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Full Length Research Paper

Pulsed-field gel electrophoresis, plasmid profile and antimicrobial resistance pattern of *Salmonella typhimurium* isolated from human and retail meats

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***Salmonella enterica* subspecies *enterica* serovar Typhimurium is one of the common food pathogens which may cause human disease and/or animal infections. In an attempt to trace the clonal relationship and to discern the possible transmission of these strains from different origins, twenty-five *Salmonella typhimurium* strains isolated from retail meats and four strains isolated from food-poisoning cases were tested for antimicrobial resistance using the standard disk diffusion method. Pulsed-field gel electrophoresis was used to identify subtypes of these *S. typhimurium* strains. The results showed that in *S. typhimurium* high genetic similarity could exist. When plasmid profiles were combined with PFGE patterns, this combination further improves the discrimination of these strains. The strains of the same pattern combination may be the most epidemic strains which circulating between contaminated animal–derived meat and human. Transmission of these strains between different areas and origins might be possible.**

Key words: *Salmonella typhimurium*, antimicrobial resistance, PFGE, plasmid profile.

INTRODUCTION

Nontyphoidal *Salmonella* infections are an important public health problem worldwide (Rocourt et al., 2003). It can be isolated from raw meats, poultry and poultry products, milk and milk products (Gorman et al., 2002). In humans, *Salmonella* is one of the most common causes of bacterial gastroenteritis (Mead et al., 1999). *Salmonella* outbreaks have been usually associated with consumption of contaminated food or water. The fecal wastes from infected animals and humans are important sources of bacteria contamination of the environment and the food chain (Thong et al., 2002). In Tunisia, the surveillance for *Salmonella enterica* is carried out by the National Centre of Enteropathogenic Bacteria (*Salmonella, Shigella*, and *Vibrio cholerae*). Annually, about 2000 *Salmonella* strains are reported from all over Tunisia to the National Centre for Enteropathogenic Bacteria (Ben and Al-Gallas, 2007). *Salmonella typhimurium* is one of the most important *Salmonella* serovars that may cause foodborne disease and human salmonellosis infection (Herikstad et al., 2002; Lin and Tsen, 1999). *S. typhimurium* has a wide range of animal reservoirs and high potential to spread, as well as ability to survive in environmental water (Gatto et al., 2006). There are few reports available on *S. typhimirium* infections and their resistance to antimicrobial agents in Tunisia.

In Africa and most other developing regions, multidrug

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resistance, particularly to commonly available antimicrobial agents, remains a major challenge for the health system and *S. typhimurium* is the predominant cause of community-acquired bacteraemic illness in both children and in adults (Kariuki et al., 2006). In addition to the use of phenotypic typing methods (serotyping and antimicrobial resistance pattern), this study has applied genotypic typing methods. Pulsed-field gel electrophoresis (PFGE) which involves cutting genomic DNA at specific sites to generate fragments of different sizes followed by determination of their molecular weights by running them through an electrically charged gel matrix (Gatto et al., 2006; Powell et al., 1994) and in particular, has been widely used in the molecular epidemiological investigation of *Salmonella* spp. (Suzuki et al., 1995). The plasmid profiling is among methods used to determine and characterise possible genetic relationships between the different serovars of the genus *Salmonella* (Gatto et al., 2006; Lukinmaa et al., 2004).

The main objectives of this study were to determine the genetic relatedness of isolates of *S. typhimurium* from humans and retail meat. The potential of antibiogram, PFGE and plasmid analysis (alone and combined) were examined to trace the clonal relationship and to discern the possible transmission of *S. typhimurium* isolates from different origins.

MATERIALS AND METHODS

Bacterial strains

Twenty-nine *S. typhimurium* strains were isolated from 2006 to 2008. The non-human isolates (n=25) were recovered from a variety of retail meats (beef, n=6; chicken, n=15; minced meat, n=4). 25 g of each sample were pre-enriched into 225 ml of buffered peptone water (BPW) (Merck) and incubated at 37°C for 20 h. After pre-enrichment, 10 ml of the BPW was transferred to 100 ml selenite cystine broth (Oxoid, Basingstoke, England) and 0.1 ml of the same pre-enrichment broth was transferred to 10 ml of Rappaport-Vassiliadis broth (Difco). The cultures were then incubated at 37 and 42°C, respectively, for 24 h. A loopful of enriched broth were taken and streaked onto Bismuth Sulphite agar (oxoid) and Hektoen enteric agar (oxoid).

Inoculated plates were incubated at 37°C for 24 to 48 h and typical colonies were selected and streaked onto nutrient agar and subjected to initial biochemical screening in triple sugar iron agar (Oxoid). The human isolates (n=4) were from food-poisoning and diarrhoea cases and were obtained from the the National Centre of Enteropathogenic Bacteria (*Salmonella, Shigella*, and *V. cholerae*), Tunis, Tunisia. These strains were mainly isolated from the stool samples of unrelated patients of food-poisoning diarrhoea cases. Cultures displaying an alkaline slant and acid butt, with or without production of H_2S were confirmed by biochemical tests using an API-20E strip (bioMérieux) and PCR analysis involving the amplification of a 284 bp fragment of the *invA* gene, according to the protocol described by Malorny et al. (2003).

Salmonella serotyping

All *Salmonella* isolates were serotyped by seroagglutination with commercial antisera (Statens Serum Institut, Copenhagen, Denmark). Polyvalent *Salmonella* O and H antisera were used to obtain a presumptive diagnosis, and the definitive antigenic designation was then assigned by using monovalent antisera.

Antimicrobial susceptibility testing

Antimicrobial susceptibility testing was done using the disk diffusion assay on Mueller-Hinton agar with commercial antibiotic disks (Oxoid, Basingstoke, Hampshire, United Kingdom) according to the CLSI guidelines (Clinical and Laboratory Standards Institute, 2005). The antimicrobials used were nalidixic acid (10 µg), ampicillin (10 µg), streptomycin (10 µg), ciprofloxacin (5 µg), trimethoprim/ sulfamethoxazole (25 µg), tetracycline (30 µg), chloramphenicol (30 µg), gentamicin (30 µg), kanamycin (30 µg), sulphonamide compound (300 µg), neomycin (10), furazolidone (15 µg), amoxicillin/clavulanic acid (30 µg), cefalotin (30 µg), ceftazidime (30 µg) and cefotaxime (30 µg) (Becton Dickinson). Interpretation of inhibition zones was according to CLSI guidelines (Clinical and Laboratory Standards Institute, 2005).

Plasmid analysis

Plasmid DNA was isolated by the alkaline lysis method as described previously (Kado and Liu, 1981). Samples were analyzed by electrophoresis in 1x TBE buffer at 150 V for 4.5 h on 0.8% agarose gels with recirculation at 20°C. Plasmid-containing *Escherichia coli* strain 39R861 and a supercoiled DNA ladder (Gibco-BRL, Paisley, United Kingdom) were used as size markers. Plasmids were compared by the use of MVSP 3.1 software (Multivariate Statistics Package for PCs, RockWare Inc^R). The molecular weights of the plasmids were calculated by comparison with the external markers, and images were normalized accordingly.

Pulsed-field gel electrophoresis

Pulsed-field gel electrophoresis was preformed according to the one-day (24 to 28 h) standardized laboratory protocol for molecular subtyping of nontyphoidal *Salmonella* by PFGE (Centers for Disease Control and Prevention, 2002). A single colony of each isolate was streaked on tryptic soy agar and incubated overnight at 37°C. Using a cotton swab, a portion of the growth on the agar plate was transferred to 2 ml of cell suspension buffer (100 mM Tris, 100 mM EDTA, pH 8.0), and the cell density was adjusted to a turbidity reading of 0.50±0.02 in a Microscan turbidity meter (Dade Behring, West Sacramento Microscan turbidity meter, Calif.). Immediately, 400 µl of adjusted cell suspension was transferred to 1.5-ml microcentrifuge tubes with 20 µl of proteinase K (20 mg/ml), subsequently mixed with 400 µl of melted 1% SeaKem Gold (Cambrex, East Rutherford, N.J.). 1% sodium dodecyl sulfate agarose prepared with TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0), and 400 µl of the agarose mixture was pipetted into reusable plug molds. Three solidified agarose plugs were transferred to 50-ml polypropylene screw-cap tubes with 5 ml of cell lysis buffer (50 mM, Tris, 50 mM EDTA, 1% Sarkosyl, pH 8.0) and 25 µl of proteinase K (20 mg/ml) and incubated in a shaking water bath at 54°C for 2 h with agitation.

Therefore, plugs were washed two times with 15 ml of sterile water for 15 min each time and four more times with TE buffer for 15 min each time in a shaking water bath. Agarose-embedded DNA plugs were cut (2.0 mm) and restricted with 50 U of *Xba*I (Promega, Southampton, United Kingdom) for 2 h at 37°C. PFGE was performed on a CHEF DRIII system (Bio-Rad, Hercules, Calif.) in 0.5X Tris-Borate-EDTA (TBE) extended-range buffer (Bio-Rad) with recirculation at 14°C. DNA macrorestriction fragments were

Table 1. *Salmonella* Typhimurium PFGE subtypes, plasmid profiles and resistance patterns.

Specimen/sample source and year	No. of *S. typhimurium* isolates	PFGE Type	Plasmid profile	Resistance patterns
Human				
2006	1	*Xb*03	P01	S3, N, SXT, K
2006	1	*Xb*02	P01	AMP, NA
2007	1	*Xb*03	P01	AMP, FR
2007	1	*Xb*02	P01	AMP, CN, NA
Subtotal	4			
Meat beef				
2006	1	*Xb*01	P02	$
2007	4	*Xb*01	P02	$
2008	1	*Xb*01	P0	$
Subtotal	6			
Chicken				
2006	5	*Xb*01	P02	$
2006	1	*Xb*01	P02	AMP
2006	1	*Xb*01	P02	AMP, FR
2006	3	*Xb*01	P02	$
2007	1	*Xb*03	P01	$
2007	2	*Xb*02	P01	$
2008	1	*Xb*03	P0	$
2008	1	*Xb*03	P01	$
Subtotal	15			
Minced meat				
2006	3	*Xb*01	P03	$
2006	1	*Xb*01	P0	$
Subtotal	4			

AMP, ampicillin; CN, gentamicin; S3, sulphonamides compound; FR, furazolidone; K, kanamycin; NA, nalidixic acid; N, neomycin; SXT, sulphamethoxazole/trimethoprim; $, sensitive.

resolved on 1% SeaKem Gold agarose (Cambrex) in 0.5 X TBE buffer. DNA from *Salmonella* Braenderup H9812 restricted with *Xba*I was used as a size marker. Pulse times were ramped from 2.2 to 63.8 s during an 18-h run at 6.0 V/cm. Gel images were obtained using a Gel Doc 1000 imager (Bio-Rad) under UV transillumination. Macrorestriction patterns were compared with the use of Gelpro 3.1 software for windows (media cybernetics). A difference of at least one restriction fragment in the patterns was considered as a criterion for discrimination between different clones. Pairwise similarities between *Xba*I profiles were calculated by the Jaccard's similarity coefficient, and a dendogram was created with the unweighted pair group method with arithmetic averages (UPGMA), using the software Program MVSP 3.1 (Multivariate Statistics Package for PCs, RockWare Inc^{R}).

RESULTS

S. typhimurium isolates from human and retail meat samples taken between 2006 and 2008 are shown in Table 1. PCR results obtained in this study indicate that all *S. typhimurium* strains tested by PCR were positive for the presence of a 284 bp fragment of the *inv*A gene. Of the 25 non-human isolates and the 4 clinical isolates, 23 of the *S. typhimurium* were sensitive to all the antimicrobial agents tested. The remaining 6 isolates were resistant to one or more antimicrobial agents and all the clinical isolates were resistant to at least one antimicrobial agent. Susceptibility results appear in Table 1. The different antibiograms were: S3, N, SXT, K (n=1); AMP, NA (n=1); AMP, FR (n=2); AMP, CN, NA (n=1); and AMP (n=1). Results of plasmid screening showed that all the resistant *S. typhimurium* carried at least one plasmid. Only four plasmid profiles were observed (Table 1, Figure 2). All the resistant strains had a high molecular weight plasmid, alone or in combination with other plasmids.

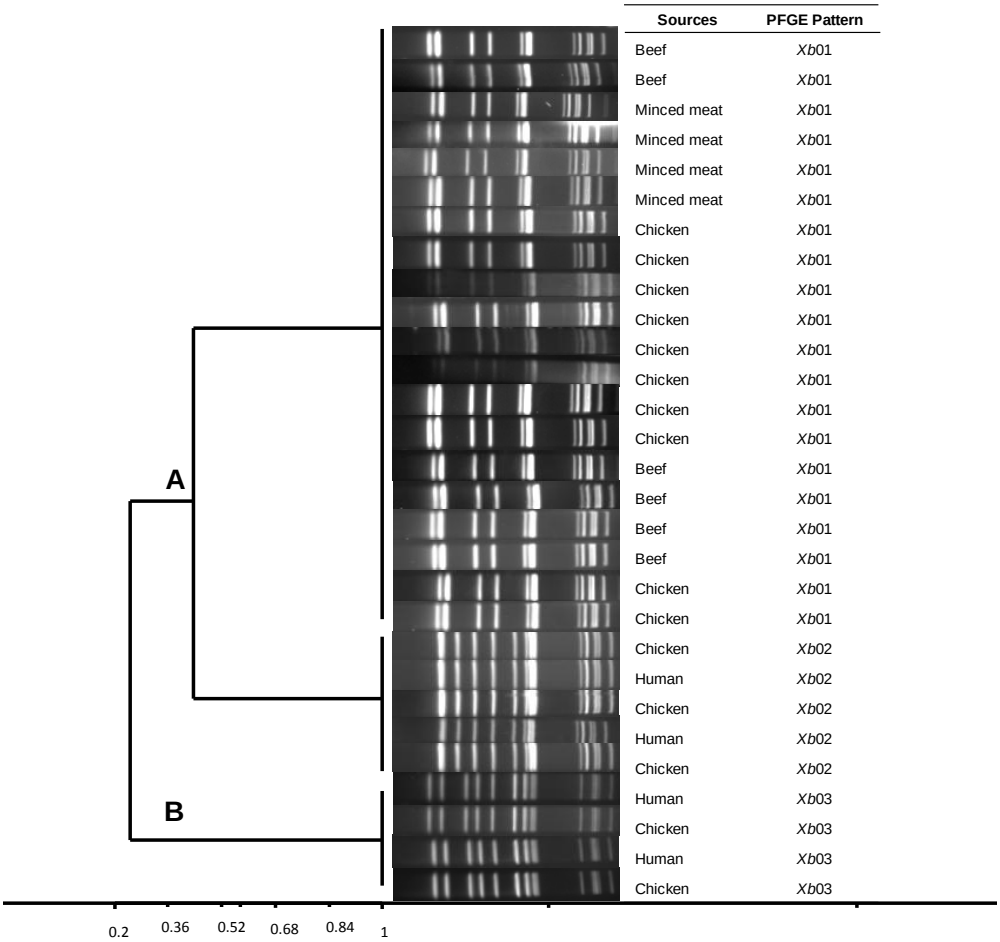
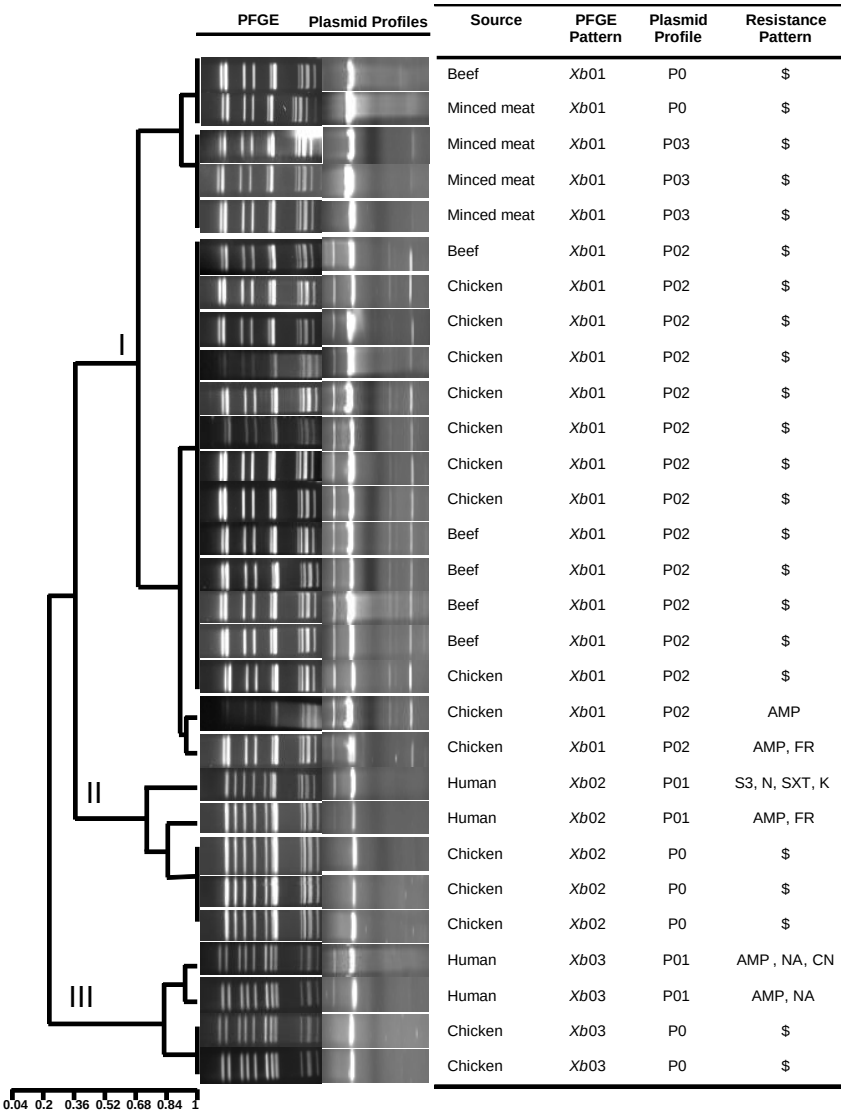


Figure 1. PFGE types for *Xba*I-digested genome DNA of *Salmonella* Typhimurium strains isolated from retail meats and and patients of sporadic cases. A total of 29 strains of serovar Typhimurium were analyzed for chromosomal DNA digestion patterns and a total of 3 PFGE types were obtained. Major clusters are marked A and B.

Overall, 15 isolates had one high molecular weight plasmid in combination with three plasmids, four isolates had one high molecular weight plasmid alone, three isolates had one plasmid and seven isolates were plasmid free.

All the 29 *S. typhimurium* isolates were typed by PFGE. Three different profiles (arbitrarily designated as *Xb*01 to *Xb*03) in two clusters A and B (Table 1 and Figure 1) were generated. Most of the strains (69%) belonged to PFGE pattern Xb01, which is the major subtype since 25 strains isolated from different locations and from different origins at different times during 2006 to 2008 showed this PFGE pattern. Also isolates from human and chicken sources showed the identical PFGE patterns. Figure 1 showed the DNA fingerprint patterns of all the *S. typhimurium* isolates. Among the two clusters (A and B); cluster A was represented by 25 strains (2 clinical strains, 13 strains isolated from chicken meat, 6 strains isolated from beef and 4 strains from minced meat). Cluster B was represented by 4 strains (2 clinical strains and 2 strains isolated from chicken meat). Thus, limited genetic diversity was found for those *S. typhimurium* and most of these strains circulating in the capital of Tunis are of very similar genetic types or are highly related.

This study has utilized a combination of antimicrobial susceptibility patterns, plasmid profiles and pulsotypes to observe the relationship among strains of *S. typhimurium* isolated from human and retail meats in the Tunisian capital. This combination further improves the discrimination of these strains. Twenty strains with a single pulsotype (*Xb*01) were further subtyped into three plasmid profiles (P0, P02, P03) and 3 resistance profiles. The results from combination of the PFGE patterns, plasmid profiling and resistance patterns were shown in Table 1 and Figure 2. A dendrogram based on unweighted pair group method with arithmetic averages (UPGMA) shows three clusters I, II and III. The main cluster consisted of 20 isolates with 5 profiles



Source	PFGE Pattern	Plasmid Profile	Resistance Pattern
Beef	*Xb*01	P0	$
Minced meat	*Xb*01	P0	$
Minced meat	*Xb*01	P03	$
Minced meat	*Xb*01	P03	$
Minced meat	*Xb*01	P03	$
Beef	*Xb*01	P02	$
Chicken	*Xb*01	P02	$
Chicken	*Xb*01	P02	$
Chicken	*Xb*01	P02	$
Chicken	*Xb*01	P02	$
Chicken	*Xb*01	P02	$
Chicken	*Xb*01	P02	$
Chicken	*Xb*01	P02	$
Beef	*Xb*01	P02	$
Beef	*Xb*01	P02	$
Beef	*Xb*01	P02	$
Beef	*Xb*01	P02	$
Chicken	*Xb*01	P02	$
Chicken	*Xb*01	P02	AMP
Chicken	*Xb*01	P02	AMP, FR
Human	*Xb*02	P01	S3, N, SXT, K
Human	*Xb*02	P01	AMP, FR
Chicken	*Xb*02	P0	$
Chicken	*Xb*02	P0	$
Chicken	*Xb*02	P0	$
Human	*Xb*03	P01	AMP , NA, CN
Human	*Xb*03	P01	AMP, NA
Chicken	*Xb*03	P0	$
Chicken	*Xb*03	P0	$

Figure 2. Relationships of *Salmonella typhimurium* strains revealed from cluster analysis of compiled data of antibiotic resistance patterns, *Xba*I-pulsotypes and plasmid profiles. Three major clusters (I, II and III) were identified. AMP, ampicillin; CN, gentamycin; FR, furazolidone; K, kanamycin; N, neomycin; NA, nalidixic acid; S3, compound sulphonamide; SXT, sulfamethoxazole-trimethoprim.

combinations while the second cluster consisted of six isolates with 3 different combinations. The third cluster consisted of 4 isolates with 3 different combinations of antimicrobial susceptibility patterns, plasmid profiles and pulsotypes. The four clinical isolates were divided into cluster 2 and 3 with non human isolates (Figure 2).

DISCUSSION

Salmonellosis continues to be one of the major food-borne illnesses worldwide (Gatto et al., 2006). The main source of human infection has been found to be contaminated food from animal origin (Ahmed et al., 2000; D'Aoust, 1994; Threlfall, 2000). The predominant serotypes change over time and differ from one geographical area to another (Zerrin et al., 2007). *S. typhimurium* is of importance in Tunisia since it showed obvious peaks in different categories: food, human, animal and environment (Ben et al., 2007). In salmonellosis, the gene *inv*A is one of a number of genes involved in the invasion of *Salmonella* into the small intestine. They are clustered in chromosomal regions called *Salmonella* pathogenicity islands. The investigation

using PCR for the presence of *inv*A gene in this study demonstrated its presence in all *S. typhimurium* isolates. This finding was similar with reports (Abouzeed, 2000; Khan et al., 2000; Torpdahl et al., 2005) that established the presence of *inv*A gene in all *Salmonella* irrespective of serovar or source.

Although, antimicrobial therapy is not essential for most *Salmonella* infection, it is necessary for treatment of invasive infections and infections in the immunocompromised patients (Bakeri et al., 2003). The selection of antimicrobial agents for the treatment of these infections has become increasingly restricted because of increasing antimicrobial resistance among *Salmonella* isolates (Bakeri et al., 2003). In the past years, ampicillin, chloramphenicol and trimethoprim-sulfamethoxazole have been the treatment of choice for *Salmonella* infections (Cheng-Hsun et al., 2004). Due to emergence of multidrug-resistant *Salmonella*, fluoroquinolones and third-generation cephalosporins are the current drugs-of-choice for the treatment of *Salmonella* infections in adults and children, respectively (Bakeri et al., 2003; Herikstad et al., 1997). The use of antimicrobial agents in animal feeding is presumed to be one of the causes for this increase (McEwen and Fedorka-Cray, 2002; Piddock, 2002) because resistance to antimicrobial agents has increased significantly in other infections with similar sources, such as campylobacteriosis (Threlfall et al., 1997; Threlfall et al., 1999).

In the present study, 25 *S. typhimurium* isolates from bovine and avian derived food products and four clinical *S. typhimurium* isolates were characterized by antimicrobial susceptibility testing, plasmid profiling and PFGE. Six of twenty-nine (0.6%) of the strains studied showed resistance to one or more antimicrobial agents. A higher resistance rate to ampicillin (83% or 5 of 6) and nalidixic acid (33% or 2 of 6) was observed. Although the level of multidrug-resistance in *S. typhimurium* remained relatively low in this study, continued surveillance for antimicrobial susceptibility remains the first approach towards detection of emergence of antimicrobial resistant strains. When PFGE patterns of the human isolates were compared with those of the non-human isolates reported in this study, identical PFGE patterns were found for strains from both origins. For example, patterns *Xb*02 and *Xb*03 were shared by human isolates and chicken meat isolates. Thus, the PFGE data may suggest that some of the sporadic cases for human salmonellosis are the results of the circulation of certain strains between contaminated animal–derived meat and human hosts. Transmission of non-typhoidal *Salmonella* isolates between human and broiler-chickens have been reported in Korea (Cheong et al., 2007). Moreover, transmission of *S. typhimurium* isolates between human, broiler chickens and human have been reported in Thailand (Tsen et al., 2002) and between human and raw-milk cheese in Northern California (Cody et al., 1999).

The results of this study show three different PFGE patterns, four different plasmid profiles and 4 different antimicrobial susceptibility profiles identified among the 29 isolates tested. There was less discriminatory power of each of these techniques used alone. These data confirm those of Sood et al. (2002) who concluded that although PFGE is a highly sensitive method for the subdivision of *Salmonella* strains within serotype, PFGE can be influenced by plasmid DNA present in the strains. In such cases, extraneous fragments will be generated which may influence the final profiles (Sood et al., 2002). Moreover, Fernandez et al. (2003) concluded that plasmid typing did not provide enough discriminatory power due to its instability and the low level of diversity of extrachromosomal DNAs harbored among *Salmonella* isolates (Fernandez et al., 2003). The gain or loss of plasmids is well established, and therefore the analysis of the plasmid profile is not a definitive typing method (Douadi et al., 2010).

The results demonstrate that the use of a combination of PFGE typing with plasmid and antimicrobial susceptibility profiling increase the ability to genetically type isolates especially when trying to separate isolates that appear clonal by a single technique. This finding agrees with the results of Tatavarthy et al. (2006) and Foley et al. (2006) who examined the genetic diversity of *Salmonella enterica* serovar Newport isolates (Tatavarthy et al., 2006) and *Salmonella enterica* serovar Typhimurium isolates (Foley et al., 2006) respectively. Strains of the same subtype have been found in meat isolates and clinical isolates from humans suffered from food-poisoning and diarrhoea. Thus, the transmissions of the strains among animal–derived meats and humans are possible. Finally, it should be mentioned that to further strengthen the above conclusions, studies with more strains collected in longer periods from different origins are necessary.

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