

Prevalence and Characterization of Multidrug-Resistant (Type ACSSuT) *Salmonella enterica* Serovar Typhimurium Strains in Isolates from Four Gosling Farms and a Hatchery Farm[∇]

Chang-You Yu,¹ Shih-Jen Chou,¹ Chia-Ming Yeh,² Maw-Rong Chao,¹ Kwo-Ching Huang,³
Yung-Fu Chang,⁴ Chien-Shun Chiou,⁵ Francois-Xavier Weill,⁶ Cheng-Hsun Chiu,^{7,8}
Chi-Hong Chu,⁹ and Chishih Chu^{2*}

Department of Veterinary Medicine,¹ and Department of Microbiology and Immunology,² National Chiayi University, Chiayi, Taiwan; Bureau of Animal and Plant Inspection and Quarantine, Council of Agriculture, Executive Yuan, Taipei, Taiwan³; Department of Population Medicine and Diagnostic Science, College of Veterinary Medicine, Cornell University, Ithaca, New York 14853⁴; Central Branch Office, Center for Disease Control, Taichung, Taiwan⁵; Centre National de Référence des *Salmonella*, Institut Pasteur, Paris, France⁶; Department of Pediatrics, Chang Gung Children's Hospital, Taoyuan, Taiwan⁷; Chang Gung University College of Medicine, Taoyuan, Taiwan⁸; and Department of Surgery, National Defense Medical Center, Taipei, Taiwan⁹

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Salmonella enterica serovar Typhimurium strains of phage types DT104 and U302 are often resistant to ampicillin, chloramphenicol, streptomycin, sulfonamides, and tetracycline (the ACSSuT resistance type) and are major zoonotic pathogens. Increased consumption of goose meat may enhance the risk of transferring *S. enterica* serovar Typhimurium and other enteric pathogens from geese to human due to the consumption of meats from infected geese or improper preparation of meats. Therefore, we characterized *S. enterica* serovar Typhimurium strains isolated from four goose farms (farms A, B, C, and D) and one hatchery farm (farm E) to determine the epidemic and genetic differences among them. Antibiotic susceptibility tests and multiplex PCR confirmed that 77.6% (52/67) of strains were ACSSuT strains isolated from farms A, C, and E. Antibiotic-susceptible strains were isolated mostly from farm B, and no strain was observed in farm D. All ACSSuT strains harbored a 94.7-kb virulence plasmid and contained one 1.1-kb conserved segment identical to that of *Salmonella* genomic island 1. Four genotypes were determined among these *S. enterica* serovar Typhimurium isolates by pulsed-field gel electrophoresis analysis of XbaI-digested DNA fragments. Most isolates (85.29%; 29/34) of major genotype Ib were ACSSuT strains isolated mainly from goslings of farm C and egg membranes of farm E, a hatchery farm, suggesting that *S. enterica* serovar Typhimurium strains in isolates from goslings might originate from its hatchery, from the egg membranes to the gosling fluff after hatching. Multiple phage types, types 8, 12, U283, DT104, and U302, were identified. In conclusion, geese were a reservoir of diverse multidrug-resistant (type ACSSuT) *S. enterica* serovar Typhimurium strains, and each farm was colonized with genetically closely related *S. enterica* serovar Typhimurium strains.

Outbreaks of salmonellosis caused by *Salmonella enterica* serovar Gallinarum result in great economic losses due to the high mortality of young chickens; however, *S. enterica* serovar Typhimurium appears to have a higher invasion capability than *S. enterica* serovar Gallinarum (11). As one of the most predominant *Salmonella* serovars isolated from poultry, *S. enterica* serovar Typhimurium can be transmitted vertically through eggs and horizontally from the contaminated eggs and meat to humans (1). In ducklings, 93% of *Salmonella* isolates are *S. enterica* serovar Typhimurium (30). The infected duckling may be dehydrated and emaciated, have difficulty breathing, and even die in opisthotonus. In the United Kingdom, *S. enterica* serovar Typhimurium and *S. enterica* serovar Enteritidis have been reported to cause septicemia in young ducklings with mixed infection with other pathogens (18). In market-ready geese, *S. enterica* serovar Typhimurium is the major serovar

isolated from 60% of fluids from carcass rinsings and 18.4% of cloacal swabs (25). In addition, *S. enterica* serovar Typhimurium also caused a few cases of chronic salpingitis in geese and ducks (6).

S. enterica serovar Typhimurium usually harbors a 94.7-kb virulence plasmid (pSTV) encoding the 8-kb *spv* operon (16) that is involved in survival in the macrophage and then assists in systemic infection (21). The *ccdAB* addition system between the *spv* operon and RepFIB in pSTV (GenBank accession number AE006471) maintains plasmid stability (34). Since multidrug-resistant (type ACSSuT) phage type DT104 was discovered in 1984 (35), a 43-kb multidrug resistance DNA region responsible for the ACSSuT type has been found and named *Salmonella* genomic island 1 (SGI1), which is inserted between the *thdF* and *yidY* genes and contains two conserved-segment (CS) regions (7). Due to the process of evolution by recombination, insertion, and deletion events, SGI1 variants have been found in several serovars and may enhance the virulence and dissemination of the host strain (27). Recently, we reported multidrug-resistant (type ACSSuT) *S. enterica* serovar Typhimurium phage types DT104, DT120, and U302 isolated from

* Corresponding author. Mailing address: Department of Microbiology and Immunology, National Chiayi University, 300 University Road, Chiayi 60004, Taiwan. Phone: 886 5 2717898. Fax: 886 5 2717831. E-mail: cschu@mail.ncyu.edu.tw.

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TABLE 1. Sampling locations, methods, and clinical conditions of geese used in the study

| Farm [test date (yr/mo)] | Sampling method | Age | Clinical body condition | Test code | No. of isolates |
|--------------------------|-----------------------|---------------------|-------------------------|-----------|-----------------|
| A (2002/6) | Necropsy | 2 wk ^a | Sick | FAN | 5 |
| B (2003/11) | Cloacal swab | 3 wk ^b | Normal | CBC | 6 |
| C (2004/8–9) | Necropsy | 2–5 wk ^a | Sick | FCN | 10 |
| | Cloacal swab | 1 wk ^b | Normal | FAC1 | 3 |
| | Cloacal swab | 4 wk ^a | Sick | FAC4 | 27 |
| D (2004/8–9) | Cloacal swab | 1 wk ^b | Normal | FBC1 | 0 |
| | Cloacal swab | 4 wk ^b | Normal | FBC4 | 0 |
| E (2004/8–9) | Cloacal swab | 0 days ^b | Normal | HCG | 0 |
| | Hatching egg membrane | | | HHE | 16 |
| | Hatching cabinet | | | HHC | 0 |

^a These geese were sick at the time of clinical examination.

^b These geese were healthy at the time of clinical examination.

human in Taiwan (13). To elucidate whether these multidrug-resistant *S. enterica* serovar Typhimurium phage types can also be isolated from geese, the prevalence of multidrug-resistant *S. enterica* serovar Typhimurium and the pSTV profile, genotyping, and phage typing of these goose isolates were analyzed. Despite the existence of phage types DT104 and U302 in human and goose isolates, other *S. enterica* serovar Typhimurium phage types, types 8, 12, and U283, were isolated from geese, which were not of human origin.

MATERIALS AND METHODS

Collection and enrichment of samples. The samples were taken from five different goose farms in Chiayi, Taiwan, from 2004 to 2005. The culled sick geese were detected by necropsy and laboratory examination on farm A and farm C according to methods described previously Zander et al. (40). A method employing cloacal swabbing was used to examine *S. enterica* serovar Typhimurium infection of 4-week-old geese with diarrhea from farms A and C and healthy birds from farms B, C, D, and E (Table 1). All broths and selected agars used for isolating and identifying *S. enterica* serovar Typhimurium were purchased from Difco (Becton Dickinson Co., Franklin Lakes, NJ). The sampled swabs were enriched in 9 ml of selenite cysteine broth and incubated at 37°C for 24 h. In farm E, 15 goose hatching eggs and three dragging swabs were collected from individual cabinets. The inner membranes of eggshells were separated from the eggshell halves with sterile forceps and then incubated in 5 ml of gram-negative (GN) broth. Before sampling, the dragging swabs were autoclaved within the culture tubes of GN broth and then used to vigorously sample an area of approximately 30 cm² on the inner surfaces of the hatching cabinets. After sampling, the dragging swabs were put back into the culture tubes, which were incubated for 24 h at 37°C. If the initial subculture was *Salmonella* negative, delayed secondary enrichment was performed as described previously (37). The negative broth was incubated at room temperature for 5 to 7 days. One milliliter of the broth was then transferred into 9 ml of Rappaport-Vassiliadis broth, and the broth was incubated for 24 h at 37°C.

Salmonella isolation. Selectively enriched samples from selenite cysteine broth and GN broth were streaked onto xylose lysine deoxycholate plates. These plates were incubated at 37°C for 24 h to select typical *Salmonella* colonies, which were further identified by biochemical tests including triple sugar iron agar and lysine iron agar slants. At least two positive isolates from each plate were maintained on brain heart infusion agar plates.

Serogrouping and serotyping of Salmonella isolates. *S. enterica* serovar Typhimurium strains were identified by using commercial *Salmonella* O and H antisera provided by Difco (Becton Dickinson Co., Franklin Lakes, NJ). All isolates were first serogrouped by the slide agglutination test with the use of O antiserum. With a positive reaction to O antigens 1, 4, 5, and 12, serogroup B *Salmonella* strains were selected and further examined for their serotypes by the tube agglutination test using H antisera (H antigens 1, 1, and 2). While the second-phase antigen was not present in the first agglutination test, strains with second-phase H antigen

were selected on the other end of a paper bridge containing first-phase H antiserum across the culture medium.

Antibiotic susceptibility test. Antimicrobial susceptibility was tested by a standard disk diffusion method, and *Escherichia coli* (ATCC 25922) was used for validating antibiotic test results (28). The antimicrobial agents used were ampicillin (10 µg), chloramphenicol (30 µg), florfenicol (30 µg), streptomycin (10 µg), trimethoprim-sulfamethoxazole (1.25 µg/23.75 µg), sulfisoxazole (250 µg), and tetracycline (30 µg). Susceptible and resistant isolates were defined according to the criteria suggested by the CLSI (formerly NCCLS) (28). Multiplex PCR was used to amplify genes including *spvC* for pSTV and the following resistance genes: *pse* for ampicillin resistance (A), *floR* for chloramphenicol resistance (C), *aadA* for streptomycin resistance (S), *sulI* for sulfonamide resistance (Su), and *tetR* or *tetG* for tetracycline resistance (T) (13). In order to differentiate the integron type, PCR was performed to amplify the CS region containing a resistance gene cassette according to a method described previously (29). After PCR amplification, the PCR product of the CS region was sequenced.

Identification of virulence plasmid by DNA-DNA hybridization. The virulence plasmid (pSTV) of *S. enterica* serovar Typhimurium isolates was checked by Southern blotting hybridization with a probe of the *spvC* DNA fragment amplified by PCR as previously described (12, 33). Plasmid DNA was subjected to gel electrophoresis as previously described (23). The plasmid DNA was then transferred onto a Zeta-Probe membrane (Bio-Rad, CA) as recommended by the manufacturer. PCR products of *spvC* were purified by use of a Wizard SV gel and the PCR Clean-Up system (Promega), labeled with digoxigenin (DIG)-11-dUTP (Roche), and used as the DNA probe. After hybridization of the DIG-labeled probe and the addition of anti-DIG antibody conjugated with peroxidase, the membrane was reacted with CSPD (Roche), a chemiluminescent substrate, and then exposed to X-ray film. If both PCR and DNA hybridization were positive for the strain tested, it was concluded that the strain harbored the virulence plasmid.

Genotyping by PFGE and phage typing. Genotypes of all *S. enterica* serovar Typhimurium strains were determined by pulsed-field gel electrophoresis (PFGE) analysis using the restriction endonuclease XbaI to digest total genomic DNA. The procedure for the PFGE analysis was described previously (13). The digested DNA was separated by use of CT Mapper XA (Bio-Rad) in 0.5× Tris-borate-EDTA at 14°C for 22 h. Due to the conserved characteristic of the *Salmonella* genome, strains with a banding pattern difference of more than three bands were designated different genotypes, and strains with at least one band difference were designated different subgenotypes. Representative strains of each *S. enterica* serovar Typhimurium genotype were phage typed at the French National Center for *Salmonella* (Institut Pasteur, Paris, France) according to methods described previously by Anderson et al. (2). Phage types and genotypes were analyzed.

RESULTS

***S. enterica* serovar Typhimurium infection in geese.** The prevalence of *S. enterica* serovar Typhimurium was 7% (36 of 513 samples) for cloacal swab samples and 10.7% (16 of 150

TABLE 2. Antibiotic susceptibilities and distributions of the virulence plasmid of 67 *S. enterica* serovar Typhimurium strains from different farms

| Type of drug resistance | Total no. of isolates | CS region | | pSTV (94.7 kb) | | Sample designation(s) for isolation source(s) |
|-------------------------|-----------------------|-----------|-----------------|----------------|-----------------|---|
| | | Presence | No. of isolates | Presence | No. of isolates | |
| ACSSuT | 52 | + | 52 | + | 52 | FAC1, FAC4, FAN, FCN, HHE |
| ACSSu | 1 | – | 1 | + | 1 | FAC4 |
| CSSu | 1 | – | 1 | – | 1 | HHE |
| SSu | 1 | – | 1 | + | 1 | FAC4 |
| S | 2 | – | 2 | + | 1 | CBC |
| C | 1 | – | 1 | – | 1 | HHE |
| None ^a | 9 | + | 0 | + | 8 | CBC, FAC1 |
| Total | 67 | | 67 | | 66 | |

^a These strains were isolated from CBC of farm B.

samples) for hatching eggshell membranes. Although differing in prevalence among sampling sources, the highest isolation rate (22.9%; 27/118) was obtained in diseased sample FAC4 (C-FAC4) isolates and not in normal FAC1 (C-FAC1) isolates of farm C (Table 1). Also, no *S. enterica* serovar Typhimurium isolate was found in normal farm D and E-HCG as well as E-HHC samples (Table 1).

Antibiotic susceptibility and SGI analysis. After antibiotic susceptibility analysis by the disk diffusion method and multiplex PCR analysis, 77.6% (52/67 isolates) of *S. enterica* serovar Typhimurium were of the ACSSuT type (Table 2 and Fig. 1). Most ACSSuT strains were isolated from C-FAC4 (25/52 isolates) and E-HHE (15/52 isolates); however, these resistant strains were not found in B-CBC. Except for one strain that appeared in the 1.3- and 1.1-kb SGI1-specific CS region, all ACSSuT strains contained the 1.1-kb SGI1-specific CS region with identical sequences, which was not amplified from non-ACSSuT strains (Table 2).

Virulence plasmid, genotype, and phage types. *S. enterica* serovar Typhimurium usually harbors a 94.7-kb virulence plasmid. Here, all ACSSuT *S. enterica* serovar Typhimurium isolates (100%; 52/52 isolates) harbored pSTV, which is missing in three strains isolated from E-HHE (Table 2). To understand the genomic variation among these *S. enterica* serovar Typhimurium isolates, PFGE analysis of XbaI-digested fragments separated 58 strains into four major genotypes (Fig. 2). Genotype distributions varied among isolation sources, and the major genotype, genotype Ib (74%; 43/58 isolates), appeared only

in E-HHE as well as C-FAC1 and C-FAC4 (Table 3). Almost all isolates showed identical genotypes, such as genotype III found only in B-CBC samples, or one major genotype at the same farm (Table 3). However, three genotypes (genotypes I, II, and IV) were observed in C-FAC4.

Phage typing further differentiated these strains and determined the existence of a DT104 strain. Although only seven of the tested strains were analyzed, five phage types, types 8, 12, DT104, U283, and U302, were obtained (Fig. 2). Among these genotypes, genotype Ib was further divided into three phage types, types 8, 12, and U302. In contrast, the same phage type (types 8 and 12) appeared in different genotypes. Moreover, similar to genotype distribution, multiple phage types were observed in C-FAC4 strains.

DISCUSSION

S. enterica serovar Typhimurium is widely distributed among diverse ranges of animals, and hence, the prevalence of the pathogen in poultry is not unexpected. In poultry, *S. enterica* serovar Typhimurium appeared to have a superior invasion capability compared to *S. enterica* serovar Gallinarum (11). Therefore, *S. enterica* serovar Typhimurium has been frequently found in young chickens, imported frozen chickens, ducklings, and geese in different countries (6, 18, 20, 25, 26, 28, 30). Although *S. enterica* serovar Typhimurium may not be a primary cause of disease, it could possibly cause septicemia in young ducklings with complications of other diseases (18). The association of *S. enterica* serovar Typhimurium with septicemia or diarrhea in young geese (farms A and C) and subclinical infection with *S. enterica* serovar Typhimurium (farm B) imply that systemic infection with *S. enterica* serovar Typhimurium with complications of other pathogens may cause the death of geese. Previous reports indicated that the 94.7-kb plasmid pSTV is involved in systemic infection (21). In the present study, most of the *S. enterica* serovar Typhimurium isolates collected from human and geese harbored pSTV (13) (Table 2), which carries the *ccdAB* addiction system (34), and the *spv* operon (21), both responsible for the existence of pSTV in *S. enterica* serovar Typhimurium and the enhancement of the systemic infection of *S. enterica* serovar Typhimurium in humans and animals, respectively.

Salmonella can cause food-borne illness in humans due to contaminated meats and/or eggs, where *Salmonella* localizes

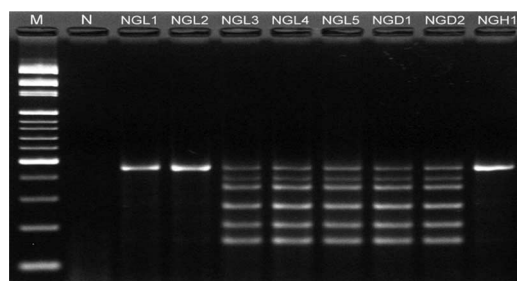


FIG. 1. Electrophoretic profiles of the PCR products of drug resistance genes and *spvC* amplified by a multiplex PCR. M, 100- to 3,000-bp DNA ladder; N, negative. The PCR product sizes are 156 bp for *pse*, 203 bp for *str*, 266 bp for *floR*, 351 bp for *sull*, 391 bp for *tetG*, and 450 bp for *spvC*.

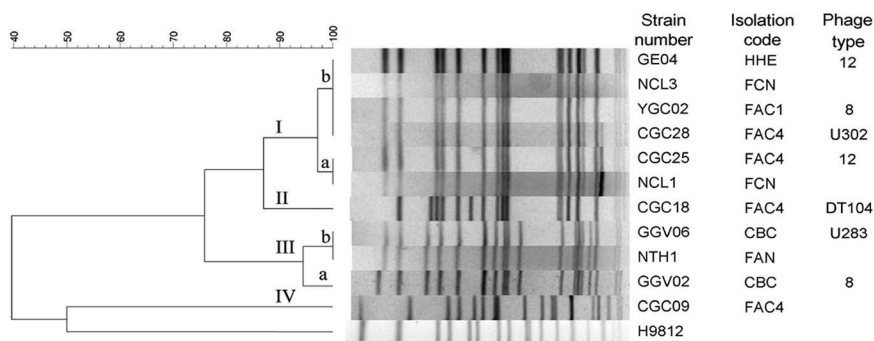


FIG. 2. Dendrogram and PFGE patterns of XbaI-digested genomic DNA of *S. enterica* serovar Typhimurium isolates. The dendrogram was constructed based on the unweighted-pair group method using average linkages algorithm and the Dice similarity coefficient by using BioNumerics software with 3% optimization and 1% position tolerance. The genotypes are indicated as I, II, III, and IV. *S. enterica* serovar Braendrup strain H9812 was the PFGE size marker.

on the inner membranes of eggshells in isthmal secretions (8), and the inner membrane also provides a physical barrier to prevent the entry of *Salmonella*. These studies revealed that *S. enterica* serovar Typhimurium existed on the inner membrane of goose eggs, as previously reported for duck egg membranes (31), but not from cloacal swabs of 0-day-old goslings and the hatching cabinet (Table 1), which are the most frequently found sites of *S. enterica* serovar Enteritidis and *S. enterica* serovar Typhimurium contamination (5, 9, 24) and a useful target to evaluate *S. enterica* serovar Typhimurium contamination (3, 4). In addition, egg contamination by salmonellae can occur through contact with *Salmonella*-contaminated equipment, personnel, and the environment and infected wild birds and rodents (14, 15), followed by the entry of *Salmonella* into hatching eggs in a hatchery. The transmission of *S. enterica* serovar Typhimurium from the hatchery to goslings was found in farm A since the goslings originated from a hatchery with high levels of contamination of *S. enterica* serovar Typhimurium on eggshell membranes (Tables 1 and 3). The XbaI-digested PFGE profile indicated that prolonged exposure to the external environment such as a longer feeding period (genotype Ib for C-FAC1 versus genotypes Ia, Ib, II, and IV for C-FAC4) can increase the prevalence of diverse multidrug-

resistant *Salmonella* strains in geese (Fig. 2 and Tables 2 and 3). In addition, identical genotypes in each farm, such as genotype IIIb in A-FAN and genotype Ib in C-FAC1 and E-HHE, and genotype differences among farms (Table 3) suggest that each farm was colonized with genetically closely related *S. enterica* serovar Typhimurium strains.

Generally, adequate sanitation can reduce the prevalence of *Salmonella*. For example, proper egg washing may minimize the problem of *Salmonella* contamination on outer eggshells (5, 22) and transmission from incubator to hatchers (9, 10). However, improper washing or rinsing of eggs, such as processing at lower temperatures, may increase the entrance of *S. enterica* serovar Typhimurium and *S. enterica* serovar Enteritidis into egg contents (22), and the washing process cannot remove *Salmonella* contamination in the inner membrane and egg contents. Therefore, a vaccine is needed to prevent *S. enterica* serovar Typhimurium and *S. enterica* serovar Enteritidis infection.

Since the discovery of a multidrug-resistant ACSSuT *S. enterica* serovar Typhimurium DT104 strain in United Kingdom (35), this strain has spread around the world, and it is prominently distributed in Europe and Northern America (17, 20), is less dispersed in Asia, such as in Korea (39) and Japan (32), and causes gastroenteritis and occasional outbreaks in human and wild or domestic animals. Type ACSSuT *S. enterica* serovar Typhimurium DT104 isolates not only have been frequently found in human (13) but have been commonly found in geese in the present study (Table 2). In Asia, several phage types of *S. enterica* serovar Typhimurium have been isolated from poultry and animals, such as phage types 13, 120, and DT104 in chickens (19) and phage type 90 in humans and animals (36). In this study, diverse phage types were found mainly in type ACSSuT *S. enterica* serovar Typhimurium isolates belonging to genotypes I and II, and some phage types, types 8, 12, and U283, were found only in geese (Fig. 2), suggesting that geese are a reservoir of diverse multidrug-resistant *S. enterica* serovar Typhimurium isolates.

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TABLE 3. Genotype distribution of 58 *S. enterica* serovar Typhimurium strains from different farms

| Genotype | No. of isolates from: | | | | | | Total no. of isolates |
|----------|-----------------------|-------|--------|--------|-------|-------|-----------------------|
| | A-FAN | B-CBC | C-FAC1 | C-FAC4 | D-FCN | E-HHE | |
| I | | | | | | | |
| Ia | | | | 3 | 1 | | 4 |
| Ib | | | 3 | 21 | 4 | 15 | 43 |
| II | | | | 1 | 0 | | 1 |
| III | | | | | | | |
| IIIa | | | 1 | | | | 1 |
| IIIb | 3 | 5 | | | | | 8 |
| IV | | | | 1 | | | 1 |
| Total | 3 | 6 | 3 | 26 | 5 | 15 | 58 |

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