

Phage type and antimicrobial susceptibility of *Salmonella enterica* serovar Enteritidis from food-producing animals in Japan between 1976 and 2004

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SUMMARY

A total of 56 isolates of *Salmonella enterica* serovar Enteritidis, including 38 isolates from poultry, 16 from cattle and two from pigs, collected between 1976 and 2004, were subjected to bacteriophage typing and antimicrobial susceptibility testing. Phage type (PT) 8 was predominant in bovine isolates, whereas PT1 and PT4 were predominant in poultry isolates. Resistance was found for 8 of 11 antimicrobials tested, at the following rates: 46.4% for dihydrostreptomycin followed by ampicillin and oxytetracycline (both 8.9%).

KEY WORDS: *Salmonella* enteritidis, Phage type, Antimicrobial resistance, Food-producing animals

Received December 31, 2007

Accepted February 19, 2008

In Japan, *Salmonella enterica* serovar Enteritidis has been the serotype isolated most frequently from patients suffering from food-borne illness since 1989 (Infectious Disease Surveillance Center, 2003). As infections with *S. Enteritidis* are closely linked with egg consumption in Japan, the Enforcement Regulations of the Food Sanitation Law (Law No. 23 of 1948) were amended for safe distribution of raw eggs and liquid egg products in 1998. Although large-scale outbreaks of food poisoning due to *S. Enteritidis*-contaminated egg products have been reduced (Infectious Disease Surveillance Center, 2003), *S.*

Enteritidis is still a predominant serovar and accounted for about 50% of all the *Salmonella* isolates from human sources between 2003 and 2005 (Infectious Disease Surveillance Center, 2006). *S. Enteritidis* is also a causative agent of salmonellosis in animals. Salmonellosis caused by *S. Enteritidis* in specific domestic animals such as cattle, sheep, pigs and poultry, is a notifiable disease regulated by the Domestic Animal Infectious Disease Control Law (Law No. 166 of 1951). Antimicrobial therapy of *S. Enteritidis* infection is generally needed for systemic infections in humans and is essential for treatment and control of salmonellosis in animals. Emergence of antimicrobial resistance in isolates of *S. Enteritidis* is a significant public and animal health concern. Antimicrobial-resistant isolates of *S. Enteritidis* from human and egg products have been reported in Japan (Murase *et al.*, 2002; Yamasaki *et al.*, 2007). In the present study, we characterized phage types and antimicrobial sus-

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ceptibility of animal isolates over the last three decades. A total of 56 *S. Enteritidis* isolates including 38 isolates from poultry such as broiler and layer chickens and geese, 16 isolates from cattle and 2 isolates from pigs, collected between 1976 and 2004, were subjected to bacteriophage typing in accordance with the methods of the Public Health Laboratory Service (PHLS), London, United Kingdom (Ward *et al.*, 1987). In cases where a tested strain reacted with some of the typing phages but did not conform to any of the schemes the isolate was denoted as RDNC ("Reacted but did not conform").

Eighteen phage types of *S. Enteritidis* were found: 11 phage types were found in poultry isolates, eight phage types in bovine isolates and two phage types in porcine isolates. Phage type (PT) 1 and PT4 were predominant in poultry isolates. PT8 was predominant in bovine isolates, and two isolates of pig origin were identified as PT8 and PT5c. The two phage types, PT1 and PT4, were the most frequently isolated from unpasteurized liquid egg between 1993 and 1998 (Murase *et al.*, 2002) and the patients with food poisoning caused by *S. Enteritidis* since 1992 (Anon, 1997. *Salmonella*, Japan, 1994-1996. IASR, <http://idsc.nih.go.jp/iasr/18/205/tpc205.html>) throughout Japan. During the period from 1997 to 2002, PT6, PT6a, PT21, PT47 and RDNC were also isolated from patients in outbreaks of food poisoning caused by *S. Enteritidis* (Izumiya *et al.*, 2003). The present study showed that a variety of phage types was observed in the isolates from food-producing animals, especially poultry, after 1989.

Antimicrobial susceptibility was determined using an agar dilution method according to the Clinical and Laboratory Standards Institute (formerly, the National Committee for Clinical Laboratory Standards [NCCLS]) recommendations (2002). The antimicrobial agents used in this study were ampicillin (ABPC), cefazolin (CEZ), dihydrostreptomycin (DSM), kanamycin (KM), gentamicin (GM), oxytetracycline (OTC), chloramphenicol (CP), colistin (CL), nalidixic acid (NA), enrofloxacin (ERFX), and trimethoprim (TMP). *Staphylococcus aureus* ATCC 29213, *Enterococcus faecalis* ATCC29212, *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 were used as quality control strains. The minimum inhibitory concentrations (MICs) except for those of OTC, DSM and CL were inter-

preted using the recommendations of the NCCLS (2003); the breakpoints used for DSM, OTC and CL were in accordance with our previous reports (Esaki *et al.*, 2004).

Of 56 isolates, 27 (48.2%) were susceptible to all the antimicrobials tested. Resistance was found for 8 of the 11 antimicrobials tested at the following rates: 46.4% for DSM, 8.9% for ABPC and OTC, 7.1% for CP, and 1.8% for KM, GM, CL and TMP. Even isolates of *S. Enteritidis* from patients and chicken food in Japan, the similar result that a half of isolates were susceptible to antimicrobials was reported (Yamasaki *et al.*, 2007). Compared with previous studies in Korea (Chung *et al.*, 2004), Spain (Cruchaga *et al.*, 2001) and southern Brazil (Dias de Oliveria, 2005), frequencies of antimicrobial resistance in *S. Enteritidis* were low except for DSM in Japan. Although tetracycline antibiotics are the most frequently used antibiotic for therapeutic purpose in food-producing animals (Asai *et al.*, 2007), streptomycin resistance was most frequently observed in *S. Enteritidis* in Japan. *S. Typhimurium* and *Infantis* are predominant serovars in *Salmonella* isolates from food-producing animals in Japan (Asai *et al.*, 2006a). High frequencies of antimicrobial resistance were found in these serovars in Japan (Asai *et al.*, 2006b; Kawagoe *et al.*, 2007). Although *S. Enteritidis* is the leading serovar in *Salmonella* food poisoning in Japan, low frequencies of antimicrobial resistance was observed in isolates of *S. Enteritidis*. In Japan, NA-resistant isolates of *S. Enteritidis* have frequently been found in imported chicken meats (Matsumoto *et al.*, 2006). Recently, Izumiya *et al.* (2005) reported an extended-spectrum cephalosporin (ESCs)-resistant isolate of *S. Enteritidis* from patients in Japan. Although no isolates of *S. Enteritidis* resistant to NA, ERFX and CEZ were found in the food-producing animals studied, continuous surveillance need to monitor emergence and prevalence of antimicrobial resistance in food-producing animals.

Nine isolates (16.1%) exhibited resistance to two or more antimicrobials (multi-drug resistance [MDR]): these included 5 (13.2%) out of 38 poultry isolates, 3 (25%) out of 16 bovine isolates, and one (50%) out of 2 porcine isolates (Table 1). In Korea between 2000 and 2002, 89.8% of human isolates, 64.7% of poultry isolates and 13.3% of pig isolates showed MDR (Chung *et al.*, 2004).

TABLE 1 - Resistance pattern of *S. Enteritidis* isolates from food-producing animals during three different collection periods since 1976.

Resistance pattern ^a	1976~1982			1989~1999			2000~2004			Total	%			
	cattle		pig	cattle	poultry		cattle	pig	poultry					
	PT8	others	PT8	PT1	PT4	others	PT8	others	PT5c			PT1	PT4	others
ABPC-DSM-KM-OTC-CP	1											1	1,8	
ABPC-DSM-OTC-CP	1											1	1,8	
DSM-OTC-CP	1											1	1,8	
ABPC-DSM						1						1	1,8	
ABPC-TMP									1			1	1,8	
DSM-OTC					2							2	3,6	
DSM-CP											1	1	1,8	
DSM-CL					1							1	1,8	
ABPC											1	1	1,8	
DSM				1	3	3	3		1		1	6	18	32,1
GM									1			1	1,8	
Susceptible	2	1	1	2	4			3	2		4	8	27	48,2
Total	4	2	1	3	10	3	4	3		1	1	4	16	56

^aABPC, ampicillin; DSM, dihydrostreptomycin; KM, kanamycin; OTC, oxytetracycline; CP, chloramphenicol; CL, colistin; TMP, trimethoprim.

Although most isolates of *S. Typhimurium* in cattle (resistance to ABPC, DSM, OTC and either CP or KM) and pigs (resistance to DSM and OTC) and *S. Infantis* (resistance to DSM and OTC) in broiler chickens showed MDR (Asai *et al.*, 2006b; Kawagoe *et al.*, 2007), MDR isolates of *S. Enteritidis* from food-producing animals remained uncommon in Japan.

Out of the nine MDR isolates, three isolates from cattle obtained between 1976 and 1982 exhibited resistance to three to five antimicrobials: two isolates of PT8 exhibited DSM-OTC-CP resistance (No. 8-3) and ABPC-DSM-OTC-CP resistance (No. 8-5) and one isolate of PT29a exhibited ABPC-DSM-KM-OTC-CP resistance. As resistance to three or more antimicrobials was found in isolates of PT8 from cattle between 1976 and 1982, all eight isolates of PT8 were subjected to pulsed-field gel electrophoresis (PFGE) analysis to clarify the relationship between resistant isolates in

1976 to 1982 and susceptible isolates in 2000 to 2004. At first, BlnI digestion was adopted because of the better discrimination among genotypes of *S. Enteritidis* strains, as previously described (Terajima *et al.*, 1998). PFGE was carried out with a CHEF-DRIII system (Bio-Rad Laboratories, Inc., Ca, USA) according to Pulse-net procedures of the Center of Disease Control and Prevention (Hunter *et al.*, 2002). The running conditions were 6 V/cm at 14°C for 19 h with pulse times ramped from 2.2 to 63.8 s. Images were prepared using Quantity One software (Bio-Rad Laboratories). BlnI-digested PFGE profiles were classified into 2 types: type 1 contained two susceptible isolates in 1981 (No. 8-4) and 2003 (No. 8-8), and type 2 contained the remaining six isolates including two MDR isolates (No. 8-3 and 8-5) with small-sized bands (less than 33Kbp) (Figure 1a). In further work, the PFGE profile of 8-3 digested with XbaI and BlnI (type 3) could be clearly distin-

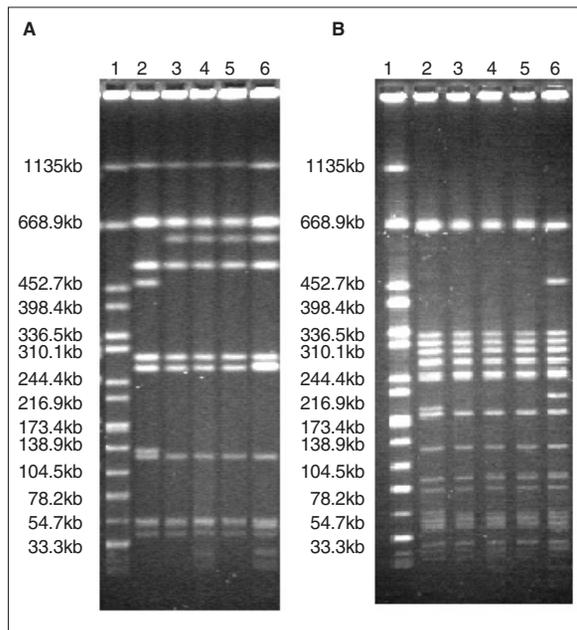


FIGURE 1 - PFGE patterns of PT8 isolates of *S. enterica* serovar Enteritidis digested with *BlnI* a) and with *XbaI* and *BlnI* b). Lanes 1, *S. enterica* serovar Braenderup H9812 digested with *XbaI* (sizes of the bands of H9812 are represented on the left of lane 1); lane 2, type 1 (no. 8-4 and 8-8); lane 3 type 2 (no. 8-1, 8-2, 8-6 and 8-7); lane 4, no. 8-5; lane 5, a susceptible clone from 8-5; lane 6, type 3 no. 8-3.

guished from other isolates of type 2 (Figure 1b). These results showed that, of three types of PFGE profiles, two types of PT8 strain have been prevalent among cattle in the last 2 decades. For plasmid curing, 1000-fold diluted cultures of isolate No. 8-5 were incubated with at 43°C for 20hr. After 3 consecutive transfers, susceptible clones were obtained. Plasmid profile tests revealed that a plasmid (about 130Kbp) was cured in the susceptible clones. *BlnI*-digested PFGE pattern of the clones derived from isolate No. 8-5 was identical to the isolates of type 2 (Figure 1a). The present study could not show genomic differences between the classic resistant isolates and the recent susceptible isolates.

ACKNOWLEDGEMENTS

We thank the member of the Livestock Hygiene Service Centers for providing *S. Enteritidis* isolates. This work was supported in part by grant in aid of the Ministry of Health, Labor and Welfare (H18-Shokuhin-Ippan-003).

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