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Phage Types and Pulsed-Field Gel Electrophoresis Patterns of *Salmonella enterica* serovar Enteritidis Isolated from Humans and Chickens

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We analyzed 66 *Salmonella* Enteritidis isolates in 2002. Thirty isolates were obtained from human patients with diarrhea, and 36 were obtained from chickens. A total of ten phage types (PT) were identified in the human and chicken isolates. PT1 and PT21 were the predominant PTs in both the human (20% and 13%) and chicken (17% and 47%) isolates. Twelve pulsotypes were generated by PFGE and divided into two major groups. Most of the PFGE types were categorized into cluster group 1. Eighteen chicken isolates in cluster group 1 showed high-level genetic association (>95%) with 22 other human isolates. Additionally, six chicken isolates from cluster group 2 showed fairly high-level genetic association (>95%) with the other seven human isolates. The highest levels of genetic association in humans and chickens were seen with A5-PT21 (11 isolates), A2-PT1 (7 isolates), and B1-PT4 (6 isolates). The Pulsed-Field Gel Electrophoresis (PFGE) and phage typing provided conclusive evidence that human *Salmonella* infections are attributable to the consumption of contaminated chicken.

***Keywords*:** *Salmonella* Enteritidis, pulsed-field gel electrophoresis, phage typing, resistance

Salmonella enterica species, known to induce salmonellosis in both humans and animals, are widely distributed in humans, animals, and the environment. Despite the phenotypic and genotypic similarities among serovars of *Salmonella enterica*, many of them have unique host specificities, epidemiological characteristics, and clinical manifestations.

One of the unique epidemiological characteristics of the serovar Enteritidis is that it is routinely transmitted to humans via intact chicken eggs (Clavijo *et al*., 2006). Poultry and poultry-derived products (meat and chicken eggs) have been identified as important sources of human infection with *S*. Enteritidis (Woo, 2005) and have been associated principally with the consumption of eggs and egg products (Khakhria *et al*., 1991). *Salmonella* Enteritidis is the predominant *Salmonella* serovar serotype found in eggs (Sarna *et al*., 2002; Tribe *et al*., 2002). *Salmonella* enteritidis has a specific advantage over other *Salmonella* serovars due to its ability to colonize the vaginal tissues of hens, and this higher affinity of *S*. Enteritidis for the vagina may play a crucial role in the production of many *S*. Enteritidis-contaminated eggs (Okamura *et al*., 2001). Therefore, the Enteritidis serotype is still considered the principal serotype that infects humans and poultry worldwide.

Phage typing has been employed with great success to trace sources of *Salmonella* Enteritidis infections in humans and animals, although many *S*. Enteritidis isolates could not be discriminated using this method alone (Hickman-Brenner *et al*., 1991). Pulsed-Field Gel Electrophoresis (PFGE) is a technique mostly used to separate macro-molecular weight DNA by length by applying alternating electric fields to DNA being electrophoresed on a flat gel agarose matrix. The resulting fingerprints are compared and indicate genetic differences among samples. Many researchers have recently reported that PFGE is the optimal method for DNA fingerprinting of *S*. Enteritidis, as well as other Gram-negative bacteria (Laconcha *et al*., 2000; Chung *et al*., 2004; Kim *et al*., 2004). PFGE is already known as a dependable and reproducible genotyping technique for the analysis of specific isolates from a putative outbreak (Lee *et al*., 2003; Liebana *et al*., 2004).

Formerly, phage typing and serotyping for epidemiological investigations were mainly undertaken to predict phenotypic characteristics. Nowadays, DNA typing techniques are used with more frequency for epidemiological investigations. Molecular epidemiology has been utilized to track specific pathogenic strains, and to identify the sources of salmonellosis outbreaks (Lukinmaa *et al*., 1999). The potential of these combined tools to identify sources of infection and transmission clearly indicates that they are vital components of surveillance programs.

The hypothesis of this study was that consumption of chickens, whether contaminated with *S*. Enteritidis or not, is the primary risk factor for human *Salmonella* infection. To address this hypothesis, we conducted a molecular epidemiological analysis and compared subtypes of *S*. Enteritidis isolated from humans and chickens using phage typing and PFGE.

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Materials and Methods

Bacterial strains

A total 66 isolates of *Salmonella enterica* subsp. *enterica* serovar Enteritidis were analyzed in this study. Thirty isolates were isolated from sporadic diarrheal patients in Korea, 2002 and 36 isolates were isolated from chicken in local poultry farms in Seoul and suburban areas between 1994 and 2002. Chicken isolates were received and analyzed at the Intron Biotechnology, Gyeonggi-do, Korea. *S.* Enteritidis was grown in Typtic Soy agar (Difco Laboratories)

Bacteriophage typing

Phage typing was conducted in accordance with a standardized methodology (Ward *et al*., 1987). Standard typing phages were obtained from the Laboratory of Enteric Pathogens, HPA (Human Protection Agency), England. Eighteen-hour cultures on Blood agar plates were inoculated into 3 ml of a phage broth (Double concentration Nutrient broth with 0.85% NaCl). After a 1.5 h incubation with vigorous shaking,

Table 1. Phage types of *S*. Enteritidis isolated from humans and chickens in 2002

PT	No. (%) of isolates	
	Human	Chicken
1	**6 (20)**	**6 (17)**
3	3 (10)	0
4	**4 (13)**	**3 (8)**
7	0	4 (11)
16	3 (10)	0
21	**4 (13)**	**17 (47)**
22	0	2 (6)
31	3 (10)	0
35	0	1 (3)
41	1 (3)	0
RDNC[a]	6 (20)	3 (8)

[a] RDNC: reacted but did not conform

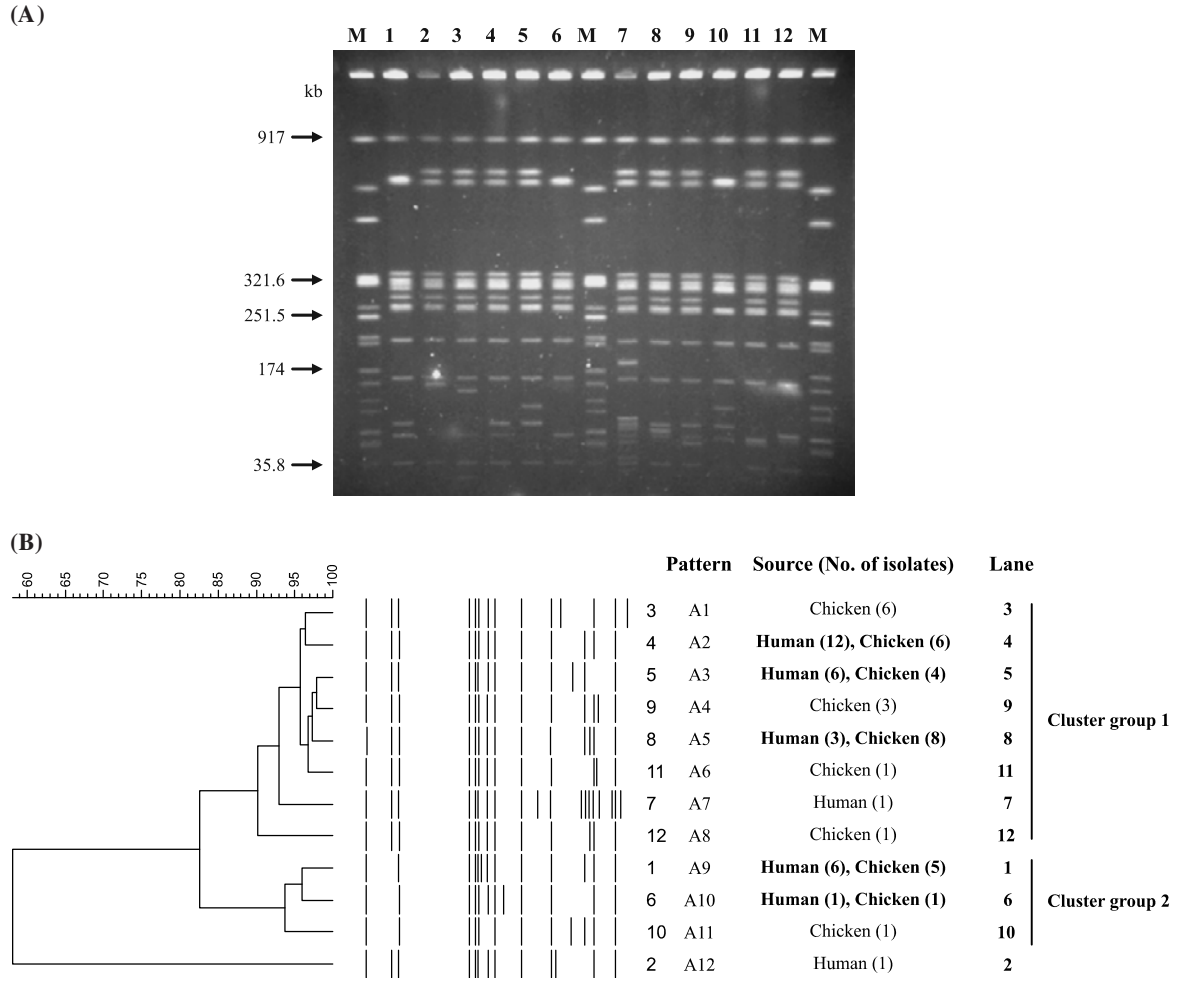

Fig. 1. (A) *Xba*I PFGE patterns of *Salmonella* Enteritidis isolates from humans and chickens. M is a *Salmonella* Newport standard size marker strain. (B) Dendrogram generated by BioNumerics software showing the relationship of 12 representative fingerprints. Analysis of PFGE banding patterns was conducted using the Dice coefficient and the unweighted pair group method with arithmetic averages (UPGMA).

the broth was poured onto phage agar plates. After the removal of excess broth from the plates, 10 typing phages were spotted per plate using a micropipette. Dried plates were incubated overnight at 37°C, and the phage lysis pattern of each culture was compared with the published pattern list. A strain with a pattern that was different from any recognized PT was designated 'RDNC'

Pulsed-Field Gel Electrophoresis (PFGE)

Agarose plugs were prepared using genomic DNA digested with the restriction enzyme *Xba*I (New England Biolabs, USA). PFGE was used to type *Xba*I-digested genomic DNA using the CHEF Mapper system (Bio-Rad Laboratories, USA). The PFGE pulsing and running conditions were an initial 2.2 sec to a final 63.8 sec for 18 h and 6 volts/cm at 14°C. *Salmonella* Newport AM01144 was utilized as a molecular size marker. After electrophoresis, the gels were stained for 20 min with ethidium bromide, destained with water for 30 min, and photographed using Gel Doc 2000 (Bio-Rad Laboratories).

Macrorestriction patterns were compared using BioNumerics software version 4.0 (Applied Maths, Belgium). A dendrogram was constructed from the PFGE types in accordance with the Dice coefficient for band matching with a 1.0% position tolerance, and an unweighted pair-grouping method with arithmetic averaging (UPGMA) cluster analysis was used to generate dendrograms describing the relationship among *Salmonella* pulsotypes (Tenover *et al*., 1995). Strains with 90% similarity or more were classified into the same cluster group.

Measurement of discriminatory power

The discriminatory power (DP) of a typing method is a measurement of its ability to distinguish between unrelated strains sampled randomly from a test population. This population can be measured with the Simpson's index of diversity (Hunter and Gaston, 1988), in which D is the index of discriminatory power, N the total number of unrelated strains tested, S the number of different types, and *nj* the number of strains belonging to the jth type, assuming that the strains can be classified into mutually exclusive categories.

Results

Phage types of *S.* Enteritidis isolated from humans and chickens

A total of ten Phage Types (PT) were identified in both the human and chicken isolates. The human isolates had seven PTs and the chicken isolates had six PTs.

The predominant PTs were PT1 and PT21 in both human (20% and 13%) and chicken (17% and 47%) isolates, followed by PT4 (13% and 8% in human and chicken respectively). PT3, 16, and 31 were all found in three. Only one human isolate was identified as PT 41. Four, two, and one isolates of PT 7, 22, and 35, respectively, were detected in chicken samples. Of the 66 isolates, nine were designated atypical (Table 1).

PFGE patterns of *S.* Enteritidis isolated from humans and chickens

To determine the epidemiological properties of *S.* Enteritidis isolates, PFGE using *Xba*I-digested genomic DNA was performed on 66 humans and chicken isolates. Restriction fragments were separated into a range of 12 to 19 fragments of 35.8~917 kb. Seven types were classified from 30 human isolates and type A2 was dominant in 12 (40%) of isolates. Chicken isolates contained ten types. The predominant type, type A5, was detected in 8 of 36 (22%) chicken isolates. PFGE generated 12 pulsed-field types in two groups. Cluster group 1 showed the highest level of genetic similarity (>90%), regardless of source. The majority of the human and chicken PFGE types were included in this cluster group. Interestingly, 18 chicken isolates in cluster group 1 showed the highest level of genetic association (>95%) with 22 other human isolates. Six chicken isolates in cluster

Table 2. The PFGE and phage type patterns for human and chicken isolates of *S.* Enteritidis

PFGE & PT	Human isolates (%)	Chicken isolates (%)	Total (%)
A1-PT21	-	4 (11.1)	4 (6.1)
A1-PT7	-	1 (2.8)	1 (1.5)
A1-RDNC	-	1 (2.8)	1 (1.5)
A2-PT1	**4 (13.3)**	**3 (8.3)**	**7 (10.6)**
A2-PT21	1 (3.3)	1 (2.8)	2 (3.0)
A2-PT22	-	1 (2.8)	1 (1.5)
A2-PT3	2 (6.7)	-	2 (3.0)
A2-PT31	1 (3.3)	-	1 (1.5)
A2-PT41	1 (3.3)	-	1 (1.5)
A2-RDNC	3 (10)	1 (2.8)	4 (6.1)
A3-PT16	2 (6.7)	-	2 (3.0)
A3-PT3	1 (3.3)	-	1 (1.5)
A3-PT31	1 (3.3)	-	1 (1.5)
A3-PT7	-	3 (8.3)	3 (4.5)
A3-RDNC	2 (6.7)	1 (2.8)	3 (4.5)
A4-PT21	-	3 (8.3)	3 (4.5)
A5-PT21	**3 (10)**	**8 (22.2)**	**11 (16.7)**
A6-PT1	-	1 (2.8)	1 (1.5)
A7-PT31	1 (3.3)	-	1 (1.5)
A8-PT21	-	1 (2.8)	1 (1.5)
A9-PT1	2 (6.7)	1 (2.8)	3 (4.5)
A9-PT16	1 (3.3)	-	1 (1.5)
A9-PT35	-	1 (2.8)	1 (1.5)
A9-PT4	**3 (10)**	**3 (8.3)**	**6 (9.1)**
A10-PT1	-	1 (2.8)	1 (1.5)
A10-PT4	1 (3.3)	-	1 (1.5)
A11-PT22	-	1 (2.8)	1 (1.5)
A12-RDNC	1 (3.3)	-	1 (1.5)
Total	30	36	66

group 2 showed the highest level of genetic association (>95%) with seven other human isolates (Fig. 1).

Combination of PFGE and phage typing

In this study, we have described the typing of 66 *S.* Enteritidis isolates using two methods. The two methods were used to identify different degrees of polymorphism in the following order: phage typing (10 types, DP is 0.8373) and PFGE (12 types, DP is 0.8476). Types obtained with each of the methods did not correspond, and thus the combination of these methods allowed for more accurate discrimination. A higher DP (0.945) was obtained using the combination of phage typing and *Xba*I PFGE analysis. A high degree of genetic association in humans and chickens was observed with A5-PT21 (11 isolates), A2-PT1 (7 isolates), and B1-PT4 (6 isolates) (Table 2).

Discussion

Salmonella contamination in chicken is a major concern in Asian countries (Lapuz *et al*., 2007; Vindigni *et al*., 2007). *S*. Enteritidis is the principal contaminating serotype in chickens and chicken products (Sarna *et al*., 2002). Recently, many countries have reported that *Salmonella* infections were caused by consumption of contaminated chickens and chicken products.

According to National Institute of Health (NIH) statistical data collected from 1998 to 2005, *S*. Enteritidis PT 21 and PT 1 were most commonly detected from human isolates in Korea between 2001 and 2005. PT 21 is known to be the prevalently detected PT in Brazil (Nunes *et al*., 2003). PT 21 has been detected in isolate from humans and poultry products between 1995 and 1997 (Nunes *et al*., 2003). Whereas PT1 is the most prevalent PT in Italy (Nastasi *et al*., 1997), Denmark (Baggesen *et al*., 1997), Finland (Lukinmaa *et al*., 2006), Spain (Soler *et al*., 2006), and Japan (Itagaki *et al*., 2004).

Phage typing has traditionally been used as the primary method for subdividing within the Enteritidis serotype and for facilitating epidemiological tracing. However, this system has some limitations. As not all organisms can be assigned to recognized types, phage conversions are still possible (Chart *et al*., 1989). Furthermore, phage typing requires access to special reagents, which are available only in reference laboratories. The discrimination power of phage typing may be too low for epidemiological analyses of *S.* Enteritidis. Additionally, the human or animal origin of *S.* Enteritidis isolates may have considerable influence on the selection of the best typing strategy for individual programs, and the use of a single method may not reliably allow strain discrimination. Therefore, a combination of phenotyping and genotyping methods is required for accurate discrimination among strain isolates.

The PFGE patterns of whole genomes of isolates from humans and chickens were quite similar. This suggests that some of the sporadic human *Salmonella* infections in Korea are due to the consumption of contaminated chicken products from Korea.

Epidemiological similarity could not be clarified solely on the basis of phage typing data. However, PFGE analysis indicated that a clonal relationship was characteristic of *S.* Enteritidis isolates. Although we did not assess the PTs of isolates from eggs and egg products within this study, we found that the PT distribution in chicken isolates was similar to that in the human isolates. Furthermore, the PFGE patterns of isolates from humans and chickens were extremely similar. This study indicates that some of the human *S*. Enteritidis infections in Korea are attributable to the consumption of contaminated chicken.

We suggest that more powerful prevention and control systems for salmonellosis be imposed nationwide on chicken and chicken products. This study confirmed the close genetic relationship among isolates of *S.* Enteritidis from humans and chickens in Korea.

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