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Original Article

Characterization of *Salmonella* Isolated in Okinawa, Japan

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SUMMARY: *Salmonella enterica* strains isolated in Okinawa between 1995 and 2005 were analyzed with respect to their serovars and antimicrobial susceptibility, and pulsed-field gel electrophoresis (PFGE) was used to examine their digestion patterns. A total of 1,071 isolates, including 610 from humans, 358 from animal rectal swabs and 103 from meat obtained at grocery stores, were examined. The first 3 most frequent serovars in human isolates were Enteritidis, Weltevreden and Bareilly, together accounting for 65% of the isolates. In isolates from the rectal swabs of laying hens, the predominant serovars were Albany, Saintpaul and Aarhus, accounting for 82% of the isolates. In broilers, 123 of 124 isolates belonged to serovar Infantis, which reflected the high ratio of this serovar in the chicken sold at grocery stores. An antibiogram of human isolates was different from that of broilers and chicken. Chromosomal DNAs of *S*. Infantis isolated from humans and from the rectal swab of broilers and chickens were examined by PFGE using the restriction enzymes *Xba*I and *Bln*I. The digestion patterns of human isolates were not coincident with those of the isolates from the rectal swab of broilers and chicken-meat samples.

INTRODUCTION

Salmonella is a member of *Enterobacteriaceae*. Human infection with *Salmonella* is usually seen in the form of gastroenteritis, but occasionally extraintestinal focal infection and some restricted serovars cause enteric fever (such as typhoid or paratyphoid fever). The genus *Salmonella* is classified into two species, *S. enterica* and *S. bongori*. *S. enterica* is one of the most common causes of human gastroenteritis. More than 2,500 serovars of *S. enterica* have been identified, depending on the combination of O and H antigens. *S. enterica* serovars Typhimurium and Enteritidis have been reported to be the most common causes of human salmonellosis (1). However, in some regions, other serovars are of greater importance (2).

In the past few decades, Japan has been among the countries with the lowest incidence of enteric fever. However, outbreaks and sporadic cases of gastroenteritis due to *Salmonella* are being seen with increasing frequency and constitute an important health problem. In Tokyo, the serovars Enteritidis, Thompson, Hadar, Infantis and Typhimurium have been reported to be predominant (3). However, in Okinawa, located about 2,000 km southeast of Tokyo, the serovar distribution of *Salmonella* has not yet been reported; nevertheless, food poisoning frequently takes place.

Most serovars of *Salmonella* are widely distributed in nature, and are found in the intestines of domestic and wild mammals, reptiles, birds and insects (4). Foods containing products from farm animals are considered to be an important source of human *Salmonella* infections. The surveillance of *Salmonella* serovars from human and animal sources will thus provide important information for public health.

To know the distribution of *Salmonella* serovars in a district or a country can be of global importance because of travel and food product trade. The purpose of this study was to characterize the *Salmonella* isolated in Okinawa, with attention focused mainly on the serovars.

MATERIALS AND METHODS

Bacterial strains: (i) Human isolates. Six hundred and ten *Salmonella* strains that were isolated and identified at hospitals between 1995 and 2004 and submitted to our laboratory (the Okinawa Prefectural Institute of Health and Environment) for further typing were used. All were first isolates from patients. Five hundred and ninety-nine of these isolates were from stool specimens and 11 were from blood. (ii) Animal isolates. From 2001 to 2003, a total of 1,487 domestic animals (100 pigs from 5 farms, 100 goats from 37 farms, 599 laying hens from 2 farms, and 688 broilers from 4 farms) were examined at a slaughterhouse and two food-processing plants. From each animal, rectal swab samples were collected, placed in selenite-cystein broth (BBL, Cockeysville, Md., USA) and enriched at 43°C for 24 h. The enriched samples were streaked onto Rambach agar (CHROMagar; Microbiology, Paris, France) and Shigella/Salmonella agar (Eiken Chemical Co., Ltd., Tokyo, Japan). *Salmonella* was identified by using triple sugar iron agar and lysine indol motility agar. Further identification was made using an API 20E (bioMerieux, Marcy I'Etoile, France). Among the 1,487 animals examined, 358 were positive for *Salmonella*. These 358 strains were further examined as animal isolates. (iii) Food isolates. From 1998 to 2005, a total of 806 food samples

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collected from a variety of grocery stores and food-processing plants were examined. They included 315 raw chicken meat parts, 97 cuts of beef, 60 portions of minced pork, 334 fresh raw vegetables (lettuce, cucumber, cabbage and tomato) and 33 oysters. Ten grams of each sample was placed in 90 ml Buffered Peptone Water (Difco Lab., Detroit, Mich., USA) and incubated at 37°C for 24 h for enrichment. Five hundred microliters of the enriched culture was inoculated in 10 ml of Rappaport-Vassiliadis medium (Oxoid Ltd., Hampshire, England) and incubated at 43°C for 24 h. Enriched samples were then processed as described above for animal samples. Finally, 103 strains were identified as to be *Salmonella* spp.

Serotyping: *Salmonella* strains were tested by slide agglutination with an O-antisera (Denka Seiken Co., Tokyo, Japan). Flagellar antigens were determined by tube agglutination with H-antisera (Denka Seiken). Serotyping was based on O- and H-group antigen, according to the Kauffman-White scheme (5).

Antimicrobial susceptibility: The antimicrobial susceptibilities of the organisms against 12 drugs were examined by the disc diffusion method using Mueller-Hinton agar according to the guidelines of the National Committee for Clinical Laboratory Standards (6). The respective quantities (μg/disc) of the active compounds were as follows: ampicillin (ABPC) 10, chloramphenicol (CP) 30, streptomycin (SM) 10, tetracycline (TC) 30, gentamicin (GM) 10, kanamycin (KM) 30, nalidixic acid (NA) 30, ciproflaxacin (CPFX) 5, cefotaxime (CTX) 30, sulfamethoxazole-trimethoprim (ST) 25 (23.75 + 1.25), fosfomycin (FOM) 30 and trimethoprim (TMP) 5. These antibiotic discs were purchased from Nippon Becton Dickinson Co., LTD., Tokyo, Japan.

Pulsed-field gel electrophoresis (PFGE): A selected number (108 strains) of *S. enterica* subsp. *enterica* serovar Infantis (*S.* Infantis) in this study were characterized by PFGE analysis. The examined strains included 15 human isolates, 40 isolates of chicken meat collected from 15 grocery stores, 22 isolates from chicken meat collected from 2 food-processing factories, 32 isolates from broilers collected from 4 farms and 1 isolate from a layer collected from 1 farm. Plug preparation, restriction digestion, electrophoresis conditions, and staining of gels were carried out according to the PulseNet standardized PFGE protocol (7) with the following modifications. The cell suspension buffer was sterile ultrapure water. The cell suspensions were adjusted to a turbidity reading of 7% transmittance in a Vitek colorimeter (bioMerieux). The agarose gel used to make the plugs was mixed using distilled water. The plug containing cells were lysed in buffer consisting of 1 mg/ml proteinase K with 1% N-laurylsarcosine in 0.5 M EDTA, pH 8.0. The lysis time was increased from 1.5 to 3 h. A total of 5 washes, 2 with sterile 4 mM Pefabloc SC (Roche Diagnostics, Mannheim, Germany) in 10 mM Tris-EDTA buffer and 3 with 10 mM Tris-EDTA buffer, pH 8.0, were used to remove excess reagents and cell debris from the lysed plugs. DNA was digested with restriction enzymes *Xba*I and *Bln*I, and fragments were separated in 1.0% agarose gel on a clamped homogenous electric field apparatus (GENEPATH System; Nippon Bio-Rad Labs, Tokyo, Japan). The initial pulse time of 2.2 s was increased linearly to 54.2 s over 19.5 h. Gels were stained with ethidium bromide, destained in water, and photographed under UV illumination with Polaroid film. The chromosomal DNA restriction patterns produced by PFGE were interpreted by the criteria of Tenover et al. (8).

RESULTS

Isolation frequency of *Salmonella*: In the animal samples, the rectal swab positive rates were 39.1% (234/599) in laying hens (hens used for egg production) and 18% (124/

Table 1. Isolation frequency of *Salmonella*

Source	No. examined	No. *Salmonella* positive cases	Isolation frequency (%)
Rectal swab			
laying hens	599	234	39.1
broilers	688	124	18.0
pigs	100	0	0
goats	100	0	0
Foods			
Chicken	315	101	32.1
(A)	(211)	(54)	(24.9)
(B)	(94)	(47)	(50.0)
pork	60	1	1.7
beef	97	1	1.1
oyster	33	0	0
vegetable	334	0	0

(A): chickens from grocery stores.
(B): chickens from food-processing factories.

Table 2. Distribution of *Salmonella* serovars

Serovars	No. of strains	Frequency (%)
Human sporadic diarrhea isolates (610 strains)		
Enteritidis	300	49.2
Weltevreden	48	7.9
Bareilly	43	7.0
Typhimurium	37	6.1
Infantis	22	3.6
Stanley	18	3.0
Montevideo	16	2.6
Newport	16	2.6
Paratyphi B	10	1.6
Agona	6	1.0
Agama	6	1.0
Heidelberg	5	0.8
Litchfield	5	0.8
Waycross	5	0.8
Others (37 serovars)	72	11.8
Laying hens rectal swab isolates (234 strains)		
Albany	113	48.3
Saintpaul	51	21.8
Aarhus	28	12.0
Bareilly	23	9.8
Oranienburg	4	1.7
Cerro	3	1.3
Agona	2	0.9
Enteritidis	2	0.9
Others (8 serovars)	8	3.4
Broilers rectal swab isolates (124 strains)		
Infantis	123	99.2
Enteritidis	1	0.8
Chicken meats isolates (101 strains)		
Infantis	94	93.1
Enteritidis	4	10.0
Harder	2	2.1
Abony	1	1.0

688) in broilers, and no *Salmonella* was isolated from 100 pigs and 100 goats. In foods, the positive rates were 32.1% in chicken (101/315), 1.7% in pork (1/60), 1.1% in beef (1/97), and no *Salmonella* was isolated from 33 oysters and 334 vegetable samples. Among the chicken-meat samples obtained at the food-processing factories, 50.0% of the samples were revealed to be *Salmonella* positive, but only 24.9% of the samples from grocery stores were found to be *Salmonella* positive (Table 1). The frequency in the human samples was not determined because we obtained organisms already identified at the hospital laboratories.

Serovars: Six hundred and ten human isolates of *Salmonella* were classified into 51 serovars. The most frequent serovar was Enteritidis, which accounted for 49.2% of the 610 isolates. The second most frequent serovar was Weltevreden, which accounted for 7.9%. Two hundred and thirty-four isolates from laying hens were classified into 16 serovars with the most frequent serovar, Albany, accounting for 48.3%. The isolation frequency of *S*. Enteritidis was only 0.9% (2/234). In broilers, 124 isolates were classified into only 2 serovars, 123 of Infantis (99.2%) and 1 of Enteritidis (0.8%). The chicken meat isolates included 4 serovars, with 93.1% (94/101) belong to Infantis, 10% (4/101) to Enteritidis, 2.1% (2/101) to Harder, and 1% (1/101) to Abony. The serovar of an isolate from pork was Havana, and that of an isolate from beef was Chester. The details of the serovar distribution are presented in Table 2.

Antimicrobial susceptibility: Different antibiograms were seen depending on the source of the organisms. In humans and laying hens, 25 and 16%, respectively, were resistant to at least 1 of the 12 drugs examined. However, in chicken and broilers, almost all isolates were resistant to at least 1 of the 12 drugs. In addition, most isolates from chicken meat and the rectal swabs of broilers were resistant to multiple drugs, such as SM, TC, KM, ST and TMP, whereas 10% of human isolates were resistant to ABPC. There were almost no resistant strains against CP, CPFX, GM, NA or FOM. Details are shown in Table 3.

Table 3. The rate of drug resistant *Salmonella* (% of the strains examined)

Antibiotics	Isolates (no. examined) from			
	humans (483 strains)	chickens (101 strains)	broilers (124 strains)	laying hens (234 strains)
ABPC	10	0	1	0.4
SM	12	94	76	15
TC	4	95	99	1
CP	0	0	0	0
KM	1	32	50	0
CTX	1	1	0	0
CPFX	0	0	0	0
ST	0.2	71	71	0.4
TMP	0.2	71	69	0.4
GM	0	0	0	0
NA	0.4	1	0	0
FOM	0.2	0	0	0
	Rate of resistant organisms to at least 1 of the 12 drugs			
	25	96	99	16

ABPC, ampicillin; SM, streptomycin; TC, tetracycline; CP, chloramphenicol; KM, kanamycin; CTX, cefotaxime; CPFX, ciproflaxacin; ST, sulfamethoxazole-trimethoprim; TMP, trimethoprim; GM, gentamicin; NA, nalidixic acid; FOM, fosfomycin.

Table 4. PFGE pattern of *S*. Infantis from laying hens, broilers, chickens and human diarrhea

PFGE types	Freq.	L.h*	Broilers				Chickens																		Human	
		farm	farm				factory		grocery stores																hospital	
		A	B	C	D	E	F	G	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	H	I
X1a/B1a	10																							1	9
X1a/B1b	1																								1
X1b/B1b	1																								1
X1c/B1f	1										1														
X1d/B1d	1							1																	
X1d/B1g	1					1																			
X1d/B1i	1							1																	
X1e/B1i	1												1												
X1e/B1j	1						1																		
X1f/B1b	1																							1	
X1f/B1d	3						2	1																	
X1f/B1i	2															1		1							
X1f/B1h	5						5																		
X1g/B1c	1																							1	
X1g/B1d	33			5	1	1	6	2	1	3					3		1		2	5	1	1	1		
X1g/B1e	1																			1					
X1g/B1g	28		15			7				1			2			1				1	1				
X1g/B1i	5		1					1		1	1		1												
X1h/B1d	5													4	1										
X1i/B1j	1																					1			
X1j/B1k	1											1													
X1k/B2	1											1													
X2/B1l	1																							1	
X3/B1g	1					1																			
X4/B3	1	1																							
Total	108	1	16	5	1	10	14	6	1	5	2	2	4	4	4	2	1	1	2	7	2	2	1	4	11

*: Laying hens.

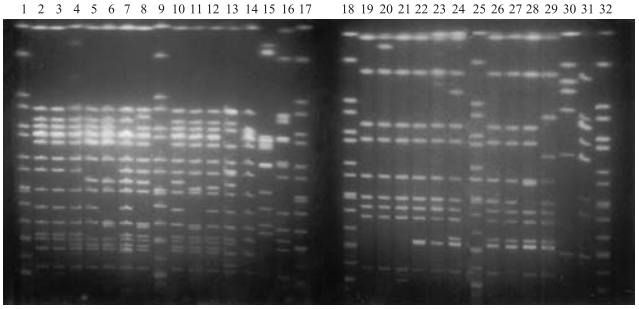

Fig. 1. *Salmonella* Infantis PFGE patterns represented as 14 types for *Xba*I and 12 types for *Bln*I. PFGE analysis of *S.* Infantis. Types and subtypes were classified as described in the Results. Lanes 1, 9, 17, 18, 26 and 32, molecular marker (*Salmonella* Braenderup H9401 *Xba*I-digestion); Lanes 2 to 16, *Xba*I-PFGE patterns, represented as X1a, X1b, X1c, X1d, X1e, X1f, X1g, X1h, X1i, X1j, X1k, X2, X3, and X4; Lanes 19 to 31, *Bln*I-PFGE patterns, represented as B1a, B1b, B1c, B1d, B1e, B1f, B1g, B1h, B1i, B1j, B2 and B3.

PFGE analysis of *S.* Infantis: Since *S.* Infantis was frequently isolated from broilers, chickens and humans, 108 isolates of these sources were analyzed by PFGE. The digestion patterns determined using the restriction enzymes *Xba*I and *Bln*I were classified as described by Tenover et al. (8). Digestion by *Xba*I produced 4 types and that by *Bln*I produced 3 types (type X1 to type X4, and type B1 to type B3, respectively). Type X1 was further classified into 11 subtypes (subtype X1a to subtype X1k); and type B1 was further classified into 10 subtypes (subtype B1a to subtype B1j). By the combination of these types and subtypes obtained using the 2 enzymes, the organisms examined were classified into 25 types (Fig. 1 and Table 4). Human isolates were classified into 6 types and the other isolates into 19 types. There was no coincidence of the digestion patterns (PFGE types) between human isolates and the other isolates. The broiler isolates from the same poultry farm tended to show the same digestion pattern.

DISCUSSION

This study presents the distribution of *Salmonella* serovars, an antibiogram of the organisms and the epidemiological features of salmonellosis in Okinawa. The three most frequent serovars in Tokyo were Enteritidis, Thompson and Harder (3), and the most frequent in Okinawa were Enteritidis, Weltevreden and Bareilly. The serovar distribution was characterized by a high frequency of the serovar Weltevreden, which is the major serotype in Thailand, Malaysia and the Philippines, but which is much less frequent in Tokyo (3,9-11). This suggests that the bacterial movement between Okinawa and Southeast Asia is more active than that between Okinawa and Tokyo. The finding that the frequency of Weltevreden was higher in Okinawa than in Tokyo might have been related to differences in climate or to differences in dietary habits, which share similarities between Okinawa and Southeast Asia. The serovar Enteritidis appears to be a major serovar in *Salmonella*, causing human gastroenteritis throughout the world, but the ratio in the total isolates is variable (3,12-14). As an example, the ratio of *S.* Enteritidis in all *Salmonella* isolates was 22.1% in Tokyo and 49.2% in Okinawa.

The antimicrobial susceptibility of *Salmonella* in Okinawa was different depending on the source of the organisms. The isolates from humans and laying hens were generally sensitive to all drugs examined, but some were resistant to ABPC or SM. On the other hand, the isolates from broilers and chickens were generally resistant to multiple drugs, suggesting that preventive medication is intensively given to the broilers. The difference of antimicrobial susceptibility between the isolates from broilers and laying hens could also be explained by a difference in the serovar distribution. Laying hens were treated by addition of antibiotics such as colistin to their feed for only the first 10 weeks, but after this period when producing eggs, no antibiotics in the feed was given in the farms. On the other hand, the broilers were treated with salinomycin, enramycin, sodium lasalocid and other antibiotics over about 60 days (antibiotics were given until 7 days before slaughter). However, this should have created no problems for the treatment of salmonellosis, because almost all isolates were sensitive to therapeutic drugs such as ABPC, CP, CTX, CPFX, NA and FOM. Drug-resistant *Salmonella* is important in *S.* Typhi as the causative agent of typhoid fever, for which antibiotic therapy is essential, but we have to bear in mind that antibiotic therapy for *Salmonella* gastroenteritis is not necessarily important, or rather, is contraindicated (15) expect in some specific cases such as young babies, immunosuppressive patients, patients with intra-organic devices, etc.

It is easy to speculate that laying hens, broilers and chicken meat could be the source of human salmonellosis because of the high contamination ratio and high consumption as daily food. Actually, some serovars, such as Infantis and Enteritidis, were isolated in both humans and chickens in grocery stores. The rectal swabs of the broilers at the slaughter house revealed 18% positivity for *Salmonella*, whereas 50% of the chicken-meat samples at the food-processing factories were positive for *Salmonella*. These results suggest that the contamination spread during the processing. However, the reason why the contamination rate in the chicken meat-samples at grocery stores decreased to 24.9% is unknown.

PFGE analysis of *S.* Infantis isolated from human, broilers and chicken meat revealed that there was no coincidence of the chromosomal DNA digestion patterns by two restriction enzymes, *Xba*I and *Bln*I. This finding was contrary to the speculation for the infection focus; in other words, the infection source of human salmonellosis due to *S.* Infantis is not likely the broilers or the chickens. Although the chickens from laying hens are not used for food, *S.* Bareilly was frequently isolated from humans and laying hens. Since the egg has been thought to be a major infection focus of human salmonellosis (16), the infection route in Okinawa must also be determined by studying the egg contamination rate and by molecular analysis of the isolates. Clarifying the source of *Salmonella* infection is vital to prevent disease, and the present study suggests that the source is not likely chickens, as far as *S.* Infantis is concerned. This study will continue to clarify the infection route of *S.* Infantis as well as *S.* Enteritidis and *S.* Bareilly, which are frequently isolated from laying hens, broilers and human salmonellosis.

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