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Pulsed-Field Gel Electrophoresis Analysis of *Salmonella enterica* serovar Typhi Isolates in the North-East Region of Peninsular Malaysia between 2002 and 2009

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Authors' contributions

This work was carried out in collaboration between all authors. Author NFK performed the experiments and wrote the first draft of the manuscript. Author JNJ managed the literature review and document formatting. Author MHH provided the state epidemiological data. Author ARZ provided the specimens and analysed the data. Author BP reviewed the manuscript. Author IA analysed the data. Author KLT provided PFGE training for author NFK and helped analysed the data. Author RG critiqued the manuscript. Author KKP designed the study, analysed the data and finalized the manuscript.

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ABSTRACT

Aim: This study was conducted to ascertain, retrospectively, the genetic diversity and distribution of *S*. Typhi strains in Kelantan, isolated from patients with acute typhoid, asymptomatic carriers and environmental samples in Kelantan between the years 2002-2009.
Methodology: Two hundred and sixty individual *S*. Typhi isolates were characterized using pulsed-field gel electrophoresis (PFGE) using XbaI restriction enzyme, and results were interpreted according to standard guidelines.
Results: Thirty-eight pulsotypes (designated X001 to X038) and six major relatedness-clusters were found. Cluster B was predominant and accounted for 78% of the total isolates. Strains X001, X002 and X009 were found in both acute and asymptomatic subjects; and strain X023, which was found in a water sample in 2008, was also found in acute patients in 2004 and 2005. *S*. Typhi strains that were found in typhoid carriers were also found in acute patients.
Conclusions: These findings suggest close circulation of a few strains of *S*. Typhi that perpetuate the disease in the state, unlike developed countries where the high diversity of strains reported was because of immigration. Since there was no difference in the strains found between carriers and acute patients, host- rather than pathogen-factors were likely to be associated with development of the typhoid carrier state. The finding of *S.* Typhi in both the environment and acute typhoid patients indicate the resilience of the pathogen and the need to improve water sanitation in order to control the transmission of the disease in this state.

Keywords: Molecular epidemiology; PFGE; Salmonella typhi; typhoid fever.

1. INTRODUCTION

Typhoid fever is a systemic infection caused by the bacteria *Salmonella enterica* subspecies *enterica* serovar Typhi (*S*.Typhi) [1,2]. A person is confirmed to have typhoid fever when he has fever (38°C and above) for at least 3 days, and a laboratory-confirmed positive blood or stool culture for *S*.Typhi [1]. *S*.Typhi is transmitted by the fecal-oral route, acquired by ingestion of food or water contaminated by human wastes containing *S*.Typhi [3,4]. The disease is restricted to humans as its host and reservoir [3]. Typhoid fever remains a public health problem in the state of Kelantan, located in the North-East region of Peninsular Malaysia (Fig. 1).

The annual incidence rate of typhoid fever in this state is between 3.3 and 56.7 per 100,000 population, and it is the state with the highest number of typhoid cases in the country (Fig. 2).

Typhoid cases are reported not only during outbreaks, but also throughout the year as 'sporadic' cases. Between January 2002 and December 2009, the Public Health Department in Kelantan reported 2057 laboratory-confirmed typhoid fever cases. During this period the number of typhoid cases per district varied widely (e.g, in 2005 only one case was recorded in the district of Gua Musang compared to 713 in the district of Kota Bharu (Fig. 3) (Data from the Public Health Department of Kelantan).

Typhoid fever is a notifiable disease in Malaysia, and each confirmed positive case must be reported to the Ministry of Health via the Public Health Department of each state. Each patient with a history of acute typhoid fever is followed-up for a year after recovery, to screen for typhoid carrier status; i.e. whether the patients are still harboring *S*.Typhi in their stool [6].

Fig. 1. Map of the state of Kelantan, Malaysia

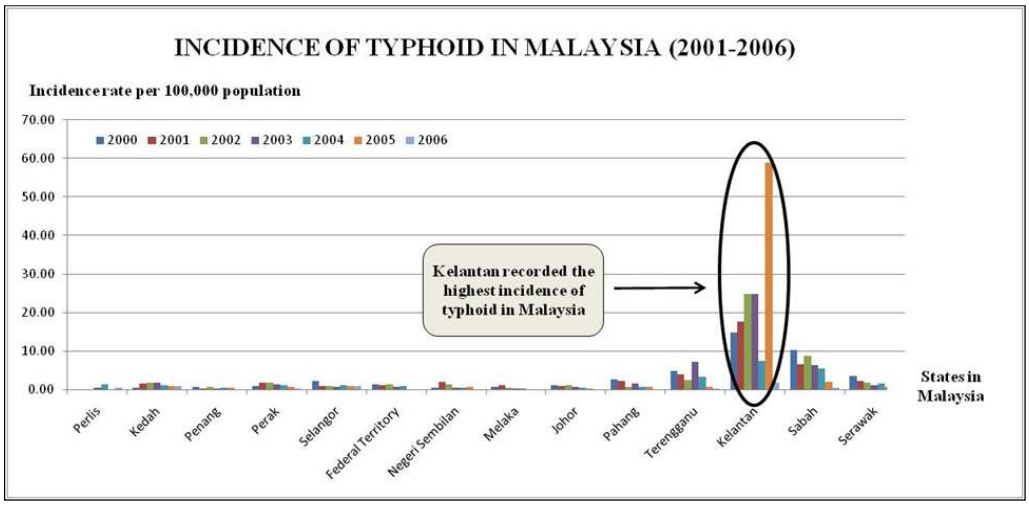

Fig. 2. Incidence of typhoid in Malaysia (Data from the Public Health Department of Kelantan, 2006 [5])

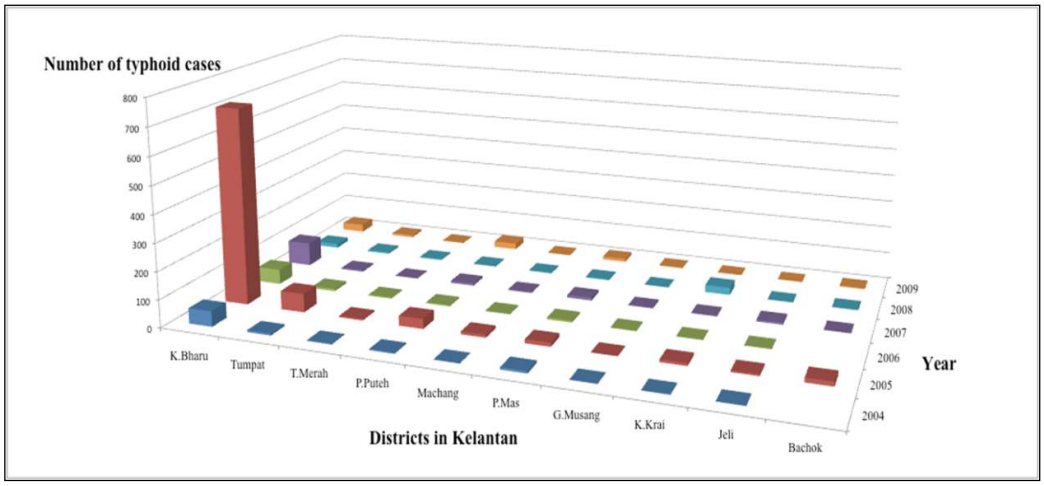

Fig. 3. Distribution of typhoid cases in the state of Kelantan between the years 2004 and 2009 (Data from the Public Health Department of Kelantan)

However, despite the burden of typhoid fever in Kelantan, detailed molecular epidemiology data is still lacking regarding the extent of genomic diversity of *S*.Typhi in this state; i.e. whether typhoid cases are due to a single strain or diverse strains of the bacteria. Pulsed-Field Gel Electrophoresis (PFGE) is considered the 'gold standard' for bacterial subtyping since it involves direct analysis of the whole bacterial genome to provide a 'finger-print' of each isolate tested. This approach has been applied to at least 40 pathogens worldwide [7] making it the most widely used method for the typing of bacterial pathogens. The method has been standardized by the Centres for Disease Control (CDC), USA, to allow global comparison of PFGE patterns for surveillance purposes [8]. Thus, the aim of this study was to ascertain, retrospectively, the genetic diversity of *S*.Typhi isolated from acute patients, asymptomatic subjects and environmental samples, in Kelantan between the years 2002 and 2009, using PFGE.

2. MATERIALS AND METHODS

2.1 Research Setting

This study was conducted retrospectively in Kelantan, a typhoid endemic state that is located in the Northeast region of Peninsular Malaysia.

All isolates available for this study were recovered between the years 2002 and 2009 including from four typhoid outbreaks that were reported by the Kelantan Public Health Department. A total of 260 individual *S*.Typhi isolates were available for this study. This included isolates recovered from acute patients (n=246), which were obtained from Hospital Universiti Sains Malaysia (HUSM), Kubang Kerian, Kelantan, and the Kuala Krai District Hospital, Kelantan, Malaysia. *S*. Typhi isolates from asymptomatic subjects (carriers) (n=13) were also obtained from INFORMM's Bacterial Bank. These were samples from routine follow-up visits by the Public Health Department staff of patients one year after recovery from acute typhoid fever, and also during routine screening of asymptomatic food-handlers in the districts following a typhoid outbreak. An environmental isolate (n=1) was also included from a screening of 27 household water samples in the district of Kuala Krai during the 2008 outbreak.

2.2 Bacterial Cultivation

All isolates were stored in glycerol stock cultures at -70°C in INFORMM's Bacterial Bank. When required, 100 µl of the bacteria glycerol stock was thawed and sub-cultured in 10 ml nutrient broth at 37°C overnight using an incubator shaker (Innova 400, USA) for 18 hours at 200 rpm. The bacterial culture was then grown in selective medium (MacConkey agar) and subsequently tested using standard biochemical and serotyping tests for confirmation of *S*. Typhi [1].

2.3 Antibiotic Susceptibility Tests

All isolates were tested for susceptibility to 6 antibiotics; Ampicillin (10 µg), Chloramphenicol (30 µg), Trimethoprim–sulfamethoxazole (25 µg), Ciprofloxacin (5 µg), Ceftriaxone (30 µg), and Nalidixic acid (30 µg), by the Kirby-Bauer disk diffusion method according to the guidelines provided by the Clinical Laboratory Standards Institute (CLSI) [9].

2.4 PFGE Procedure

PFGE protocol for molecular typing of *S*.Typhi isolates was based on the PulseNet, CDC, protocol [8]. In brief, DNA samples for PFGE analysis were prepared in agarose gel blocks, digested using restriction endonuclease *Xbal* (Fermentas, USA) and then separated using pulsed-field gel electrophoresis equipment (CHEF DR-III, Bio-Rad Laboratories). *Salmonella* serotype Braenderup strain H9812 was used as the molecular size marker. The PFGE patterns were analyzed using Fingerprinting Quest Software v.4.5 (Bio-Rad Laboratories). Due to the highly clonal nature of *S*. Typhi isolates in this study, PFGE pattern differences of a single band or more were considered different strain types. 'Relatedness Clusters' were assigned based on a Dice Coefficient of similarity (F value) ≥ 0.85 (i.e. 1-3 bands difference), consistent with the interpretation guidelines of Tenover et al. [10].

3. RESULTS

3.1 PFGE Analysis

Analysis of the 260 *S*.Typhi isolates using PFGE identified 38 PFGE patterns which were arbitrarily designated as X001 to X038 (Fig. 2). Each pattern consisted of between 13 to 18 bands within the range of 20.5 and 668.9 kilobases (kb). Isolates with different PFGE patterns were considered distinct strains of *S*.Typhi, according to the recommendation of Tenover et al. [9]. The 5 most commonly identified *S*.Typhi strains were X001, X002, X009, X011, and X037. All but one (X011) were grouped in relatedness-Cluster B. Together, these accounted for 77% (199/260) of the total isolates. The predominant strain was X001 seen in 44% (114/260) of the total isolates. The remaining 33 strains consisted of between 1-5 isolates each. *S*. Typhi strain X001 was consistently found in every year and in nearly all districts in Kelantan, with the highest number of cases recorded in the districts of Kota Bharu and Bachok (Figs. 3 & 4).

This strain also predominated in the outbreak of 2005, where 888 people were infected. For these reasons, *S*. Typhi strain X001 was considered as an endemic strain in this state.

As shown in Fig. 5, *S*. Typhi strain X002, which differed from strain X001 by only 1 band in its PFGE pattern (F= 0.93), accounted for 6% of the total isolates found in all years except 2004 and 2008.

S. Typhi strain X009 was found in all years except for 2002 and 2005 and accounted for 6% of the total isolates. *S*. Typhi strain X011 was only found in the year 2002, 2003 and 2009, and accounted for 12% of the total isolates. *S*. Typhi strain X037 was only found in the year 2008 and accounted for 9% of the total isolates in the year 2008. Kota Bharu, the capital city and district

which recorded the highest number of typhoid cases in Kelantan, displayed the most diverse number of strains (Fig. 6). Of 38 *S*. Typhi strains identified in this state, 29 strains were found in this district. The district of Bachok which is located next to the district of Kota Bharu, also showed genetic diversity with 12 different *S*. Typhi strains found.

As shown in Fig. 7, the dendrogram analysis of the 38 strains found in this study revealed 6 relatedness-Clusters designated A to F.

The majority of the strains (87%) identified were from the 4 main Clusters; A, B, C and F. However, as noted earlier, Cluster B was predominant with 12 strain types accounting for 78% (203/260) of the total *S*. Typhi isolates analysed in this study (Fig. 5). The majority of the 13 *S*. Typhi isolates from asymptomatic subjects were from Cluster B with strain types X001 (n=5), X009 (n=4), and X002 (n=1), but also included single isolates of strains X024, X033 and X038. Strains X001, X002, X009 and X024 were also found in acute typhoid subjects, whereas strains X033 and X038 were only found in the asymptomatic subjects. Interestingly, the *S*. Typhi strain X023 which was found in a household tap water sample in the district of Kuala Krai in 2008 was also found in isolates from 2 acute typhoid fever patients in the district of Kota Bharu, but in the year 2004 and 2005.

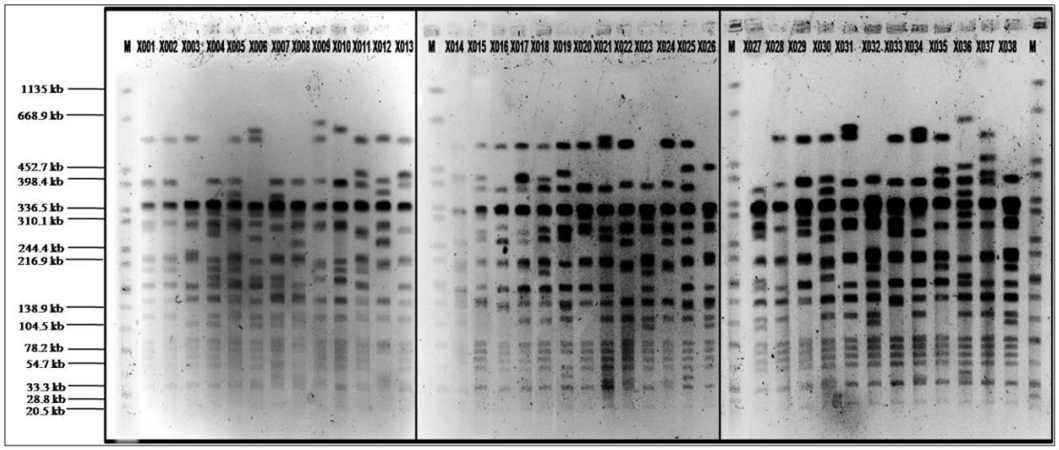

Fig. 4. Representative PFGE patterns X001 to X038 identified from a total of 260 individual *S.* Typhi isolates in the current study

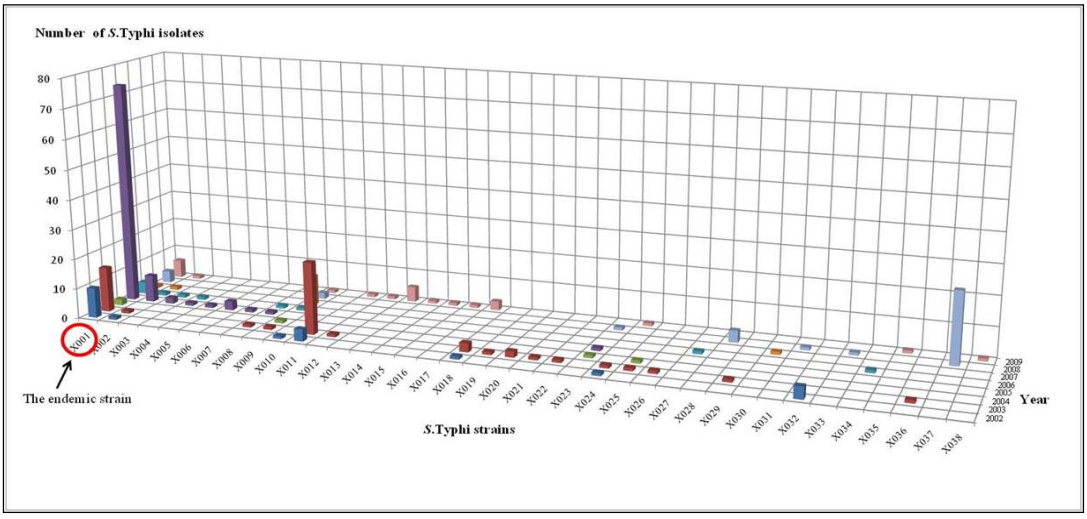

Fig. 5. Distribution of *S.* Typhi strains from 260 isolates characterized using PFGE between the year 2002 and 2009 in the state of Kelantan

3.2 Antibiotic Susceptibility Tests

Of 260 *S*. Typhi isolates tested, 28% (73/260) were sensitive to all 6 antibiotics used. The remaining 72% (191/260) isolates were resistant to at least 1 antibiotic. Of the 6 antibiotics tested, resistance to Nalidixic acid was the most common in which 12% (31/260) and 56% (146/260) of the isolates showed resistance and intermediate susceptibility, respectively. Resistance towards Ciprofloxacin was found in 2% (6/260) of the isolates and 12.0% (32/260) showed intermediate susceptibility towards this antibiotic. For Ceftriaxone, 8.8% (22/260) of the isolates showed resistance or intermediate susceptibility towards this antibiotic. Only 1 (0.4%) *S*.Typhi isolate showed resistance to 4 antibiotics; Ampicillin, Trimetophrim-Sulfametoxazole, Ciprofloxacin and Nalidixic acid, and thus was considered a multi-drug resistant (MDR) strain.

PFGE analysis on this MDR isolate showed that it belonged to strain X001. Isolates with resistance towards Nalidixic acid belonged to multiple strains of *S*. Typhi, including X001 (n=16), X002 (n=1), X003 (n=1), X009 (n=1), X011 (n=5), X033 (n=1), and X037 (n=6). Based on these findings, there was no specific PFGE pattern that could be associated with the MDR trait and resistance towards Nalidixic acid for *S*. Typhi isolates in this study.

4. DISCUSSION

Analysis of all 260 *S*. Typhi isolates in this study found 38 distinct *S*. Typhi strain types which were grouped into 6 predominant Clusters. These results suggest that typhoid fever in Kelantan, over the past 8 years, has been due to an abundance of typhoid carriers that may have contaminated food or water systems in this state. Of the 38 strains found in this study, only 4 strains were consistently found throughout the duration of the study; i.e. X001, X002, X009 and X011. Except for strain X009, all belonged to Cluster B, which is the major cluster identified in the dendrogram. This finding showed that typhoid endemicity in this state is due to the circulation of a group of closely related strains. 'Close circulation' of these strains was also supported by the finding that *S*. Typhi strains found in acute patients were also found in asymptomatic subjects, suggesting that these asymptomatic subjects may have spread the bacteria to the community. These carriers may be important reservoirs that perpetuates typhoid endemicity in this state.

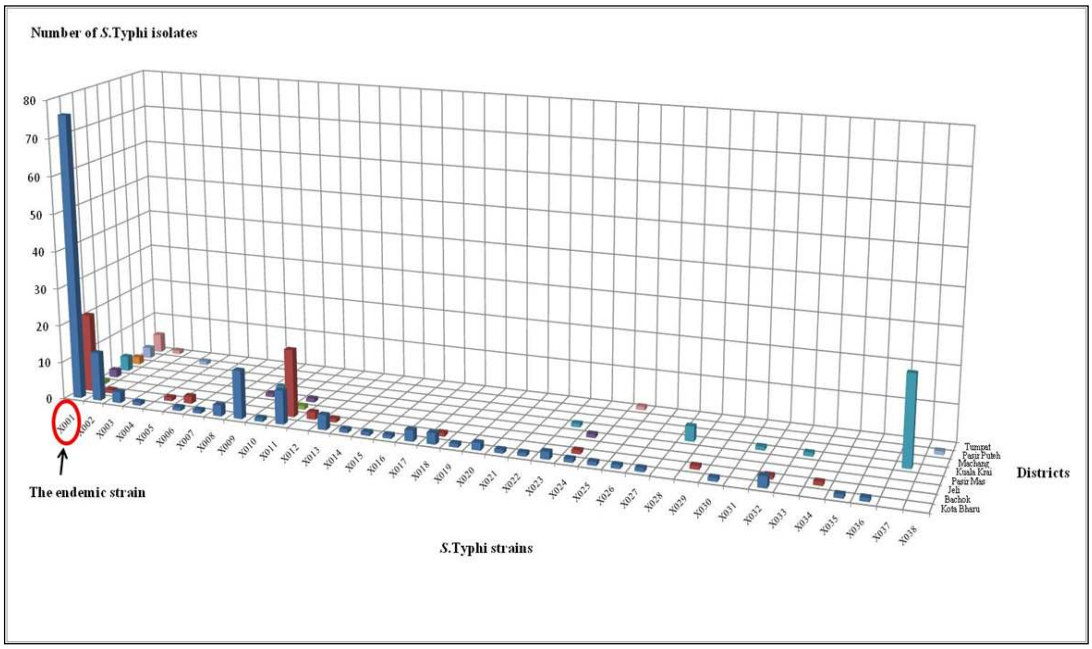

Fig. 6. Distribution of *S.* Typhi strains in 8 major districts in the state of Kelantan

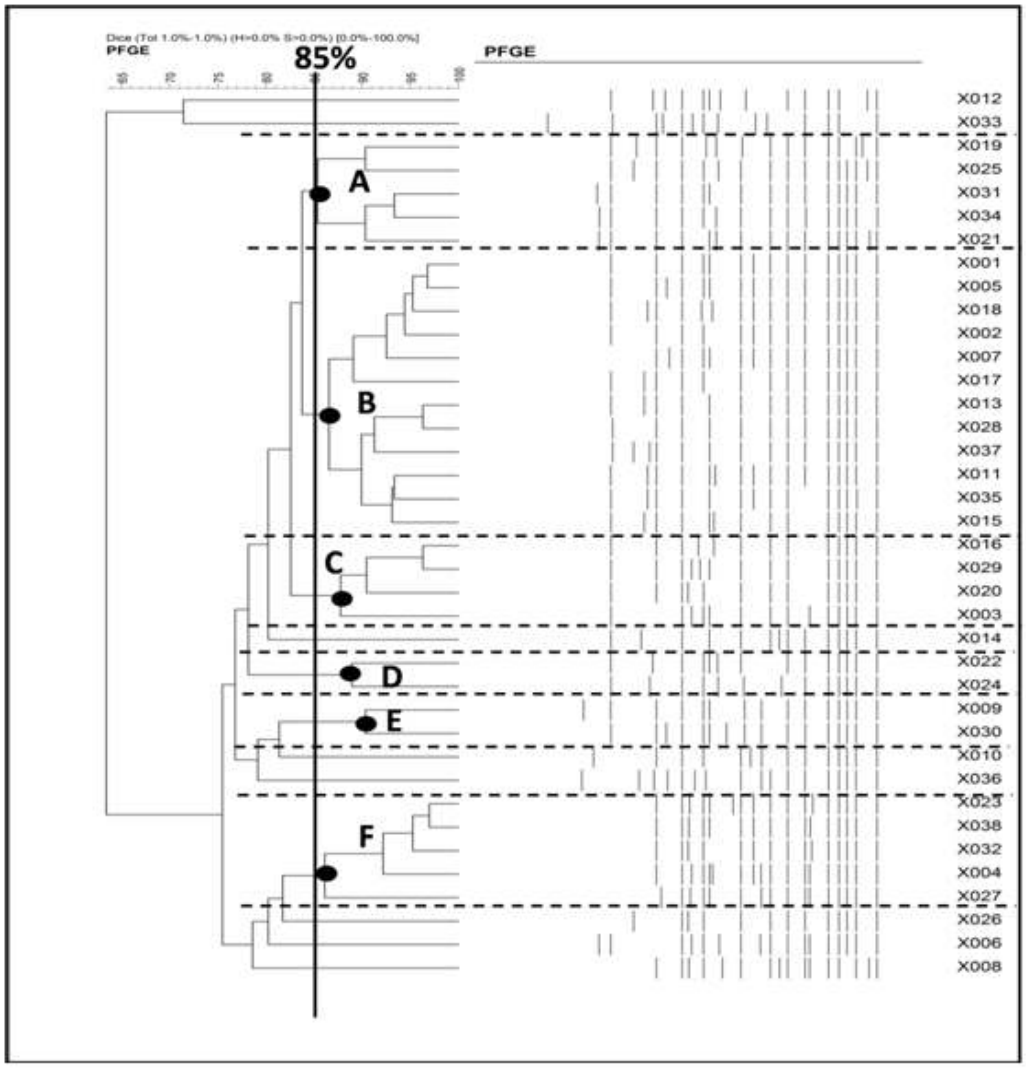

Fig. 7. Dendrogram showing the existence of 6 relatedness-Clusters from 38 distinct *S.* Typhi strains identified in this study

'Close circulation' could be the reason for the low genetic diversity of *S*. Typhi observed in this state over the 8-year period, compared to studies conducted elsewhere. Molecular epidemiology studies of *S*. Typhi conducted in Canada found 91 different strains between the periods of 2000 to 2006 [11], while a study conducted in Hong Kong between the period of 2000 to 2004 found 66 different PFGE strains of *S*. Typhi [12]. Characterization of *S*. Typhi isolates from Colombia and Argentina also exhibited high genetic diversity of *S.* Typhi, where 83 strains were found circulating in both countries [13]. A previous study on *S*. Typhi isolates from different parts of Malaysia (without mentioning the origin of the *S*. Typhi isolates) found 48 strains from 60 isolates tested [14]. The genetic diversity of *S*. Typhi strains reported in these studies were, however, attributed to foreign-acquired cases of typhoid fever, which is unlikely to be the case for typhoid endemicity in this state. In addition, more recent studies of *Salmonella* genetic diversity in Malaysia, including a limited number of *S*. Typhi isolates from Kelantan, did not identify the X001 strain type, thus underscoring the value of documenting the contribution of this endemic strain to typhoid disease in this region [15].

The identification of strain X001 in Cluster B that predominates in this state, whether in sporadic or

outbreak cases, suggests that strain X001 has a consistent route for reaching and infecting the people in the state. The typhoid outbreak in 2005, which occurred after a major flood at the end of 2004, could have resulted from contamination of drinking water sources by faecal material. The fact that strain X001 predominated during the outbreak, suggests that this strain has the ability to persist in the environment, probably through the water system, before infecting humans. This observation highlighted the problem with contaminated water systems. The single isolate from the water specimen in Kuala Krai in 2008 showed PFGE pattern X023, which did not match with the outbreak strain (X037) in the same locality. Thus, while this strain was not the cause of the 2008 outbreak in Kuala Krai, 2 isolates of X023 were obtained from acute patients in Kota Bharu in the year 2004 and 2005, suggesting that *S*.Typhi-contaminated water systems (together with the pool of typhoid carriers) could be an important factor that perpetuate the disease in the state.

In this study, the finding of a predominant *S*.Typhi strain in this region is unique compared with the results reported in other countries. For example, in the typhoid endemic country of Chile, multiple *S*.Typhi strains coexisted simultaneously to cause sporadic cases of typhoid fever [11] as has also been seen in Hong Kong, where no single dominant strain was identified from 134 *S*.Typhi isolates during the 5-year period of study [12].

For the state of Kelantan, the data suggests that *S.* Typhi was acquired locally; being transmitted in one cycle, either by typhoid carriers or contaminated water systems, rather than foreign-acquired as suggested elsewhere. These findings highlight the need for effective identification and treatment of typhoid carriers to reduce the rate of transmission in this state. Knowing that water systems could be bacterial reservoirs underscores the importance of water systems surveillance by the Public Health Department. To accomplish this, well constructed drainage systems are needed especially in the rural areas, where currently untreated sewage water is directly channeled into the river. Hygiene awareness campaigns should also be promoted to ensure that the public is aware of the dangers. This effort should be complemented with further surveillance of the water systems using rapid screening and typing methods. Improvement of the water system, as well as public health education and hygiene, has dramatically reduced the incidence of typhoid fever in the developed countries, such as the United States [16].

Despite increasing typhoid cases caused by MDR-*S*. Typhi reported worldwide, only 1 in 260 isolates was found to be MDR in this study. This indicates that the MDR strain has not spread widely in this state. However, the fact that MDR strains have arrived in this state and the continued influx of foreign labourers from Third World countries, underscores the urgent need for Public Health monitoring to ensure that such strains do not spread further. The finding of Nalidixic acid resistance in this country is concordant with other typhoid endemic places, such as in Vietnam, China, Bangladesh, India, Nepal and Pakistan, which showed a rising trend. Patients infected with Nalidixic acid-resistant *S*. Typhi strains were reported to have poor clinical response, high relapse rates and prolonged fecal carriage [17]. Thus, information on the extent of resistance toward the current drug of choice for typhoid treatment (resistant towards Nalidixic acid is used as the marker to predict susceptibility towards fluoroquinolones) is needed as this will provide knowledge on the efficacy of the drugs used to avoid failure of typhoid treatment in the future.

Due to the small size of this study, we could not correlate the MDR trait to a specific PFGE pattern as reported by Shanahan et al. [18] and Kubota et al. [19]. Shanahan et al. [18] found a specific PFGE pattern for MDR isolates that carry plasmid-encoding antibiotic resistance genes. Kubota et al. [19] reported that MDR-*S*.Typhi isolates lack genetic diversity compared to drug-susceptible isolates, and suggested that the MDR phenotype must have emerged relatively recently. The lack of correlation between PFGE pattern and Nalidixic acid resistance in *S*. Typhi isolates in this study, was contradictory to the findings reported by Hong Le et al. [20] who correlated a specfic PFGE pattern with isolates resistant to this antibiotic.

5. CONCLUSION

In conclusion, we have successfully utilized PFGE to document the molecular epidemiological profile of *S*. Typhi in this typhoid endemic state. The present study, however, poses some limitations, as it was not conducted in a population-based setting, and represented only a small snapshot of all the typhoid cases in this state. Since this is a retrospective study, missing epidemiological data prevented the

inclusion of more isolates from the INFORMM Bacterial Bank. However, the PFGE patterns identified in this study were successfully catalogued in the *S.* Typhi Bacilli Bank, thus laying the foundation for a national molecular epidemiological database for monitoring transmission of *S*. Typhi in this state. This would allow for routine molecular typing to monitor the geographical spread of such bacteria in this state, that would assist in the identification of typhoid carriers and thus, pave the way for the ultimate eradication of the disease.

ETHICAL APPROVAL

Authors declare that ethical approval has been acquired from Universiti Sains Malaysia's ethics committee and all standards laid down have been complied as stipulated.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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