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Short communication

Antibiotic susceptibility and molecular characterization of *Salmonella enterica* serovar Paratyphi B isolated from vegetables and processing environment in Malaysia

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ABSTRACT

Salmonella enterica serovar Paratyphi B (*S.* Paratyphi B) is a major foodborne pathogen distributed all over the world. However, little is known about the antibiotic resistance, genetic relatedness and virulence profile of *S.* Paratyphi B isolated from leafy vegetables and the processing environment in Malaysia. In this study, 6 *S.* Paratyphi B isolates were recovered from different vegetables and drain water of processing areas obtained from fresh food markets in Malaysia. The isolates were characterized by antibiogram, Pulsed-field gel electrophoresis (PFGE) and virulence genes. Antibiotic susceptibility test showed that 3 of the isolates were resistant to the antibiotics. These include *S.* Paratyphi B SP251 isolate, which was resistant to chloramphenicol, ampicillin, sulfonamides and streptomycin; Isolate SP246 which was resistant to chloramphenicol, sulfonamides and streptomycin and Isolate SP235 showing resistance to nalidixic acid only. PFGE subtyped the 6 *S.* Paratyphi B isolates into 6 distinct *XbaI*-pulsotypes, with a wide range of genetic similarity (0.55 to 0.9). The isolates from different sources and fresh food markets location were genetically diverse. Thirteen (*tolC*, *orgA*, *spaN*, *prgH*, *sipB*, *invA*, *pefA*, *sofB*, *msgA*, *cdtB*, *pagC*, *spiA* and *spvB*) out of the 17 virulence genes tested were found in all of the *S.* Paratyphi B isolates. Another gene (*lpfC*), was found only in one isolate (SP051). None of the isolates possessed *sifA*, *sitC* and *ironN* genes. In summary, this study provides unique information on antibiotic resistance, genetic relatedness, and virulotyping of *S.* Paratyphi B isolated from leafy vegetables and processing environment.

1. Introduction

Enteric fever is an acute bacterial infection caused by *Salmonella* Typhi (Typhoid fever) and *Salmonella* Paratyphi A, B and rarely C (Paratyphoid fever). Enteric fever continues to be an important source of morbidity and mortality, particularly among immunocompromised adults and children in developing countries (Sánchez-Vargas et al., 2011). Infection can be acquired by ingestion of contaminated food or water and transmission via hand-to-hand especially by food handlers that are asymptomatic carriers (Bhan et al., 2005). Over the years, an estimated 5 million cases of paratyphoid fever and 26 million cases of typhoid fever occur annually worldwide, causing 215, 000 deaths (Brunette, 2017).

In Malaysia, the incidence of *S. enterica* serovar Paratyphi B is relatively unknown compared to other serovars such as *S. enterica* serovar Typhimurium, *S. enterica* serovar Typhi and *S. enterica* serovar Enteritidis. Nevertheless, from 1990 to 2000, *S.* Paratyphi B was ranked fourth common serovar isolated from humans and the reported cases occurred sporadically (Goh et al., 2003; Thong et al., 2002). Besides, the total number of *S.* Paratyphi B isolates received by *Salmonella* Reference Unit at Medical Research Institute in Kuala Lumpur has considerably increased from 37 isolates in 2006 to 105 isolates in 2009 (Norazah et al., 2012). A remarkable rise in the incidence of *S.* Paratyphi B was also reported in other countries such as Germany, Canada and Italy (Mammina et al., 2002; Miko et al., 2002; Stratton et al., 2001). This demonstrates that this serovar has the potential to be an important foodborne pathogen in the near future. In addition, this serovar is widely distributed geographically and has been isolated from numerous sources such as Indian pennywort (Salleh et al., 2003), alfalfa sprouts (Stratton et al., 2001), poultry and poultry products (Miko et al., 2002), cheese from goat milk (Desenclos et al., 1996) and smoked fish (Kühn et al., 1994).

Fresh food markets are very common in Asian countries including Malaysia, providing a large variety of raw and fresh produces. The products are sold at ambient temperature and exposed to the environment, usually under deprived hygienic condition (Gorton et al., 2011).

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In Malaysia, a variety of fresh vegetables are consumed raw, locally known as 'ulam' which could impose food safety concern due to the uncertain hygienic condition of the fresh food markets.

Over the years, the emergence of multiple antibiotic-resistant *S*. Paratyphi B has been reported in the Netherland, Scotland and Canada mainly as a result of increased selective pressure. The majority of the isolates exhibited pentaresistance phenotype designated as ACSSuT showing resistance to ampicillin, chloramphenicol, streptomycin, sulfonamides and tetracycline (Djordjevic et al., 2009; Mulvey et al., 2004).

The molecular typing of *S*. Paratyphi B is important for tracking the source of infection and contamination as well as providing a better epidemiological understanding of the human host, animal and the environment. The Pulsed-field gel electrophoresis (PFGE) is a gold standard technique for molecular subtyping of pathogenic bacteria, highly discriminatory and reproducible to detect a high degree of genetic relatedness for epidemiological purposes (Donado-Godoy et al., 2015; Oloya et al., 2009; Weill et al., 2005).

The virulence genes in *Salmonella* are mostly carried in plasmids, prophages and *Salmonella* pathogenicity islands (SPIs) which are often disseminated among the bacterial population. This may be a health concern, as a number of severe *Salmonella* infections related to these virulent elements were frequently reported (Ammar et al., 2016; Li et al., 2017; Tamang et al., 2014). As a matter of fact, a few studies have reported the occurrence of *S*. Paratyphi B in vegetables, ready to eat foods (RTE) and humans in Malaysia, but the virulence genes possessed by this pathogen were rarely investigated (Abatcha et al., 2018; Salleh et al., 2003; Thong and Ang, 2011).

In this study, *Salmonella enterica* serovar Paratyphi B isolated from vegetables and processing environments in Malaysia were characterized with respect to their virulence genes as well as their PFGE profiles and antibiotic resistance.

2. Materials and methods

2.1. Bacterial strains

A total of 6 strains of *S*. *enterica* serovar Paratyphi B isolated from leafy vegetables (2 laksa leaves, 1 wild parsley, 1 sweet basil, 1 winged bean) and processing environment (drain water) were analysed. All the strains were recovered in five different fresh food markets (Kulim, Kubang Pasu, Bayan Lepas, Desa Mayang and Kuala Perlis) in the northern states of Malaysia during routine investigation within a one year period. All the fresh food markets were located distance (approximately 65–110 km) from each other. The organisms were isolated, maintained, and identified by standard biochemical and serotyping methods (Cowan and Steel, 1974).

2.2. Antibiotic susceptibility test

Kirby Bauer disk diffusion methods for antimicrobial susceptibility testing were performed on all isolates based on Clinical and Laboratory Standards Institute guideline (CLSI, 2013). The 12 antimicrobial agents tested include amoxicillin-clavulanic acid (30 μg), ampicillin (10 μg), chloramphenicol (30 μg), gentamicin (10 μg), streptomycin (10 μg), kanamycin (30 μg), nalidixic acid (30 μg), ciprofloxacin (5 μg), cephalothin (30 μg), trimethoprim-sulfamethoxazole (1.25/23.75 μg), tetracycline (30 μg) and sulfonamides (300 μg). *Escherichia coli* ATCC® 25922™ was used for quality control purposes.

2.3. PFGE analysis

Pulsed-field gel electrophoresis (PFGE) was done according to the Standardized Laboratory Protocol for Molecular Subtyping of *Salmonella* (CDC, 2013). The chromosomal DNA was digested with 20 units of *XbaI* for 2 h at 37 °C followed by separation on CHEF Mapper (Bio-Rad, USA) in 0.5 × Tris-borate-EDTA at 14 °C with a pulse ramped times of 2.2 s to 63.8 s for 19 h. Clustering was based on the unweighted pair group method with arithmetic mean (UPGMA) and was performed with BioNumerics software version 7.0 (Applied Maths, Kortrijk, Belgium).

2.4. Virulence genes detection

Crude DNA of the bacteria was prepared by boiling method. The polymerase chain reaction (PCR) for detection of virulence genes was performed by multiplex PCR (mPCR) method using primers and cycling conditions as described by Skyberg et al. (2006). Then, 3 mPCR were used to amplify 17 known virulent genes. mPCRI consisted of 6 primer pairs for *tolC*, *orgA*, *spaN*, *prgH*, *sipB* and *invA*. mPCRII consisted of another 6 primer pairs for *sifA*, *iroN*, *lpfC*, *sitC*, *pefA* and *sopB*, whereas mPCRIII consisted of another 5 primer pairs for *pagC*, *spvB*, *spiA*, *msgA* and *cdtB*.

3. Results and discussion

For the antibiotic susceptibility test, all isolates were susceptible to 7 out of 12 antibiotics tested. These include amoxicillin-clavulanic acid, gentamicin, kanamycin, ciprofloxacin, cephalothin, trimethoprim-sulfamethoxazole and tetracycline. Three isolates were resistant to other antimicrobial agents tested (Table 1); of which, *S*. *enterica* serovar Paratyphi B SP251 isolated from winged bean showed resistance to chloramphenicol, ampicillin, sulfonamides and streptomycin, while Isolate SP246 from laksa leaves exhibited resistance to chloramphenicol, sulfonamides and streptomycin. Isolate SP235 from drain water showed resistance to nalidixic acid only. The occurrence of antibiotic resistance among these isolates may be due to the excessive use of antibiotics in agriculture for the plant treatment and animal diseases (McManus et al., 2002). The emergence of resistant isolates from vegetables is of concern because they may serve as a reservoir and vector for antimicrobial resistance. Therefore, consumption of minimally processed leafy vegetables could be a source of infection with antimicrobial resistant *Salmonella* Paratyphi B. This has apparent public health implications since multidrug resistance limits the possible therapeutic treatments.

After *XbaI* digestion and electrophoresis, 6 PFGE profiles (pulsotypes) consisting of 15–19 bands were observed with F-values of 0.55 to 0.9 (Fig. 1). The dendrogram at 80% similarity generated 2 clusters of isolates with high genetic heterogeneity. The first cluster consists of 4 *S*. Paratyphi B isolates isolated from different vegetables, sampling time and locations. Each consists of different PFGE pattern differing in one to four bands (F = 0.80 to 0.90). Among these, two closely related clones

Table 1
Strains characteristics of *Salmonella enterica* Paratyphi B from vegetables and processing environment.

Strain no.	Source	Virulence genes profiles	Antibiogram
SP051	Laksa leaves	*tolC*, *orgA*, *spaN*, *prgH*, *sipB*, *invA*, *pefA*, *sofB*, *msgA*, *cdtB*, *pagC*, *spiA*, *spvB*, *lpfC*	$
SP066	Wild parsley	*tolC*, *orgA*, *spaN*, *prgH*, *sipB*, *invA*, *pefA*, *sofB*, *msgA*, *cdtB*, *pagC*, *spiA*, *spvB*	$
SP235	Drain water	*tolC*, *orgA*, *spaN*, *prgH*, *sipB*, *invA*, *pefA*, *sofB*, *msgA*, *cdtB*, *pagC*, *spiA*, *spvB*	Na
SP251	Winged bean	*tolC*, *orgA*, *spaN*, *prgH*, *sipB*, *invA*, *pefA*, *sofB*, *msgA*, *cdtB*, *pagC*, *spiA*, *spvB*	$AmpCSS_3$
SP246	Laksa leaves	*tolC*, *orgA*, *spaN*, *prgH*, *sipB*, *invA*, *pefA*, *sofB*, *msgA*, *cdtB*, *pagC*, *spiA*, *spvB*	CSS_3
SP269	Sweat basil	*tolC*, *orgA*, *spaN*, *prgH*, *sipB*, *invA*, *pefA*, *sofB*, *msgA*, *cdtB*, *pagC*, *spiA*, *spvB*	$

Abbreviations for antimicrobials: NA, nalidixic acid 30 μg; Amp, ampicillin 10 μg; C, chloramphenicol 30 μg; S, streptomycin 10 μg; S_3, sulphonamide 300 μg. $, susceptible isolates.

Fig. 1. Dendrogram showing the results of cluster analysis of the PFGE patterns of *XbaI*-digested DNA from *Salmonella enterica* serovar Paratyphi B. The isolates' codes, sources, locations (fresh food markets), dates (sampling) and pulsotypes are indicated.

were observed X1/SP246 from laksa leaves and X2/SP241 from the winged bean, both were recovered at the same time in Kulim fresh food markets. Interestingly, the 2 isolates displayed MDR resistotypes with some common antimicrobial resistance pattern. The high degree of similarity suggests that the isolates might be originated from a single clone. Another isolates in the cluster were Isolate SP051/X1 recovered from laksa leaves and SP066/X3 from wild parsley, obtained from Bayan Lepas and Kubang Pasu fresh food markets, respectively. The second cluster comprised of 2 isolates; SP235 from drain water of processing areas and SP269 from sweet basil, all recovered at a different time in Desa Mayang and Kuala Perlis fresh food markets showing diverse PFGE profiles and pulsotypes. The presence of *S*. Paratyphi B isolates from various fresh food markets with different genetic patterns and diversity possibly reflects the microbiological difference of the contamination source. Similarly, the fresh food markets environment may represent the most important source of cross-contamination of fresh produce and vegetables with *Salmonella* Paratyphi B. The PFGE analysis of *S*. Paratyphi B isolates has provided some unique insights into the molecular epidemiology of gastroenteritis and enteric fever in Malaysia as isolates from different fresh food markets exhibited wide genetic relatedness.

Thirteen (*tolC*, *orgA*, *spaN*, *prgH*, *sipB*, *invA*, *pefA*, *sopB*, *msgA*, *cdtB*, *pagC*, *spiA* and *spvB*) out of 17 virulence genes were detected among all *S*. Paratyphi B isolates as shown in Table 1. While *lpfC* detected in only 1 isolate (SP051), none of *sifA*, *sitC* and *ironN* genes were detected in all of the isolates. Many of the virulence genes found in nontyphoidal *Salmonella* spp. were shared between all *S*. Paratyphi B isolates. These include *sipB*, *pagC*, *invA*, *orgA*, *prgH* and *spaN* which are known to reside in the Pathogenicity Island, *spvB* and *pefA* in plasmids, and the *cdtB* and *tolC* located elsewhere in the *Salmonella* genome (Bäumler et al., 1996; Geng et al., 2015; Jiang et al., 2017). By comparison, Skyberg et al. (2006) detected *tolC*, *orgA*, *spaN*, *prgH*, *sipB*, *msgA*, *spiA*, *sopB*, *lpfC*, *pefA*, *pagC* and *invA* genes in various *Salmonella* isolates (100%) of birds from the United States. They were proved to play a significant role in *Salmonella* cellular invasion, survival within a cell and adhesion (Skyberg et al., 2006; Zishiri et al., 2016). The *invA*, *sipB*, *prgH*, *spaN and spiA* genes are also associated with type III secretion systems (TTSSs or Injectisome), complex structures proteins appendages that are used in effectors protein delivery across the eukaryotic cellular membrane (Hueck, 1998). Another gene detected in this study; *cdtB,* play a key role in *Salmonella* pathogenesis, such as toxin biosynthesis. According to Fardsanei et al. (2017), some nontyphoidal *Salmonella* isolates of food origin were recognized as having a certain epidemiological relationship with infections, clinical diarrhoea and human salmonellosis. The high number of virulence genes detected highlights the pathogenic potential of the studied *S*. Paratyphi B strains, which have been causing disease in humans and contaminating food for years in Malaysia. To the best of our knowledge, this is the first study that investigated the virulence genes in *S*. Paratyphi B in Malaysia.

In conclusion, the occurrence of antibiotic resistance and virulence genes in *S*. Paratyphi B isolates in vegetables is worrying and is of health and food safety concern. The subtyping data showed that the leafy vegetables and chicken processing environment isolates were genetically diverse and heterogeneous. However, there is a need for functional genomics studies to investigate the relationship between the persistence and pathogenicity genes of these isolates.

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