a widely used molecular subtyping tool in *Salmonella* spp. Previous studies have shown that useful restriction enzymes for *Salmonella* spp were *Xba*I, *Spe*I and *Avr*II (Thong *et al*, 1994). In this analysis *Xba*I was used, as it is more discriminative. PFGE could further subtype the 3 ribotypes into 10 profiles. This proves that PFGE is more discriminative than

ribotyping in distinguishing the closely clonal strains of *S. welterveden*. Moreover, PFGE technique is more rapid and convenient to use as compared to ribotyping as it does not involve Southern transfer, hybridization or probe preparation.

In addition, it was noted that both PFGE and ribotyping were able to distinguish strain SW11, which was distinctively different from the other *S. weltevreden* strains. Strain SW11 was isolated from a patient who had a bone marrow transplant for beta thalassemia. This could imply that strain SW 11 was genetically different from the other strains and probably from a different source. The present study reiterates the usefulness of molecular based techniques in subtyping and distinguishing strains of the same serotype.

Both ribotyping and PFGE showed 100% typeability and reproducibility and the resultant banding patterns were relatively easy to analyze and interpret. Ribotyping and PFGE are based on the same principle that is the distribution of particular restriction enzyme sites on chromosomal DNA. While ribotyping examines the distribution of restriction sites within rRNA genes, or within the sequences surrounding this particular conserved chromosomal region, PFGE typing examines the distribution of particular restriction enzyme sites throughout the entire chromosome, and may therefore offer greater discriminative power with certain organisms. The precise value of the different techniques will depend on the specific microorganism being studied. It is therefore, important that ribotyping patterns should be interpreted in conjunction with total DNA fingerprint patterns, since the later may enable better discrimination between closely related strains in certain cases (Towner and Cockayne, 1993).

CONCLUSION

Macrorestriction analysis of 20 *S. weltevreden* isolates from sporadic cases of salmonellosis in Malaysia showed that considerable heterogeneity existed at the DNA level among *S. weltevreden* isolates (Dice coefficient, F=0.44-1.0). PFGE analysis with *Xba*I digestion can distinguish ten different banding patterns among the 20 *S. weltevreden* isolates. Ribotyping analysis of 16 *S. weltevreden* isolates using 16S rRNA probes showed limited genetic diversity, *ie* three ribotypes with *Pst*I digestion. In this study, PFGE was found to be more discriminative than ribotyping.

Overall, PFGE of *S. weltevreden* chromosomal DNA digested with infrequently cutting restriction endonuclease was found to be a useful method for comparing and differentiating *S. weltevreden* isolates and may complement other molecular approaches for epidemiological purposes.

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