DNA fingerprinting of human isolates of *Salmonella enterica* serotype Paratyphi B in Malaysia

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**ABSTRACT**


**Aims:** DNA fingerprinting of *Salmonella enterica* serotype Paratyphi B isolated in Malaysia during 1982–83, 1992 and 1996–2002 was carried out by pulsed-field gel electrophoresis (PFGE), antimicrobial susceptibility tests and d-tartrate utilization tests to assess the extent of genetic diversity of these isolates in Malaysia.

**Methods and Results:** Eighty-six human isolates and one food isolate of *Salmonella* Paratyphi B were analysed by PFGE, antimicrobial susceptibility tests and d-tartrate utilization tests. Sixty-five strains were d-tartrate-negative (d+1-) while 22 strains were d-tartrate-positive (d+1+). Thirty-seven per cent of the *Salmonella* Paratyphi B strains were resistant to one or more antimicrobial agents. PFGE analysis clearly distinguished the dT- and dT+ strains into two clusters based on the unweighted pair group average method (UPGMA). Twenty-two XbaI-pulsotypes were observed among the 65 dT- strains while 17 XbaI-pulsotypes were observed among the 22 isolates of *Salmonella* Paratyphi B dT+.

**Conclusions:** The present study showed that PFGE was very discriminative with 33.7% of the strains yielding distinct fingerprint. Paratyphoid fever in Malaysia is probably caused by one predominant, endemic clone of *Salmonella* Paratyphi B dT- with various subtypes. There was no association between the pulsotypes and the severity of the disease indicating that the severity of the disease is probably multifactorial.

**Significance and Impact of the Study:** The findings of the present study verify the usefulness of PFGE in characterizing strains of *Salmonella* Paratyphi B. This is the first report on the application of PFGE on a large collection of *Salmonella* Paratyphi B in Malaysia.

**Keywords:** DNA fingerprinting, pulsed-field gel electrophoresis, *Salmonella* Paratyphi B

**INTRODUCTION**

Enteric fever continues to be a public health problem in many parts of the world especially in the developing countries including Malaysia. Enteric fever (i.e. typhoid and paratyphoid fever) is a systemic disease resulting from infection with *Salmonella enterica* subsp. *enterica* serotype Typhi and *Salmonella* enterica subsp. *enterica* serotype Paratyphi A, B or C. Enteric fever is typically acquired by ingesting food or water that has been contaminated by these organisms and asymptomatic carriers, particularly food handlers who are the major source of these organisms (Hu and Kopczko 2002).

The epidemiology of *Salmonella enterica* subsp. *enterica* serotype Paratyphi B (Salmonella Paratyphi B) in Malaysia is relatively unknown compared with the more well-known serotypes such as Typhi, Typhimurium and Enteritidis. However, recent reports of increased incidence of *Salmonella* Paratyphi B in France (Descoblo et al. 1996), Canada (Stratton et al. 2001), Italy (Mannoni et al. 2007) and...
Germany (Miko et al. 2002) indicate that this serotype could be of importance in the near future. In Malaysia, only two studies reported *Salmonella Paratyphi* B as the second highest serovar isolated from children with non-typhoid gastroenteritis (Lee et al. 1998) and those with extra-intestinal nontyphoidal *Salmonella* infections (Lee et al. 2000). Thus, it is obvious that there is an urgent need for effective epidemiological surveillance as a basis for the development of rational control strategies.

*Salmonella* Paratyphi B (1,4,5,12: b, 1,2) can be differentiated by the use of deoxorotatory t-arabinose (d-tartrate acid) (Larkar 1982). Isolates from enteric fever patients typically fail to ferment d-tartrate and form a mucoid (milky) well, whereas most strains causing acute gastroenteritis are able to utilize d-tartrate and do not produce mucoid wells (Schander et al. 1990). It was also noted that d-tartrate-negative (d*- ) strains are infrequently isolated from animals, whereas d-tartrate-positive (d* + ) strains (formerly called *Salmonella enterica* subspp. enterica serotype Java) are commonly recovered from both humans and animals (Cherry et al. 1933; Van Pelt et al. 2003).

*Salmonella* spp. are ubiquitous pathogens and can be found in many human and animal hosts. The easy adaptability of this micro-organism requires close monitoring and surveillance. Detailed strain identification is essential for useful investigation of *Salmonella* outbreaks. For epidemiologic investigations, the traditional methods of strain identification and differentiation are serotyping and phage typing, supplemented by recognition of a number of phenotypic properties such as antimicrobial (Threlfall and Frost 1990). In contrast, molecular-based methods have been widely applied to study the genetic relatedness of pathogens in order to track the source of infections, and investigate the incidence of the strains. Macrogenetic analysis by pulsed-field gel electrophoresis (PFGE) has been widely used for molecular epidemiological investigations of infections caused by a range of bacterial pathogens, and is currently the gold standard for molecular subtyping of microbial pathogens including *Salmonella* spp. (Tenover et al. 1995; Mislav and Mullah 1996).

In Malaysia, based on laboratory surveillance by the Institute for Medical Research, infection caused by *Salmonella Paratyphi* B is relatively important. From 1990 till 1995, *Salmonella Paratyphi* B was ranked fourth (Ministry of Health, Malaysia). Given the relative importance of this *Salmonella* serovar, we reviewed the incidence of cases caused by *Salmonella Paratyphi* B by PFGE to determine the prevalent pulsortypes in Malaysia. To the best of our knowledge, this is the first study to report the genetic variation of chromosomal *Salmonella Paratyphi* B in the study area. The DNA fingerprints will provide a baseline data for future molecular epidemiology and surveillance of *Salmonella enterica* spp. in humans in this country.

**MATERIALS AND METHODS**

**Bacterial strains**

A total of 86 human strains of *Salmonella enterica* serotype Paratyphi B obtained from sporadic cases of enteric fever or acute gastroenteritis (during 1982-83, 1992 and 1996-2002) in different parts of Malaysia were analysed. Fourteen strains were obtained from University Hospital, Kluang, Johor, 74 strains were from various government hospitals that were submitted by microbiologists or laboratory technologists to the Salinomma Reference Centre in the Institute for Medical Research, Kuala Lumpur, Malaysia, for serotyping. Isolates from 1991 to 1995 were not available for analysis. Older cultures from 1982 to 1983 were recovered from frozen stock (−70°C) stored at the laboratory. ATCC 8759 strain of *Salmonella Paratyphi* B and one food isolate from the Food and Quality Control Laboratory, Malaysia, were included for comparison. Serotyping of these strains was performed according to the Kauffmann-White scheme, and strains were named serotype Paratyphi B when they exhibited the antigen formula (1,4,5,12: b, 1,2) (Popoff and Le Minor 1997). Further differentiation of the strains as d-tartrate-positive or negative was carried out according to the method described by Barker (1985). Stocks cultures of the strains were stored in glycerol stock at −70°C.

**Antimicrobial resistance testing**

Antimicrobial resistance tests were performed by modified Kirby-Bauer disk diffusion methods (NCCLS 2000c). The following antimicrobial discs (Oxoid Ltd, London, UK) were used: streptomycin (10 μg), tetracycline (30 μg), ampicillin (10 μg), chloramphenicol (30 μg), kanamycin (30 μg), gentamicin (10 μg), trimethoprim-sulphamethoxazole (25 μg) and ciprofloxacin (5 μg). *E. coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 were used as controls. Interpretation of the zone of inhibition was according to the National Committee for Clinical Laboratory Standards (NCCLS 2000b) procedure.

**PFGE**

Genomic DNA for PFGE were prepared according to protocols previously described (Thong and Pang 1996; Thong et al. 2002b). A slice of the DNA-agarose plug was digested with 10 U of *XhoI* for 4 h at 37°C and the restricted DNA fragments separated by using the CHEF DR II/III at ramped pulsed times of 1-40 s for 20 h at 200 V. Interpretation of the DNA banding patterns was according to the criteria of Tenover et al. (1995). Dice coefficient of similarity was calculated to compare the macrorestriction patterns (Thong et al. 2002a). The coefficient, $F$, represents...
the proportion of shared DNA fragments in two isolates and was calculated by the following formula: 
\[ p = \frac{2n_{xy}}{(n_x + n_y)} \]
where \( n_x \) is the total number of DNA fragments from isolate \( X \), \( n_y \) is the total number of DNA fragments from isolate \( Y \), and \( n_{xy} \) is the number of DNA fragments that were identical in the two isolates. Clustering was based on the unweighted pair group average method (UPGMA) and was performed with GenCompar II, Version 2.0 (Applied Maths, Kortrijk, Belgium).

RESULTS

Table 1 shows the numbers and percentage of Salmonella Paratyphi B strains received at the Salmonella Reference Centre at the Institute for Medical Research, Kuala Lumpur, Malaysia, each year from 1990 to 2000. Salmonella Paratyphi B constituted about 4-5% of the total numbers of Salmonella enterica and this represented the fourth most common serotype prevalent in humans in Malaysia. Eighty-six strains isolated during 1982-83, 1992 and 1996-2002 were randomly selected for antimicrobial susceptibility tests and PFGE analysis. All the isolates were then further tested for utilization of d-tartrate and the results showed that 74-8% (64 of 86) of the Salmonella Paratyphi B isolates were d-tartrate-negative, whereas 25-6% (22 of 86) were d-tartrate-positive.

Among the 64 Salmonella Paratyphi B dT- isolates, 58 were isolated from stools, five were isolated from blood and one was isolated from other tissue (site unknown). The clinical details were very limited. However, most of the strains were from patients with fever, diarrhoea, abdominal cramp, and nausea and vomiting. From the total of 22 Salmonella Paratyphi B dT+ isolates, 18 were isolated from stools and four were isolated from blood. These four blood isolates of Salmonella Paratyphi B dT+ were obtained from a 66-year-old male and three children ranging from 10 months to 9 years old. All the patients had varying degree of acute gastroenteritis.

Overall, 63% (54 of 86) of the Salmonella Paratyphi B isolates were susceptible to all antimicrobial agents tested while the remainder (22 of 86) were resistant to one or more antimicrobial agents (Table 2). Seventy-five per cent (24 of 32) of the resistant strains were Salmonella Paratyphi B dT- and 44% of these strains were resistant to three or more antimicrobial agents, with the highest resistance to ampicillin (62.5%), streptomycin (56.5%), chloramphenicol (43.8%) and tetracycline (37.5%) (Table 2). All the clinical isolates obtained from 1982 to 2001 remained sensitive to ciprofloxacin. However, resistance to ciprofloxacin was detected in four Salmonella Paratyphi B dT+ strains isolated in 2002.

The PFGE subtyped the 86 strains into 39 reproducible and distinct profiles (palatypes) (\( F = 0.55-1.0 \)) (Table 3) with each profile consisting 12 to 20 DNA fragments ranging in size from 20 to 700 kbp. Twenty-two XbaI palatypes were observed among the 64 Salmonella Paratyphi B dT- isolates.
Cluster analysis of all the _Salmonella_ Paratyphi B strains based on the matrix of F values (at 75% similarity) generated two clusters, with each cluster comprising some subclusters (Fig. 2). Cluster I consisted of 18 pulotypes from 19 isolates of _Salmonella_ Paratyphi B dT- whereas cluster II comprised 22 pulotypes from 21 dT+ strains. Overall, cluster analysis of the Xhol-pulotypes clearly differentiated the dT- and dT+ strains. At 90% similarity, pulotypes X1, X2 and their subtypes X1a, X1b, X2a and X2b were grouped into one subcluster Ia whereas pulotypes X3, X4 and their subtypes X3a, X3b, X4a and X4b were grouped into another subcluster Ib. It was noted that these two subclusters were generally differed by one 480 kbp fragment, therefore, although X3a and X2b were differed by only two bands, they were grouped into different subclusters at 90% similarity.

**DISCUSSION**

There is a paucity of reports on the prevalence of human _Salmonella_ Paratyphi B in Malaysia, because epidemiologically this serotype has not been significant except for the dT- negative strain, which mainly causes paratyphoid fever. Paratyphoid fever is clinically very similar to typhoid fever and it can only be distinguished from mild typhoid fever by identification of the causative organism (Hu and Kopecko 2002). _Salmonella_ Paratyphi B dT- and _Salmonella_ Paratyphi B dT+ (formerly called _Salmonella_ Java) are phenotypically close and share the same antigenic formula 1, 4, [5], 12, b: 1, 7 (Chant 2003). Historically, the dT- strain is known to be a virulent human pathogen whereas dT+ strain is generally less virulence for humans (Barker et al. 1988; Schniter et al. 1989). _Salmonella_ Paratyphi B dT+ usually causes gastro-
enteritis with the symptoms of vomiting, abdominal pain and diarrhea, the last of these is usually the main presenting symptom but its severity varies from the very slight to a violent diarrhoea, which leaves the patient severely prostrated. Rarely, the infection becomes septicaemic, most often in infants, the elderly or highly susceptible persons as evidenced in this study. However, a recent study by Chant (2003) proposed that strains of *Salmonella* Paratyphi B (dT-) are not a major cause of enteric fever but primarily a cause of gastroenteritis, in common with *Salmonella* Java (dT+) strains.

In Malaysia, from 1990 to 2000, *Salmonella* Paratyphi B was the fourth common serotype isolated from humans and the reported cases occurred sporadically. However, this may be an underestimate of the problem, as patients with salmonellosis may not visit a doctor and thus no specimen may be obtained or the laboratory findings are not notified to the Ministry of Health. Although the relative frequency of the various *Salmonella* serovars may change, the relative frequency of *Salmonella* Paratyphi B remains rather stable. It is expected that *Salmonella* Paratyphi B will be a predominant organism causing salmonellosis in Malaysia in years to come caused by contaminated food, poultry and meat products. Therefore, it would be useful for further investigations to compare human and nonhuman isolates of *Salmonella* Paratyphi B in order to confirm the source(s) of the organism. Recently, the 2003 Eurosurveillance report showed that there was an increase in the isolation of *Salmonella* Paratyphi B var Java (dT+) from poultry isolates in the Netherlands (Vos Pelt et al., 2003) and that these poultry isolates were possibly associated with human infections (Brown et al., 2003). However, in this study, whether an increase in Java infections in the poultry is associated with human infection in Malaysia remains to be determined.

In this study, 57% of the *Salmonella* Paratyphi B isolates from Malaysia were resistant to commonly available antimicrobial agents and only one *Salmonella* Paratyphi B dT- isolate (pulsotype X1) obtained in 1998 was resistant to all three first-line antibiotics, chloramphenicol, ampicillin and trimethoprim-sulphamethoxazole. In addition, four dT- strains (pulsotype X2) isolated in 2002 were multiple resistant to ciprofloxacin, gentamicin, ampicillin and chloramphenicol.

The emergence of fluoroquinolone-resistant strains is of serious concern as this class of antimicrobial agent is the drug of choice in cases of acute salmonellosis due to multiresistant strains, and the introduction of fluoroquinolones into veterinary medicine have been reported to contribute to the emergence of fluoroquinolone-resistance in *Campylobacter* spp., *E. coli* and *Salmonella* spp. (Giraud et al., 1999). No distinct association between the resistant phenotypes and *XbaI*-pulsotypes was observed in this study as both resistant and sensitive strains shared similar pulsotypes. This suggests that the resistant isolates could have derived from a sensitive progenitor strain in Malaysia that was already endemic in the country. It is also possible that differences in susceptibility may be due to point mutations or minor genetic changes that were insufficient to alter the PFGE patterns; in these cases only a large alteration in the DNA or a mutation occurring within the recognition sites for the restriction enzyme used may alter the PFGE patterns (Thong et al., 2002a).
The PFGE analysis of *Salmonella Paratyphi* B strains has provided some valuable insights into the molecular epidemiology of enteric fever and gastroenteritis in Malaysia as isolates from different localities shared wide genetic diversity. Thus, PFGE proved to be highly discriminatory, with 33-75% (29 of 86) of the isolates yielding distinct fingerprints indicating that many PFGE subtypes of *Salmonella Paratyphi* B were responsible for human infections in Malaysia.

Strain typing is an integral part of molecular epidemiology to discern the clonality involved in local epidemics or global epidemiology (Thrash et al. 2002a). Comparative analysis of clinical strains of *Salmonella Paratyphi* B DT- obtained in various years (1982–83, 1992, 1996–2002) indicated that the predominant pulsotypes X1, X2 and X2a are stable and persist over a considerable period of time. These predominant pulsotypes and their subtypes were grouped into one subcluster (based on 90% similarity) suggesting that these pulsotypes could have originated from a single clone. The emergence of new pulsotypes (X3–X12) was observed among the recent strains of *Salmonella Paratyphi* B DT- obtained during 1998–2002. Thus, our assumption is that as time passes and organisms spread, divergence may increase. Cluster analysis showed that *Salmonella Paratyphi* B DT- is clonal and less diverse with F values ranging from 0.73 to 1.0. These results concurred with the multilocus enzyme electrophoresis data reported by Selander et al. (1990), who showed that most of the DT- strains comprised a globally distributed clone, Pbl1, with highly polymorphic phenotypes. They also suggested that clones of human-adapted serotypes, for example, *Salmonella Typhi* and *Salmonella Paratyphi* C are fewer in number and have less diverse genotypes than those with a broad host range such as *Salmonella Typhimurium* and *Salmonella Enteritidis*.

Among the 22 clinical *Salmonella Paratyphi* B DT+ isolates, 17 XbaI-pulsotypes were generated as opposed to 22 XbaI-pulsotypes among the 64 *Salmonella Paratyphi* B DT- isolates. A greater number of subtypes were noted among the *Salmonella Paratyphi* B DT+ strains indicating a greater genetic variation among these strains. A total of 19 DT+ strains (with 14 pulsotypes) were grouped into cluster B with F values ranging from 0.70 to 1.0. Another two DT+ strains were grouped together with a *Salmonella Paratyphi* B ATCC strain with F values ranging from 0.79 to 1.0. One DT+ isolate exhibited pulsotype X25 which was very distinct from others suggesting that this particular isolate could have been derived from a different lineage. Selander et al. (1990) concluded that DT+ strains represented seven clonal lineages. However, Mikolajczyk et al. (2002) showed that a new distinct DT+ clonal group emerged and has replaced almost all other clonal groups. Whilst the total number of DT+ strains in the present study was relatively small, one major cluster was observed (Fig. 2).

Macromolecule fingerprinting reflects the structural organization of the bacterial chromosome, detecting the distribution of restriction sites throughout the entire length of the genome (Maslow and Mulligan 1996). PFGE analysis also clearly distinguished *Salmonella Paratyphi* B strains of DT- and DT+ as demonstrated by cluster analysis. DT- and DT+ strains can also be distinguished by IS200 profiling (Elzawida et al. 1993), but PFGE is a much simpler and shorter technique as there is no necessity for performing Southern hybridization and preparation and labelling of IS200 probe. Other studies involving the use of multilocus enzyme electrophoresis technique (Selander et al. 1990b), lipopolysaccharide and outer membrane protein analysis (Clark et al. 2003) failed to differentiate *Salmonella Paratyphi* B DT- and DT+ (Java) strains. This is not surprising as these phenotypic characteristics are known to be less discriminatory than molecular characteristics (Maslow and Mulligan 1996).

Human salmonellosis is usually caused by consumption of contaminated food and/or water. The effluents from infected animals and humans are important sources of environmental contamination and that of the food chain. In addition, *Salmonella Paratyphi* B has a wide geographical distribution and has been isolated from a variety of sources such as tropical fish (Riley et al. 1990), goat milk cheese (Theorell et al. 1994), abattoir samples (S breakdown 2001), poultry and poultry products (Miko et al. 2002; Van Peet et al. 2003). The pulsotype X3 of the single food isolate of DT- *Salmonella Paratyphi* B was also present among the human strains. Food hygiene is therefore of utmost importance in the prevention of salmonellosis, as it is impossible to eradicate *Salmonella* primarily because it survives as a normal part of the animal's gut (Thrash et al. 2002a). Thus, there is an urgent need for the establishment of accepted criteria for microbiological control of food and food hygiene.

Four *Salmonella Paratyphi* B DT+ blood isolates exhibited pulsotypes X18a, X19, X19b, and X22 which were grouped together in cluster I. Another five blood isolates and one tissue isolates of DT- isolates exhibited pulsotypes X1, X1a, X2, X2a and X12, which were grouped into cluster II. It was noted that pulsotypes X1, X1a, X2a and X2b were shared by isolates recovered from stools and blood. Therefore there was no association between the pulsotypes and the severity of the disease. The severity of the disease may be due to multifactors such as host susceptibility and age of the patient. Our study also showed that there was no distinct correlation between the molecular subtypes and different locality in Malaysia (data not shown), implying mobility and movement of strains in the country. This finding is perhaps not surprising due to the extensive mobility of the population due to improved infrastructure.

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 characterizing *Salmonella* spp to obtain...
baseline information about particular emergent serotypes and to estimate the contribution of animal reservoirs to human cases of salmonellosis. For effective surveillance, a rapid, reproducible and reliable method is PFGE. The present study has shown that PFGE is discriminatory and takes only 2 days, starting from a pure culture, to obtain suitable chromosomal DNA for analysis (Thong et al. 2003b). This is the first report on the application of PFGE on a large collection of Salm. Paratyphi B in Malaysia. This method will be applied to other Salmonella serotype for future surveillance and control of salmonellosis in both humans and animals.

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