ORIGINAL ARTICLE

Genotyping, Plasmid Analysis, and Antimicrobial Susceptibility of *Salmonella enterica* Serotype Enteritidis Isolates from Humans and Chickens in Central Taiwan

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Background/Purpose: *Salmonella enterica* serotype Enteritidis (SE) is the most frequent etiological agent of human salmonellosis. The molecular epidemiology and antimicrobial susceptibility of human and chicken isolates of SE were examined.

Methods: A total of 27 human and 40 chicken isolates of SE were collected in 2005–2006. We examined these isolates by antimicrobial susceptibility testing, pulsed-field gel electrophoresis (PFGE), and plasmid analysis.

Results: Most isolates were susceptible to the seven antibiotics tested, except chicken isolates in 2005, which showed 70% resistance to streptomycin and 75% to tetracycline. There were six plasmid profiles identified among these isolates. Almost all isolates (97%) harbored the 60-kb serotype-specific virulence plasmid. PFGE using *Xba*I digestion separated human isolates into eight subtypes (1a–1h) and chicken isolates into four subtypes (1a–1c and 1g). In 2005, 1a and 1c were predominant for human isolates and 1a for chicken isolates. However, in 2006, 1a and 1c remained predominant for human isolates and 1b and 1c for chicken isolates. Most 1b and 1c isolates belonged to plasmid type 2 or 4. Correlation between plasmid patterns and PFGE subtypes was obtained between a 36-kb plasmid and 1b and between another 3.6-kb plasmid and 1a.

Conclusion: Plasmid profiling and PFGE were efficient for discriminating SE isolates from different sources. Our data support the notion that SE is transmitted from chickens to humans, presumably through the food chain, but it appears that chickens are not the sole reservoir for human infection with SE in Taiwan. SE remained susceptible to most antimicrobial agents. [*J Formos Med Assoc* 2009;108(10):765–771]

Key Words: antibacterial drug resistance, bacterial typing techniques, genotype, plasmids, *Salmonella enterica* serotype Enteritidis

Salmonella enterica serotypes Typhimurium (ST) and Enteritidis (SE) are the two most frequent etiological agents of human salmonellosis. In the United States, SE once exceeded ST as the most common pathogen for *Salmonella*-related food-borne illnesses.[1] According to the Center for Disease Control in Taiwan, SE and ST were the two most prevalent serovars isolated from humans in

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Received: September 17, 2008
Revised: March 31, 2009
Accepted: April 2, 2009

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2003–2007. SE is capable of colonizing the reproductive tract, and can be deposited inside eggs and penetrate the yolks;[2] thus, improper handling and refrigeration of eggs are associated with outbreaks of SE.[3] Other factors such as eating contaminated chicken or undercooked eggs, and contact with infected pets also play a role in SE dissemination.[1,3,4]

As a broad-host-range pathogen, diverse SE genotypes and phage types have been reported in different geographic areas and animal species. Changes in phage-type profiles of human isolates, such as a decrease in PT4 and an increase in PT8 and PT21, have been reported from 1998 to 2003 in Europe.[5] In addition to phage typing, ribotyping, pulsed-field gel electrophoresis (PFGE) analysis and plasmid analysis have been used to characterize SE isolates.[6] In some studies, transmission of *Salmonella* from contaminated broiler meat to humans has been confirmed by PFGE.[6,7]

Human salmonellosis is usually self-limiting and antimicrobial treatment is seldom required.[8] Nontyphoidal salmonellosis has been rampant in Taiwan and caused 5.6% of food-borne outbreaks in 1986–1995.[8–12] Increased infection with SE, especially PT4, has been observed in Taiwan.[13] Most SE isolates are drug-susceptible and harbor a virulence plasmid,[14] which encodes the 8-kb *spv* operon that is responsible for enhancing *Salmonella* survival in macrophages.[15]

To evaluate the current status of SE in Taiwan, isolates were collected from two chicken farms and one hospital in central Taiwan. Plasmid analysis and genotyping were employed to study the correlation between chicken and human isolates.

Methods

Bacterial isolates

We examined 67 isolates of SE, including 27 from humans, which were collected from China Medical University Hospital, and 40 chicken isolates from National Chung Hsing University (both located in Taichung, Taiwan). These isolates were collected in 2005–2006 (Table 1). To collect chicken isolates, we randomly selected five chickens from two farms on each occasion and examined them for carriage of *Salmonella* in ovaries and cloacae. SE was identified by biochemical and O-antigen (1, 9, 12) and H-antigen (g, m; -) agglutination tests. Both antisera were purchased from Difco (part of Becton Dickinson Co., Franklin Lakes, NJ, USA). SE was maintained routinely on blood agar plates (BBL; Becton Dickinson Co.).

Antibiotic susceptibility and analysis of conserved sequence (CS) region

All isolates were examined by the disc diffusion method for their susceptibility to seven antibiotics: ampicillin (AMP), ceftriaxone (CRO), chloramphenicol (CHL), ciprofloxacin (CIP), streptomycin (STR), sulfamethoxazole–trimethoprim (SXT), and tetracycline (TET), according to the NCCLS (National Committee on Clinical Laboratory Standards) method.[16] Integrons are gene expression elements that play an important role in the recruitment of antimicrobial drug resistance determinants via site-specific recombination events catalyzed by the integron-encoded integrase. We checked the presence of integron in these isolates by detecting the CS region, using polymerase chain reaction (PCR) as described previously.[17]

Plasmid analysis and identification of virulence plasmid

Plasmid profiles were determined by the modified alkaline method.[18] A single bacterial colony was added into 5 mL Mueller–Hinton Broth (MHB; Difco) at 37°C overnight. After centrifugation of 0.5 mL overnight bacterial broth, the pellet was mixed with lysis buffer (1.5% SDS, 0.2 N NaOH, pH 12.6), and an equal amount of phenol/chloroform/isoamyl alcohol (25:24:1) (Amresco, Solon, OH, USA) was added to denature proteins. After centrifugation at 13,000 rpm for 5 minutes, 20 μL DNA solution was loaded into wells of 0.6% agarose gel and separated at 50 V for 2 hours. Plasmid profiles were visualized and recorded after staining with ethidium bromide and UV irradiation. Furthermore, DNA–DNA hybridization was conducted by using a 450-bp purified

Table 1. Characteristics of *Salmonella enterica* serotype Enteritidis strains

Isolate number	Plasmid profile	Genotype	STR/SXT/TET	Isolation			
				Species	Source	Year	Farm
1	1	1a	S/S/S	Chicken	Ovary	2005	B
1	1	1a	S/S/R	Chicken	Ovary	2005	B
8	1	1a	R/S/R	Chicken	Ovary	2005	B
1	1	1a	S/S/S	Chicken	Cloaca	2005	A
1	1	1a	S/S/R	Chicken	Cloaca	2005	A
6	1	1a	R/S/R	Chicken	Cloaca	2005	A
1	4	1c	S/S/S	Chicken	Cloaca	2005	A
7	2	1b	S/S/S	Chicken	Ovary	2006	A
5	2	1b	S/S/S	Chicken	Cloaca	2006	A
1	4	1b	S/S/S	Chicken	Ovary	2006	A
1	4	1b	S/S/S	Chicken	Cloaca	2006	A
2	4	1c	S/S/S	Chicken	Ovary	2006	A
3	4	1c	S/S/S	Chicken	Cloaca	2006	A
1	4	1c	S/S/S	Chicken	Ovary	2006	B
3	1	1a	S/S/S	Human	Blood	2005	
1	1	1a	S/R/R	Human	Blood	2005	
1	1	1a	R/S/R	Human	Blood	2005	
1	1	1b	S/S/S	Human	Blood	2005	
1	1	1d	S/S/S	Human	Blood	2005	
1	2	1f	S/S/S	Human	Blood	2005	
1	3	1a	S/S/S	Human	Blood	2005	
1	3	1a	R/S/R	Human	Blood	2005	
1	3	1b	S/S/S	Human	Blood	2005	
4	4	1c	S/S/S	Human	Blood	2005	
1	4	1h	S/S/S	Human	Blood	2005	
1	5	1c	S/R/R	Human	Blood	2005	
1	6	1g	S/S/S	Human	Blood	2005	
1	1	1e	S/S/S	Human	Blood	2006	
2	3	1a	S/S/S	Human	Blood	2006	
2	3	1a	R/S/R	Human	Blood	2006	
4	4	1c	S/S/S	Human	Blood	2006	

STR = streptomycin; SXT = sulfamethoxazole–trimethoprim; TET = tetracycline; S = susceptible; R = resistant.

PCR product from *spvC* as a probe, to confirm the presence of the virulence plasmid, according to the manufacturer's instructions (Roche, Basel, Switzerland). The primers used were: SPVC-1 5′-gag gcg ctg gat gtg cct gac t-3′ and SPVC-2 5′-gaa cgt ctg act cag gac act gt-3′.[19]

Genotype determination by PFGE

Five milliliters of overnight bacterial MHB was centrifuged at 3000 rpm (Eppendorf Centrifuge 5415D; Eppendorf, Hamburg, Germany) for 10 minutes. The bacterial pellet was suspended in 500 μL TE Buffer (10 mM Tris–HCl, pH 7.5, 0.1 mM EDTA), and then an equal amount of 1.5% TE agarose (Amresco) was added to form DNA plugs. These were treated with ES Buffer (0.5 M EDTA, pH 9, 1% sodium lauroylsarcosine) that contained 1 mg/mL proteinase K (Roche) at 50°C for 14–16 hours. Furthermore, the plugs were washed 10 times with TE buffer at 50°C for 30 minutes. DNA plugs (15 mm^2) were digested with 40 U *Xba*I (New England BioLabs, Ipswich,

MA, USA) at 37°C for 14–16 hours. Enzyme-digested DNA was loaded into 1% agarose and separated using the multi-state mode of the CHEF-Mapper XA system (Bio-Rad Laboratories, Hercules, CA, USA) at 14°C, with two continuous running conditions: Block 1, 6 V/cm for a 4–70-second switching period and ±60° separation angle for 20 hours; and Block 2, 4 V/cm for a 4–70-second switching period and ±60° separation angle for 6 hours. Phylogenetic relationships among these strains were analyzed by Bioprofil software (Vilber Lourmat, Marne La Vallée, France) using the unweighted pair group method with arithmetic mean (UPGMA) with 3% tolerance.

Statistical analysis

Unpaired Student's *t* test was used to analyze the difference in isolation rate between 2005 and 2006.

Results

Isolation and antibiotic susceptibility of SE

Among 160 chickens, the average prevalence of SE was 24.4% (39/160), and there was no difference between ovary and cloaca samples: 26.3% (21/80) for the former and 23.8% (19/80) for cloaca samples ($p = 0.9$) (Table 1). However, a significant reduction in SE was found from 2005 [40% (20/50)] to 2006 [22.2% (20/90)] ($p = 0.008$). All SE isolates were susceptible to AMP, CRO, CHL and CIP, while some resistance to STR, SXT and TET was observed. Most chicken isolates were resistant to STR (70%) and TET (75%) in 2005, but appeared susceptible to both antibiotics in 2006. Also in 2005, 11.1% (2/19) and 22.2% (4/19) of human isolates were resistant to STR and TET, respectively, and 11.2% of human isolates were resistant to SXT. In 2006, 2/9 human isolates were resistant to STR and TET simultaneously.

Plasmid profiles and virulence plasmid (pSEV) analysis

The plasmid patterns, antimicrobial susceptibility, and genotypes of the isolates and their sites of isolation are shown in Table 1. According to plasmid number and size ranging from 3.6 to 100 kb, 67 isolates were separated into six groups (Table 2). All isolates except for two harbored the 60-kb pSEV, which suggests that pSEV is important for maintenance of SE in its environmental hosts. Almost all human isolates belonged to plasmid types 1, 3 and 4. In contrast, plasmid types 1, 2 and 4 were the major types in chicken isolates. Type 1 harbored only the 60-kb pSEV and type 4 carried a 36-kb plasmid in addition to pSEV. Human-specific plasmid type 3 also harbored an extra 6-kb plasmid, and chicken-specific plasmid type 2 contained a 3.6-kb plasmid. Most 1b and 1c isolates belonged to plasmid types 2 or 4, which indicated that plasmid profiling and PFGE were efficient in the discrimination of SE isolates. No CS region (integron) was found in these isolates.

Table 2. Plasmid profiles, including the presence of virulence plasmid (pSEV), of human and chicken *Salmonella enterica* serotype Enteritidis isolates

Type	pSEV size (kb)					Human isolates		Chicken isolates		Total	
	100	60	36	6	3.6	Number	pSEV	Number	pSEV	Number	pSEV
1		+				8	8	18	18	25	25
2		+			+	1	1	12	12	14	14
3		+		+		7	7	0	0	7	7
4		+	+			9	9	9	9	18	18
5	+	+	+			1	1	0	0	1	1
6			+			1	0	1	0	2	0
Total	1	65	21	7	14	27	26	40	39	67	65

Genotypes of human and chicken isolates

Using single band differences, eight subtypes were found in human isolates and four in chicken isolates. Figure 1 demonstrates the *Xba*I-digested PFGE patterns of some representative chicken isolates. PFGE patterns appeared more diverse in human than chicken isolates (Figure 2). Although subtype 1a was the major subtype among human and chicken isolates, this subtype was distributed evenly in 2005 and 2006 for human isolates, and only appeared in 2005 for chicken isolates (Figure 2). A similar distribution was observed for subtype 1c, but it only appeared in 2006 in chicken isolates. There was a significant increase in subtype 1b in 2006 in chicken isolates. This subtype was isolated only rarely from human sources.

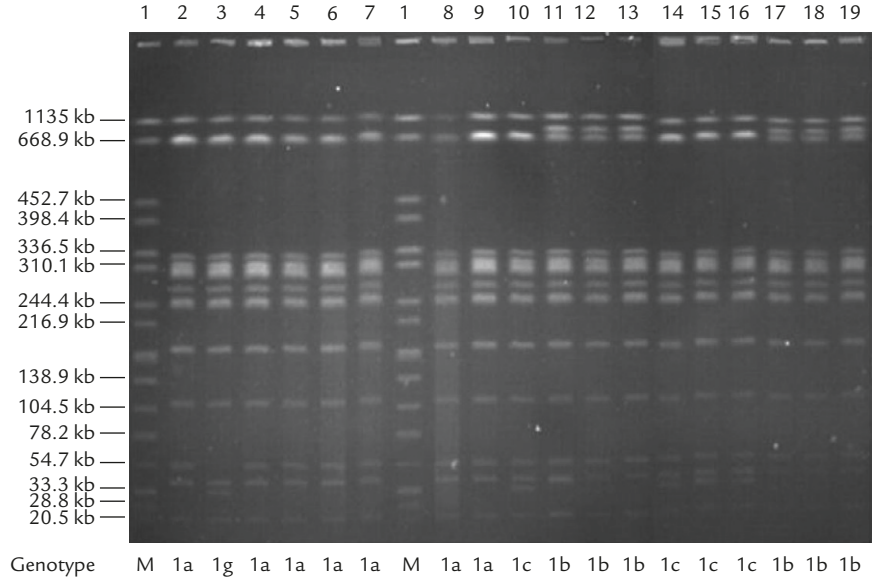
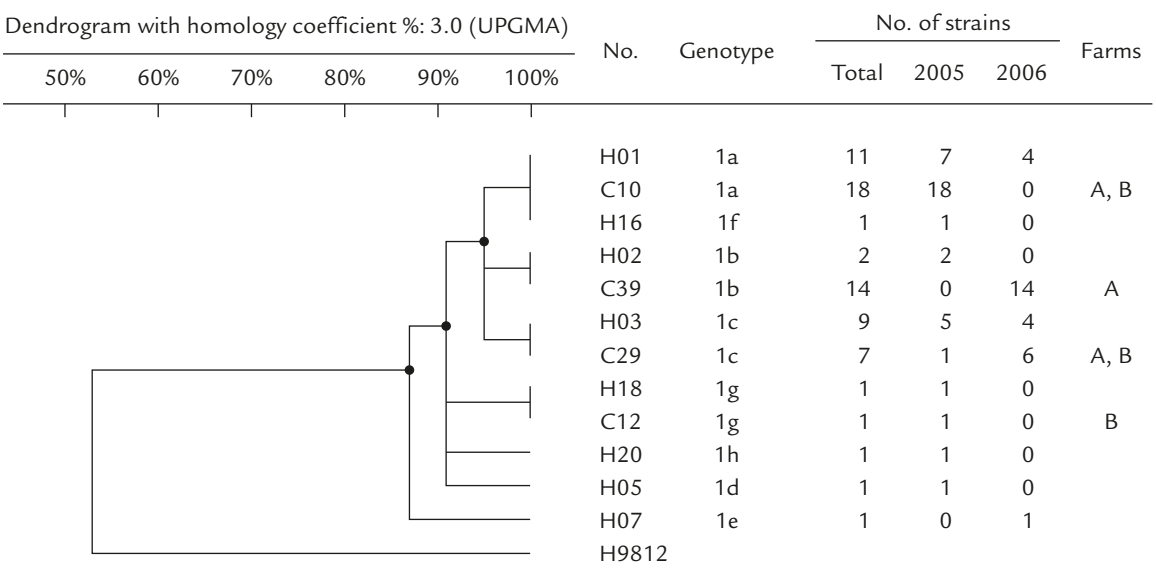


Figure 1. PFGE of *Xba*I-digested chromosomal DNA patterns of representative chicken *Salmonella enterica* serotype Enteritidis isolates. Lane 1 = *Salmonella* Braenderup strain H9812 used as DNA size marker; Lane 2 = C1; Lane 3 = C5; Lane 4 = C9; Lane 5 = C10; Lane 6 = C11; Lane 7 = C12; Lane 8 = C15; Lane 9 = C17; Lane 10 = C19; Lane 11 = C22; Lane 12 = C26; Lane 13 = C27; Lane 14 = C33; Lane 15 = C32; Lane 16 = C35; Lane 17 = C37; Lane 18 = C38; Lane 19 = C30; C = chicken.



No.	Genotype	No. of strains			Farms
		Total	2005	2006	
H01	1a	11	7	4	
C10	1a	18	18	0	A, B
H16	1f	1	1	0	
H02	1b	2	2	0	
C39	1b	14	0	14	A
H03	1c	9	5	4	
C29	1c	7	1	6	A, B
H18	1g	1	1	0	
C12	1g	1	1	0	B
H20	1h	1	1	0	
H05	1d	1	1	0	
H07	1e	1	0	1	
H9812					

Figure 2. Dendrogram of *Salmonella enterica* serotype Enteritidis isolated from humans and chickens constructed by the unweighted pair group method with arithmetic mean (UPGMA).

The majority of strains that were resistant to at least one antibiotic belonged to subtype 1a. In both farms, subtype 1a was predominant in 2005, while in 2006, the predominant subtypes shifted to 1b and 1c in farm A, and to 1c in farm B. Both subtypes were also found in human isolates, but no significant change was observed between the 2 years.

Discussion

Plasmid analysis indicated that most isolates harbored the 60-kb pSEV and a few carried an extra 36-kb plasmid, which was not present in our earlier study.[14] Furthermore, a strong correlation was found between plasmid type and genotype: plasmid type 1 (60 kb) and genotype 1a; plasmid type 2 (60 and 3.6 kb) and genotype 1b; and plasmid type 4 (60 and 36 kb) and genotype 1c. This phenomenon suggests clonal propagation of SE in human and chicken isolates. An earlier study has observed that most SE clinical isolates are drug-susceptible.[14] This situation has not changed. Except for some STR, SXT and TET resistance in chicken isolates collected in 2005, our SE isolates from humans or chickens generally remained drug-susceptible. Furthermore, STR- and/or TET-resistant strains are associated with genotype 1a in SE. Fortunately, these two agents are not used commonly in humans to treat *Salmonella* infection. There was a shift of predominant clones from 1a to 1b and 1c among chicken isolates in 2006, which indicates that the emergence of such pan-susceptible clones (1b and 1c) may have resulted from prohibition of antibiotic use in chicken feed between 2005 and 2006. Although SE is one of the most common serotypes of *Salmonella* that causes human infection in Taiwan, this serotype has remained relatively more drug-susceptible than have other common serotypes derived from human sources, such as ST.[11,14,19]

Genotypic analysis revealed a diverse origin of human isolates, which suggests that chicken isolates are not the only source for human infections. Although chicken and human SE isolates shared the same common genotypes 1a and 1c, the genotype distribution has been changing over time. For example, genotype 1c was distributed mainly in human isolates in 2005 and in chicken isolates in 2006. Furthermore, the simultaneous appearance of genotype 1c in chicken and human isolates in 2006 indicates a common reservoir or possible transmission of SE between humans and chickens.

Molecular subtyping has enhanced greatly the sensitivity and specificity of *Salmonella* surveillance.[6,7] It has allowed us to detect outbreaks that would not have been detected by traditional surveillance methods, as well as allowing us to identify rapidly specific clones of importance to public health. Traditional phage typing is time-consuming, and can be hampered by a shortage of phage suspensions. In this study, we demonstrated that plasmid profiling and PFGE were useful tools for discriminating SE isolates from different sources. Although our data support the notion that SE is transmitted from chickens to humans, presumably through the food chain, it seems that chickens are not the sole reservoir for SE infection of humans. Indeed, previous studies have shown that contact with birds and reptiles can also lead to SE infection.[20] SE infection is considered a global pandemic.[1,4,5] Continued studies to expand our knowledge about the animal reservoirs of SE, the contaminated vehicles, and any other contributing factors (travel, food or imports) that have allowed these clones of SE to proliferate and circulate in Taiwan, as well as in other areas of the world, are necessary.

Acknowledgments

This work was supported by grants from the Council of Agriculture [ASC96-AS-14.6.1-BQ-B4(3)], the National Science Council (NSC 96-2314-B-415-001), and Chang Gung Memorial Hospital (CMRPG460101), Taiwan.

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