Molecular subtyping of *Salmonella enterica* serovar Paratyphi B from animal and food isolates in Malaysia

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ABSTRACT   The genetic diversity of 16 animal and food strains of *Salmonella enterica* Paratyphi B from different animal hosts between the years 1979-2001 was determined by pulsed-field gel electrophoresis (PFGE), ERIC-PCR and REP-PCR analysis. Fourteen isolates were tested positive for dextrorotary-tartrate tests (*S*. Paratyphi B dT+, also known as *S*. Java) while two were d-tartrate negative (*S*. Paratyphi B dT-). Most of the strains tested were sensitive to the commonly used antibiotics. Both REP-PCR and ERIC-PCR gave five different profiles with a diversity index (DI) of 0.67 and 0.69 respectively. PFGE of *Xba*I digested chromosomal DNA from these isolates showed that there was a high degree of heterogeneity (F= 0.59-1.0) and most of the strains had unique profiles with a DI of 0.99. Strains that were isolated from different sources and years were genetically distinct. A pair of *S*. Paratyphi B dT+ strains isolated from cattle and pheasant were similar, indicating that these two different animals were probably infected with the same strain. A dendrogram based on the unweighted pair group average method (UPGMA) algorithm showed that the bovine isolates recovered from cattle in 1984 were in one cluster indicating that the strains were probably derived from a single clone. PFGE is more discriminative that PCR-based methods in determining the clonality and genetic diversity of *Salmonella* Paratyphi B. To the best of our knowledge, this is the first study to report the genetic variation of chromosomal *S. enterica* Paratyphi B var Java from animal and food in Malaysia.

ABSTRAK   Diversiti genetik bagi 16 strain *Salmonella enterica* serovar Paratyphi B yang diasingkan daripada haiwan dan makanan di antara tahun 1979 – 2001 telah dianalisis oleh pulsed-field gel electrophoresis (PFGE), ERIC-PCR dan REP-PCR. Empat belas isolat telah diuji positif untuk ujian dextrorotary-tartrate (*S*. Paratyphi B dT+) sementara dua isolat diuji negatif (*S*. Paratyphi B dT-). Kebanyakan strain adalah sensitif terhadap antibiotik yang biasa diguna. Kedua-dua REP-PCR dan ERIC-PCR memberi lima jenis profil dengan indeks diversiti (DI) 0.67 dan 0.69 masing-masing. Analisis PFGE dengan *Xba*I bagi isolat tersebut menunjukkan tahap heterogeniti yang tinggi (F=0.59-1.0) dan kebanyakan strain mempunyai profil yang unggul dengan DI=0.99. Strain yang diasingkan dari tahun berlainan dan berjenis-jenis punca adalah berlainan dari segi genetik. Sepasang strain *S*. Paratyphi B dT+ yang diasingkan daripada lembu dan satu strain daripada burung (pheasant) mempunyai profil yang sama menunjukkan bahawa dua jenis haiwan yang berlainan mungkin dijangkiti dengan strain yang sama. Satu dendrogram berasaskan "unweighted pair group average method (UPGMA)" menunjukkan bahawa isolat daripada lembu pada tahun 1984 adalah dalam satu kelompok. Dengan itu, strain-strain ini berkemungkinan berasal daripada sejenis klon. PFGE adalah lebih diskriminasi daripada kaedah yang berasaskan PCR dalam penentuan diversiti genetik dan klonaliti *S*. Paratyphi B. Untuk pengetahuan kami, kajian ini adalah kajian pertama mengenai genetik variasi *S*. Paratyphi B var Java yang diisolasi daripada haiwan dan makanan di Malaysia.

(molecular subtyping, *Salmonella* Paratyphi B, PFGE, animal isolates)

INTRODUCTION

Salmonellosis is one of the commonest forms of zoonosis with infection being transmitted directly or indirectly from animals to humans, or from infected food products (meat, eggs, and dairy products) to humans. Salmonellosis occurs in most countries and affects all animal species.

There is no breed predisposition to infection, but animals kept in fecally contaminated environments (e.g. Farm livestock) or that eat feces, groom companions or share feeding and drinking bowls are at high risk to contract the infection. Young animals, animals with poor immune response, animals subjected to stress and animals suffering from malnutrition or obesity are at greater risk from exposure to *Salmonella*.

Epidemiologically, *Salmonella enterica* Paratyphi B (herewith *S*. Paratyphi B) is relatively unknown as compared to the more well-known serotypes such as Typhimurium and Enteritidis. *S*. Paratyphi B can be differentiated into two biotypes based on the ability to metabolize tartrate. Strains that are dextrorotary tartrate positive (dT+) are known as *S*. Paratyphi B variant Java (formally known as *S*. Java) while strains that are d-tartrate negative (dT-) are *S. enterica* Paratyphi B (*S*. Paratyphi B). However, recent reports of increased incidence of *S*. Paratyphi B dT+ in France [1], Germany [2], Italy [3], Canada [4], and Netherlands [5] indicate that this serotype could be of importance in the near future. In addition, this serotype has wide geographical distribution and has been isolated from a variety of sources such as poultry and poultry products [2], alfafa sprouts, tropical fish [6] and raw goats' milk cheese [1].

Molecular subtyping of *Salmonella* isolates is an invaluable epidemiological tool that can be used to track the source of infection, contamination as well as to provide an epidemiological link between the animal and human hosts and the environment. Genotypic methods that have been applied for molecular epidemiology include plasmid fingerprinting, ribotyping, PCR based– typing and pulsed field gel electrophoresis (PFGE) [7, 8]. In Malaysia, there is no documentation of the characterization of *S. enterica* Paratyphi B and therefore the study was carried out to determine the molecular subtypes of these animal isolates by PFGE, REP-PCR and ERIC-PCR.

MATERIALS AND METHODS

Bacterial strains: A total of 16 strains of *S*. Paratyphi B isolated from cattle (n=7), sheep (n=1), pheasant (n=1), monkey (n=1), horse (n=1), tiger (n =1), shrew (n=1), buffalo meat (n=1) and imported raw meat (n=2) were analyzed (Table 1). All the biochemical and serological tests were performed at the Bacteriology Unit, Veterinary Research Institute, Malaysia. All, except 2 strains (shrew and buffalo meat) were tested d-tartrate positive. All the strains were tested for their antibiotic susceptibility according to the Kirby-Bauer method [9] with commercial antibiotic disks (Oxoid Ltd.). The antibiotic agents used were: amikacin (30 µg), ampicillin (10 µg), chloramphenicol (30µg), kanamycin (30µg), nalidixic acid (30µg), streptomycin (25µg), ciprofloxacin (5µg), ceftriaxone (30µg), trimethoprim-sulfamethoxazole (25µg), and trimethoprim (25µg).

PCR analysis: Crude DNA was prepared by boiling bacterial cell suspension. The PCR was performed in a 25 µl volume containing 1 × PCR buffer with $(NH_4)_2SO_4$ (Fermentas), 2.5 mM $MgCl_2$, 200 µM concentrations of each dNTP (Promega, Madison, WI, USA), 50 pmol of each primer, 1 unit of *Taq* DNA polymerase (Fermentas) and 4 µl of template DNA. The primers used for rep-PCR were REP I (5' IIICGICGICATCATCTGGG-3') and REP II (5'-ICG ICT TAT CIG GCC TAC-3'). The primers used for ERIC-PCR were ERIC I (5'-CACTTAGGGGTCCTCGAATGTA-3') and ERIC II (5'-AAGTAAGTGACTGGGGTGAG CG-3') [10]. Reactions were overlaid with mineral oil and amplified in a DNA Thermal Cycler 480 (Perkin Elmer, Main Ave, Norwalk, CT, USA) as follows: one cycle of 5 min at 94°C followed by 35 cycles of 30 s at 94°C, I min at 40°C and 2 min at 72°C. The cycling was concluded with 5 min at 72°C and the reaction products stored at 4°C prior to analysis. Amplification products were resolved on a 1% (w/v) agarose (Analytical grade agarose, Promega, Madison, WI, USA) by horizontal electrophoresis in 0.5 × Tris-borate buffer. Gels were stained with ethidium bromide (0.5 µg/ml), visualized under UV transillumination and photographed. The fingerprints obtained by PCR were visually compared.

PFGE: Genomic DNA for pulsed-field gel electrophoresis (PFGE) were prepared according to protocols previously described [11, 12]. A slice of the DNA-agarose plug was digested with 10 Units of *Xba*I for 4 hours at 37°C and the restricted DNA fragments were separated by using the CHEF DR II/III at ramped pulsed times of 1 to 40 sec for 26 hours at 200V. Interpretation of the DNA banding patterns was according to

the criteria of Tenover et al. [13]. Dice coefficient of similarity was calculated to compare the macrorestriction patterns [12]. Clustering was based on the unweighted pair group average method (UPGMA) and was performed with GelCompar, Applied Maths, Kortrijk, Belgium.

Statistical analysis: The discrimination index (DI), the probability that two unrelated isolates would be placed into different typing groups, was calculated by Simpson's index of diversity (DI) [14].

Table 1. Strains characteristics of *Salmonella enterica* Paratyphi B from animal and food isolates in Malaysia

Strain No	Host/source	Place /year of isolation	*Xba*I pulsotypes	Rep – PCR Profiles	ERIC-PCR Profiles
3060/83	Cattle	Ipoh/1983	X3a	R2	E2
3852/83*	Monkey	PJ/1983	X3b	R2	E2
3907/83*	Pheasant	PJ/1983	X3a	R2	E2
2599/84	Frozen meat	PJ/1984	X4a	R3	E3
3647/84	Cattle	PJ/1984	X1a	R1	E1
4684/84	Cattle	JB/1984	X1d	R1	E1
4688/84*	Cattle	JB/1984	X1e	R1	E1
474784	Cattle	JB/1984	X1b	R1	E1
4869/84	Cattle	Kluang/1984	X5	R5	E2
4960/84	Cattle	JB/1984	X1c	R1	E1
3651/85^	Sheep	BT/1985	X2	R2	E2
3132/86^	Imported meat	PJ/1986	X4b	R4	E3
4714/95	Tiger	Taiping /1995	X8	R1	E5
9428/01	Horse	Sabah/2001	X9	R2	E4
659/79*^	Shrew^d	Ipoh/1979	X6	R3	E3
1961/95	Buffalo meat^d	BT/1995	X7	R1	E3

* Resistant to sulfamethoxazole
^ Resistant to streptomycin
^d *S.* Paratyphi B dT-

RESULTS

Overall, ten *S.* Paratyphi B var Java isolates were sensitive to all the antibiotics, three isolates of variant Java were resistant to sulfamethoxazole while two isolates of variant Java were resistant to streptomycin. One *S.* Paratyphi B dT- isolated from shrew was resistant to both sulfamethoxazole and streptomycin.

Fingerprinting with primer pair REP I and REP II generated five patterns (R1-R5) with six major bands between 100 bp and 1.5 kbp. Pattern R1 was the most common (44%). The patterns generated were very homogeneous which differed by 1-3 bands only with a discriminatory index, DI of 0.73 (Figure 1).

PCR analysis with primer pair ERIC I and ERIC II also generated five patterns (E1-E5) with 6-10 major bands between 200 bp and 1.5 kbp. Two

profiles (E1 and E3) were most common (31% each). The profiles were slightly more diverse with 1-4 bands difference giving a DI of 0.78 (Figure 2).

It was noted that five *S.* Paratyphi B var Java strains isolated from cattle in 1984, were indistinguishable by REP-PCR and ERIC-PCR (pattern R1 and E1 respectively). Three *S.* Paratyphi B var Java strains isolated in 1983 and one *S.* Paratyphi B var Java strains isolated in 1985 also gave identical fingerprints with REP-PCR and ERIC-PCR and the banding pattern were R2 and E2 respectively.

PFGE of *Xba*I digested chromosomal DNA subtyped the 16 strains into 15 reproducible and distinct PFGE profiles (pulsotypes) (Table 1). The pulsotypes consisted of 11 to 19 DNA fragments ranging in size from 20 to 600 kb (Figure 3). Among the 14 *S.* Paratyphi B var Java

strains, 13 *Xba*I pulsotypes were observed (Table1, Figure 3). The pulsotypes from the bovine isolates differed mostly in 1 to 3 bands, indicating their close association. The two *S.* Paratyphi B var Java isolates recovered from imported meat from India were unique and were not observed in the Malaysian strains. A bovine isolate (#3960/83) was indistinguishable to another isolate from pheasant (profile X3a), both obtained in 1983. Strains from the various animal hosts recovered from different years were uniquely different, indicating considerable genetic variation among the strains.

A dendrogram, based on the matrix of F-values of pulsotypes was constructed using a clustering algorithm of the unweighted pair group arithemetic means method (UPGMA) (Figure 3). Based on 75% similarity, two major clusters were observed. The first cluster consisted of twelve *S.*

Paratyphi B var Java strains and one *S.* Paratyphi B strain while the second cluster is composed of two *S.* Paratyphi B var Java strains and one *S.* Paratyphi B strain. Within cluster I, eleven *S.* Paratyphi B var Java strains were grouped together at 80% similarity indicating a limited genetic diversity among these strains. The cattle isolates were in one subcluster, albeit minor variations (a difference of 1-3 bands, F= 0.94-0.97), probably from a common infecting clone. Isolates from the imported meat (profiles X4a/X4b), both obtained from different years (1984 and 1986, respectively) were more closely related to the *S.* Paratyphi B strains isolated from shrew (Figure 2). It was noted that these strains which were grouped in cluster II at 75% similarity had a deletion in the 30 kb fragment but an addition of a large fragment (approximately 400 kb) in their pulsotypes as compared to other strains.
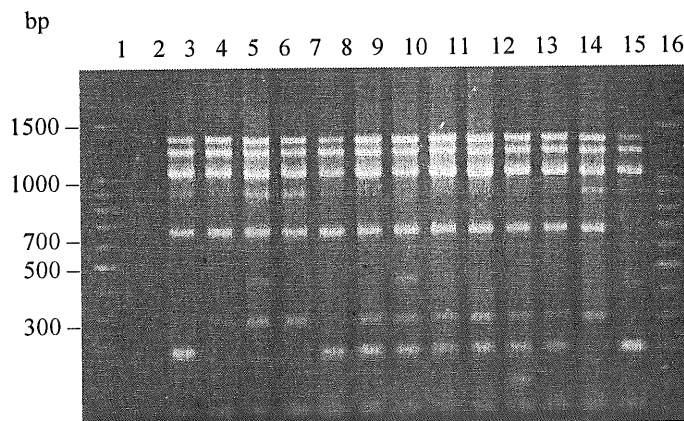
Figure 1. REP-PCR profiles of 13 animal isolates of *Salmonella* Paratyphi B. Lane 1 & 16, 100 bp DNA ladder (Promega); lane 2, negative control without template DNA; lane 3 to 15, isolates 659/79, 3060/83, 3852/83, 3907/83, 2599/84, 3647/84, 4684/84, 4688/84, 4747/84, 4869/84, 4960/84, 3651/85, and 3132/86 respectively.
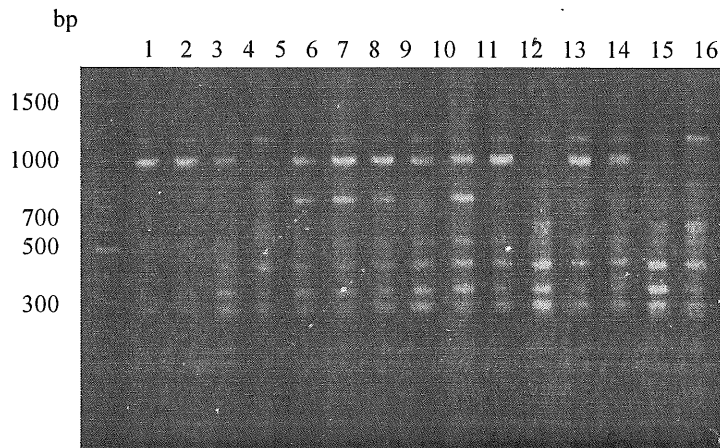
Figure 2. ERIC-PCR profiles of 15 animal and food isolates of *Salmonella* Paratyphi B. Lane 1, 100 bp DNA ladder (Promega); lane 2 to 16, isolates 3060/83, 3852/83, 3907/83, 2599/84, 3647/84, 4684/84, 4747/84, 4869/84, 4960/84, 3651/85, 3132/86, 4714/95, 9428/01, 659/79, and 1961/95 respectively.
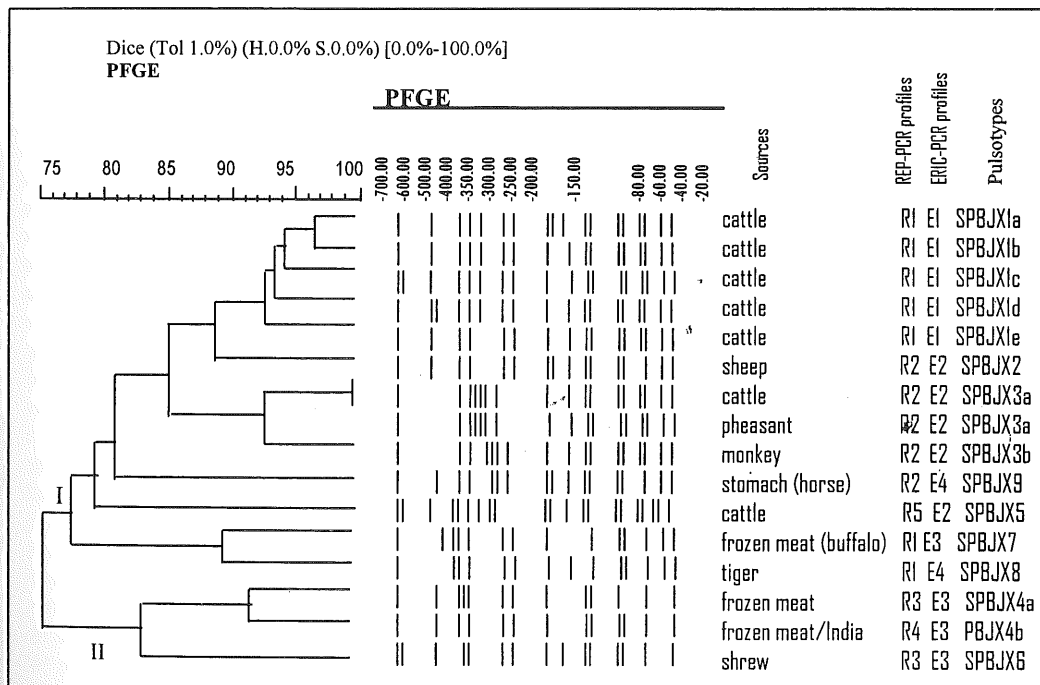
Figure 3. Dendrogram showing the cluster analysis of the different *Xba*I pulsotypes from 16 animal and food isolates of *Salmonella* Paratyphi B, generated by the GelCompar program using the UPGMA method, based on the matrix of F values.

DISCUSSION

Most of the *S.* Paratyphi B var Java strains isolated from animals and food in Malaysia are generally antibiotic-sensitive. However, *S.* Paratyphi B var Java isolates found in poultry in the Netherlands are generally antibiotic-resistant [5]. The potential for poultry meat to act as a vehicle for multiresistant strains is a matter of concern for public health. The development of resistance could be related to the high selection pressure due to vaccination and the intensive use of antibiotics in poultry farming. Therefore, continued surveillance of antimicrobial resistance in *Salmonella* spp. is needed.

In this study, *S.* Paratyphi B var Java collected from a number of different sources were shown to be homogeneous by PFGE, REP-PCR and ERIC-PCR. Such observation was in accordance with those reported by Brown et al. [15]. It has recently been reported that a particular clone of *S.* Paratyphi B var Java has become predominant in poultry production in Germany [2]. In this study, *S.* Paratyphi B var Java recovered from the cattles in 1994 probably originated from a common source as these strains exhibited pulsotypes that differed in less than three DNA bands. The *S.* Paratyphi B var Java strain isolated from the pheasant had a pulsotype indistinguishable from that isolated from the cattle in 1983 indicating that these two animals were most probably infected with a similar clone of *S.* Paratyphi B.

PFGE was shown to be very useful in delineating the genetic variability of the strains and was an invaluable epidemiological tool, as the DNA fingerprints generated were stable and reproducible and all the strains were typeable. The methodology is now relatively simple and it takes only 2 days to prepare the chromosomal DNA for PFGE analysis [12]. Overall, the pulsotypes of most of the *S.* Paratyphi B var Java strains from different animal sources were probably closely related and this indicated that these isolates belonged to a limited number of clones.

In this study, PFGE was able to differentiate the two very closely related biotypes of *S.* paratyphi B dT- and *S.* Paratyphi B dT+. Ezquerra et al. [16] also showed that *S.* Paratyphi B strains dT- and dT+ could be distinguished by IS 200 profiling. However, PFGE is a much simpler and shorter technique as there is no necessity of performing Southern hybridization and IS 200 probe preparation and labeling. Identification of the strains as *d*-tartrate positive or negative is usually done by using the sodium-potassium tartarate salt. Sometimes, this test gave ambiguous result.

PCR-based techniques such as RAPD, ERIC-PCR and REP-PCR are commonly applied in the epidemiological studies of bacterial pathogens. These methods are generally rapid and technically less demanding than most other molecular typing methods and no DNA sequence information is necessary. However, the main drawbacks of such techniques are its lack of reproducibility and stability of the DNA banding patterns.

In this study, both REP-PCR and ERIC-PCR delineated the 16 *S.* Paratyphi B from different sources into 5 different profiles. Although these two techniques were less discriminative than PFGE, there was a good correlation among the three molecular markers in the clustering of the strains. For example, bovine strains which had X1 series profiles were clustered together with E1 and RI. Similarly, the second cluster consisted of strains with X2, X3a/3b and the R2/E2 combination.

The ubiquitous distribution of *Salmonella* in the environment and in a variety of animal hosts and its prevalence in the global food chain necessitates the need for continued surveillance and monitoring. Numerous genotypic methods have been applied for subtyping of *Salmonella* spp for the purpose of surveillance that is to obtain baseline information about a particular emergent serovars and to estimate the contribution of animal reservoirs to human cases of salmonellosis. For effective surveillance, a rapid, reproducible and reliable method like PFGE is a suitable method. Although PCR based technique is rapid and provide a fast turnover time, its inherent irreproducibility problem makes this technique difficult for interlaboratory comparison. The present study showed that PFGE is a very discriminatory method and it takes only 2 days, starting from a pure culture, to obtain suitable chromosomal DNA for analysis [12]. The method developed for this study will be further applied to other *Salmonella* serovars for further surveillance and control of salmonellosis in the animals. Due to a relatively small sample size in this study, the present analysis was not

able to provide some valuable insights into the molecular epidemiology of *S.* Paratyphi B in animal and food. To the best of our knowledge, this is the first report on the molecular characterization of animal and food isolates of *S.* Paratyphi B in Malaysia.

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