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Finding the Sources of Korean *Salmonella enterica* Serovar *Enteritidis* PT4 Isolates by Pulsed-field Gel Electrophoresis

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In previous studies, it has been reported that both *S. enteritidis*, the most common serotype, and *S. enteritidis* Phage Type 4 (SEPT 4) isolates were identified as the most prevalent PT in domestic poultry and also in humans in Korea until 2002. The aim of this study was to analyze the genetic diversity and epidemiological properties of both PT isolates, and also to trace the source of SEPT 4 isolates from domestic poultry and humans by Pulsed-field gel electrophoresis (PFGE). In order to understand the molecular epidemiologic properties of SEPT 4 isolates, which have very similar phenotypic properties to our preliminary investigations (serotyping, phage typing, large plasmids and antibiograms), PFGE analysis with *Xba*I enzyme was performed on the representative SEPT 4 isolates. Thirty-six SEPT 4 isolates were analyzed and differentiated with 10 pulsed-field profiles (PFP) expressing very high discriminative ability (SID: 0.921). In PFP, SEPT 4 isolates from human patients showed a perfect genetic match with those from broiler chickens and meats. Therefore, this study was able to successfully trace the major source of SEPT 4 isolates and also to determine the usefulness of the PFGE method for genetic analysis of epidemic strains.

Key words: genetic analysis, human, poultry, PFGE, SEPT 4

Salmonella is a major zoonotic pathogen of food-borne illness in Korea (Woo *et al.*, 2000 (a), (b); Kim *et al.*, 2004). In general, poultry, poultry products, cattle and dairy products have been known to be the major sources of *Salmonella*-contaminated food products that cause salmonellosis in humans (Humphrey, 1994; Liebana *et al.*, 2004). In order to understand the spread of infection during outbreaks of salmonellosis, diverse typing tools have been developed to characterize *Salmonella* isolates from individual outbreaks. These tools include traditional typing methods such as serological and phage typing and antibiotic resistance patterns (Hickman-Brenner *et al.*, 1991; Boonmar *et al.*, 1998). These techniques are currently supplemented with molecular genetic techniques such as plasmid profiling (Threlfall *et al.*, 1990; Pignato *et al.*, 1996), and DNA fingerprinting (Powell *et al.*, 1994; Lee *et al.*, 2003; Liebana *et al.*, 2004). The United Kingdom has experienced a dramatic increase in human infections of *S. enteritidis* phage type 4 (SEPT 4). SEPT 4 has been associated almost exclusively with chickens. Both poultry meat and shell eggs have been implicated as sources of infection (Threlfall *et al.*, 1990; Liebana *et al.*, 2004). Phage typing has been used with great success to trace the source of *S. enteritidis* (SE) infection in humans, although many SE isolates could not be discriminated by this method alone (Hickman-Brenner *et al.*, 1991; Humphrey, 1994).

Since 1993, our research team has regularly surveyed the isolation frequency, serotyes and phage types of *Salmonella* isolates from domestic poultry, poultry meats, eggs and environmental samples (Feeds, Feces, House rats, and Water), which has been supported by MAF (Ministry of Agriculture and Forestry) research funds and projects (Woo *et al.*, 2000a, 2000b). Based on survey results, we observed a dramatic increase of SE serotypes in both domestic poultry and their meats for the first time in 1995. Similar to some southern European countries (England, Italy, and France), this study also revealed the highest number of SE isolates to be PT 4 (Table 1 and 2) (Woo *et al.*, 2000a). However, it was not clear whether this new trend of domestic SE isolates was associated with that of human infections in Korea, since definitive epidemiological information had not been elucidated until this study, with the exception of data based on phenotypic properties and PCR based small DNA fragments (Kim *et al.*, 2004).

Diverse DNA-based analysis methods have been developed in order to differentiate molecular epidemiologic characteristics within the same *Salmonella* serotype. Because it was already known that SE had shown high

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homogenous phenotypic and genotypic properties such as serotype, PT, large plasmid (SSP: species specific plasmid), and antibiogram patterns, we could understand that the establishment of a highly discriminative genotyping method was very important to determine the definitive epidemiological characteristics.

Although a specific gene encoding certain phenotypic properties is in their genome, it may not be fully expressed under unfavorable conditions (Powell *et al.*, 1994; Liebana *et al.*, 2004). For this reason, a more stable and discriminating genotyping tool is needed to differenciate molecular epidemiologic characteristics within the same *Salmonella* serotype isolates.

Although diverse methods used to analyze SE isolates such as ribotyping, insertion sequence (IS) 200 fingerprinting, PCR-based methods, and pulsed-field gel electrophoresis (PFGE) have been developed, many researchers have recently reported that PFGE is the best choice for DNA fingerprinting of SE as well as other *Salmonella* serotypes (Kim *et al.*, 2004). PFGE is already considered to be a reliable and reproducible genotyping method for analyzing specific isolates from a putative outbreak (Powell *et al.*, 1994; Lee *et al.*, 2003; Liebana *et al.*, 2004).

In this study, PFGE was performed to analyze the genetic diversity and epidemiological properties, and also to trace the source of SEPT4 isolates from domestic poultry and humans.

Materials and Methods

Salmonella isolation and serotyping

A pre-enrichment broth for *Salmonella* isolation was prepared with tetrathionate broth base (TTB) (GibcoBRL, USA), brilliant green dye, iodine-crystal, and novobiocin (Sigma, USA), which was included to inhibit other contaminated organisms in the samples. Each sample was incubated with the TTBN-broth in a 1:10 sample broth ratio at 42 for 24 to 48 h. The TTBN-broth was cultured on a *Salmonella-Shigella* agar (SS agar) plate and incubated at 37 for 24 to 48 h. A C_8 esterase spot test (Biolife, Italy) was used to rapidly screen the *Salmonella*-specific colonies directly on the selective agar media (Olsson *et al.*, 1991). The typical blue colored fluorescent *Salmonella* colonies were collected and confirmed by standard laboratory biochemical tests. Serotype was identified using the *Salmonella* antiserum (Difco, USA) following the Kauffmann-White method.

Bacterial strains and samples

A total of 392 strains of *Salmonella* were isolated from animals (layer and broiler chicken and duck), and environmental samples (feed and water) from 1993 to 2002 (Woo *et al.*, 2000a, 2000b). In particular, rats were trapped in the area of poultry farms and were examined for *Salmonella* species microbiologically. A total of 245 SE isolates were randomly selected to cover a variety of geographical locations (n = 9 provinces) in Korea including the Jeju Island. A variety of animal species and humans were used [layer (n = 28), broiler (n = 93), duck (n = 18), swine (n = 4), and humans (n = 14)]. The 14 human SE isolates were from human patients with sporadic diarrhea in 1995. The *Salmonella* isolates were serotyped using the both plate and tube micro-agglutination method.

Phage typing (PT) of S. Enteritidis

Phage typing was performed by the method described by Ward *et al.* (1987). Standard typing phages were obtained from the Laboratory of Enteric Pathogens, Public Health Laboratory Service (PHLS), in England. Briefly, 24 h cultures on agar plates were inoculated into 3 ml of a phage broth. After incubation for 2 h with vigorous shaking, the broth was poured onto a phage agar plate. After the excess broth was removed from the plate, 10 typing phages were spotted onto an agar plate with micropipette. The dried plates were incubated overnight, and the phage lysis pattern of each culture was compared with published patterns. Strains showing a pattern that did not conform to any recognized PT were designated as "reacted but did not conform" (RDNC). Strains that did not react with any of the standard typing phages were designated as "untypeable" (UT).

Pulsed-field gel electrophoresis (PFGE)

The extraction of genomic DNA of SE and the conditions for PFGE were described previously (Lee *et al.*, 2003; Lopes *et al.*, 2004; Kim *et al.*, 2004). The conditions for PFGE and the criteria for the recognition of pulsed-field profile (PFP) types were described by Powell *et al.* (1994). Genomic DNA was digested with 10 U of restriction endonuclease *Bln*I (Boehringer Mannheim, UK) or *Xba*I (Boehringer Mannheim) at 37°C overnight. PFGE was performed with a 1% agarose gel using a CHEF Mapper XA system (Bio-Rad, USA) in a 0.5 × Tris-borate-EDTA buffer (Bioneer, Korea) at 14°C and 200V. For separation of whole genomes, a linearly ramped switching time of 5 to 50 s was applied for 24 h. After PFGE, the gel was stained with ethidium bromide (0.2 ug/ml) and photographed under a UV transilluminator. Fragment sizes were estimated by comparing them with a Lambda ladder (Bio-Rad, USA). The discriminatory ability of the PFGE method was calculated and evaluated by Simpsons' index of diversity (SID) method (Hunter and Gaston, 1988).

Results

In order to obtain the epidemiological properties of SEPT 4 isolates, PFGE using two enzymes (*Bln*I and *Xba*I) was performed on 13 SEPT 4 isolates, which were

selected from animals and humans in Korea from 1993 to 1999. PFGE produced six (*Bln*I) and five (*Xba*I) pulsed-field profiles (PFP). On PFP profiles, *Bln*I-enzyme separated into a range of 14 to 21 fragments (48.5 - 540 kb), while *Xba*I enzyme separated into a range of 9 to 16 fragments. In particular, the M-27 strain cultured from rat intestine appeared with a high degree of genetic association (>80% similarity) with two chicken strains (Yong-in and China-SE) on BE-PFP (Fig. 1). In addition, similar associations were found between SED-50 and SED-61 strains on XE-PFP (>80% similarity) (Fig. 2). These results revealed that a different enzyme produced a different PFP on the same *Salmonella* isolates by PFGE analysis. In comparison with the discriminative ability (DA) between the two enzymes, BE-PFP (SID; 0.802) produced the higher DA value (SID) than that of XE-PFP (SID; 0.747). This suggests that *Bln*I enzyme has the higher discriminatory ability on SEPT 4 isolates from Korean animals and humans than *Xba*I enzyme. However, *Xba*I was determined to be the more efficient enzyme when considering our laboratory conditions (cost and ability to procure products, etc).

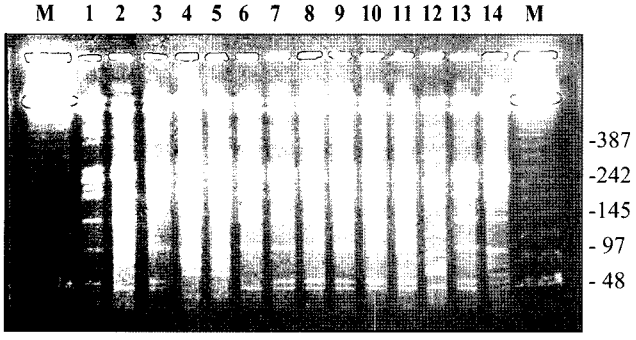

Fig. 1. Representative PFGE fingerprint patterns of *S. enteritidis* strains from domestic animals and humans in Korea after *Bln*I digestion. M, Bacteriophage lambda ladder PFGE markers (kb; Bio-Rad, Korea); lane 1 to 14, China-SE, M-27-SE, Yongin-SEPT7, D-13, D-22, D-35-SEPT1, D-37-SEPT4, D-38-SEPT4, D-39, D-40-SEPT1, D-45, D-50, D-55, and D-61-SEPT1.

Thirteen representative SEPT 4 strains from humans and poultry were analyzed with *Xba*I enzyme and were differentiated with three PFP types (from XE-I to XE-III) and in addition, three subtypes within XE-II (Fig. 2). Interestingly, four chicken strains in the XE-II group (Gj-23, AyM-A-3, Jn-23, and Dj-4-3) showed the highest genetic similarity (100%) regardless of their sources, regions and time originated. These four chicken strains also showed a very high genetic association (>90%) with SE-2-Jb and three other human strains (SE-6-Dj, SE-5-Jn, and SE-1-S). On the other hand, SE isolates from duck produced a different PFP from those of chickens (<30% similarity) (Fig. 2). This revealed that there was a big genetic difference between duck and chickens SEPT 4 isolates. In particular, SEPT 4 strains from humans showed a very high genetic association with those of broiler chicken. Therefore, this finding suggests that broiler chicken and meat is the most probable origin of human SEPT 4 isolates in Korea.

In more expended PFGE analysis by *Xba*I enzyme, 36 SE isolates [(humans; n = 8), (duck; n = 14), (chickens;

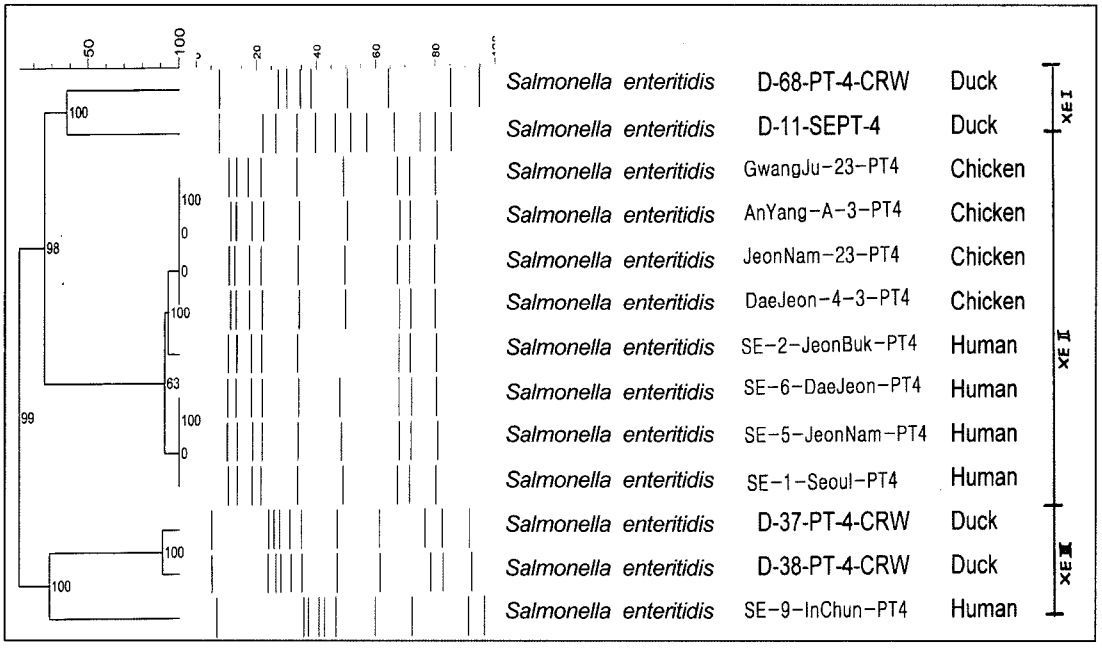

Fig. 2. Dendrogram generated by the GelCompar II software showing the relationship of 14 representative fingerprints (*Xba*I-PFGE or XE types) for 36 *Salmonella enteritidis* isolates from humans and poultry in Korea. The analysis of the bands generated was performed using the Dice coefficient and unweighted pair group method with arithmetic averages (UPGMA).

Table 1. Proportions of major phage types among *Salmonella enteritidis* isolates of domestic animals and humans from 1993 to 2000 in Korea [Woo *et al.* (a)]

PT	Phage types (PT) of *Salmonella enteritidis*			Total
	1	4	7/7a	
Number	10	128	51	245
Percentage	4.1	52.2	20.8	100

Table 2. Distribution of *S. enteritidis* phage type 4 (SEPT 4) isolates of domestic animals and humans in Korea from 1993 to 2000 [Woo *et al.* (a)]

Host	Number of identified / Number of total tested (percentage)					Total
	Layer	Broiler	Duck	Swine	Humans	
SEPT 4	11/28 (39.3)	60/93 (64.5)	10/18 (55.5)	3/4 (75.0)	8/14 (57.1)	128 (100)

n = 8), (foreign chickens; n = 4) and (mice and other; n = 2)] were differentiated into 10 PEP (XET 1 to XET 10). Nine isolates (25%), including five human isolates, were determined as the most predominant PEP (XET 9). Depending on the dendrogram, it was found that *Xba*I-PFP expressed very reliable and high DA (SID; 0.921) on the submitted isolates. Consequently, *Xba*I enzyme was determined to be a reliable and economic for PFGE of *Salmonella* species in our laboratory conditions.

Discussion

Infection of human with SE is significant for public health worldwide since SE is the most frequently reported serotype associated with gastroenteritis. In Korea, salmonellosis has increased since the early 1990's as well as Fowl typhoid, Pullorum disease and Paratyphoid infections. Furthermore, until 1998 when PT data was reported for the first time (Woo *et al.*, 2000a), we were unable to use the PT data of SE isolates from both animals and humans in official research reports. Investigating the causative materials or foods associated with gastroenteritis is difficult in Korea since there are deficiencies in the basic survey data and analysis systems. Infection of human with SE has been increasing worldwide since 1980 and have been shown to be related mainly to the consumption of eggs and egg products (Poppe *et al.*, 1992; Humphrey, 1994). *S. hadar* was a causative agent in the 1970s but currently, SEPT 4 is the major pathogen for human infections (Hickman-Brenner *et al.*, 1991; Humphrey, 1994). Understanding the factors that lead to human outbreaks of salmonellosis is essential for designing effective public-health educational programs and for improving food-processing procedures (Lopes *et al.*, 2004). The USDA-FSIS pathogen-reduction Hazard Analysis Critical Control Point (HACCP) system - a strategy based on knowledge of potential hazards - has been implemented for meat and poultry slaughter and processing plants (Lopes *et al.*, 2004). Both understanding of the risk factors and subsequent reduction of *Salmonella* transmission on farms may decrease the risk of contamination throughout the rest of the food chain. The HACCP system has been in effect in Korea since 2000 in domestic animal products processing plants. Based on preliminary examination on the prevalence of *Salmonella*, our study on *Salmonella* risk factors in domestic poultry is considered to be useful epidemiologic data for successful setting of both HACCP and *Salmonella* control programs on farms nationwide (Woo *et al.*, 2000b). To date, on-farm critical control points (CCP) for most pathogens including *Salmonella* are not well known in Korea due to the lack of epidemiologic data.

SE is known to have a wide range of animal reservoirs, high potential spread and the ability to survive in environmental water (Poppe *et al.*, 1992). In addition to poultry and poultry products, SE can be transferred to humans through fresh vegetables, raw grains, spices, and cheese (Powell *et al.*, 1994). When the animal reservoirs for *Salmonella* were traced, rats trapped near poultry farms were shown to have the highest isolation incidence rate (60%) and be the major reservoir for the *S. typhimurium* serotype. Therefore, this study showed that a systematic hygienic control program on poultry farms is preferential, and a rat control program is also needed to successful control and eradicate *Salmonella* on poultry farms in Korea (Woo *et al.*, 2000b).

Salmonella contamination in poultry is also a major problem in Asian countries. Data from Japan revealed the most common *Salmonella* serotypes from chickens in the period from 1980 to 1995 to be *S. agona, S. hadar,* and SE, in that order (Itagaki *et al.*, 2004). Data from Thailand also revealed that *Salmonella* was isolated from 6.7% (19/285) of feces from broiler chickens and SE was the most common *Salmonella* serotype. In Malaysia, *Salmonella* was isolated from 14.3% (14/98) of intestinal samples from broiler chicken and 35.5% (158/445) from broiler carcasses (Boonmar *et al.*, 1998). Compared with results from other Asian countries, the incidence rate of *Salmonella* (60.2%; by farm test) in Korean poultry was much higher level. In addition SE was the most commonly isolated serotype in both animals and humans in Korea (Woo *et al.*, 2000b). While most of the *S. muenchen* isolates were cultured from broiler chickens and meats, which were considered to be the major reservoir of this serotype, a small number of isolates were also isolated from rats.

Phage typing is a useful phenotypic analysis technique used to trace the sources and understand the epidemiological information of SE. SEPT 4 is already known to be the most common PT in England (Ward *et al.*, 1987), Germany (Schroeter *et al.*, 1994), and Italy (Pignato *et al.*, 1996), while SEPT 8 is common PT in the United States (Hickman-Brenner *et al.*, 1991), Canada (Poppe *et al.*, 1992), and the Slovak Republic (Humphrey, 1994). It is interesting that SEPT 8 was not found in either animals or

humans in Korea until this study, although SEPT 4 was the most commonly isolated PT (Table 1 and 2). The epidemiologic data presented in this study on SEPT 4 is consistent with those of some European countries such as England, France, Italy, and Germany. Although parent broiler and layer chickens have been imported from England and US similar to Japan, this epidemiologic similarity could not be clarified by phage typing data alone. In 2002, domestic SEPT 4 isolates from both animals and human were analyzed to determine their genetic diversities using the PFGE (*Xba*I) technique. The results of this analysis revealed that a clonal relationship was characteristic of SE isolates, since PFGE patterns generated from the *Xba*I enzyme are very similar among the SE isolates. However, results of the genotyping method were different from those of the phenotypic methods (serotype and phage type). This suggests that combined analysis by both phenotypic and genetic methods is required for efficient and reliable result of epidemic *Salmonella* isolates. According to the above results, SEPT 4 isolates from humans and poultry showed a very close relationship (>90% similarity) based on the dendrogram profile (Fig. 2). We were also able to find that a different genotype was produced by digestion with a different restriction enzyme (*Bln*I). In Fig. 2, *Bln*I successfully discriminated into additional subtypes among the XE4-2 group isolates. Therefore, this revealed that the selection of a suitable restriction enzyme was very important for efficient epidemiological analysis of epidemic outbreak isolates. When considering our laboratory conditions, *Xba*I enzyme was determined to be the most suitable and economic enzyme, although *Bln*I enzyme expressed more powerful discriminative ability (SID; 0.802). As the number of isolates tested increased, DA (*Xba*I; 0.912) of the PFGE method increased much more than previous results (*Xba*I; 0.702).

Conventional typing methods such as plasmid patterns, antibiograms and phage typing have been performed for further typing of diverse *Salmonella* serotypes. Since poultry products are one of the major sources of animal proteins in Korea, contaminations by zoonotic pathogens such as *Salmonella* have caused a serious economic loss for both consumers and producers. Therefore, we suggest more powerful prevention and control programs nationwide for salmonellosis should be established for the poultry industry. This study not only confirmed the usefulness and reliability of PFGE in the molecular sub-typing of SEPT 4 isolates, but also revealed the differential ability of PFGE to trace the sources of human salmonellosis.

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