NOTE *Avian Pathology*

Salmonella Isolated from the Feces of Migrating Cranes at the Izumi Plain (2002–2008): Serotype, Antibiotic Sensitivity and PFGE Type

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ABSTRACT. From November 2002 to February 2008, 2,251 crane feces were collected at the Izumi Plain in Kagoshima Prefecture. *Salmonella enterica* was isolated from 359 feces (15.9%), of which 332 (92.5%) were *Salmonella* Typhimurium (ST), 9 were *S*. Hvittingfoss/II, 4 were *S*. Abaetetuba, 3 were *S*. Enteritidis, 2 were *S*. Konstanz, 1 was *S*. Pakistan and 8 were untyped isolates, respectively. Against 12 antimicrobial agents, no resistant strains were found in 154 isolates examined, but one was found to be resistant to ampicillin. By pulsed-field gel electrophoresis (PFGE), all but one of the 68 ST isolates tested showed indistinguishable banding patterns; one had a different pattern. The results suggest that ST strains from the same origin would spread in crane flocks during their stay at Izumi Plain every winter.

KEY WORDS: antibiotic resistance, *Salmonella* Typhimurium, wild crane.

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Beginning in 1990, over10,000 cranes have flown from Siberia and Northern China to the Izumi Plain in northern Kagoshima Prefecture via South Korea each year during winter. Approximately 70% are Hooded Cranes (*Grus monacha*), and the remaining 30% are comprised of White-naped Cranes (*G.vipio*); there are also a small number of other species that migrate across the plains, such as the Common Crane (*G. grus*).

The cranes reside in the Izumi Plain approximately 4–5 months every year, from mid-October to the middle of March the following year. Post-crop rice fields provide a place of rest for the cranes; they set up their nests filled with water from one section within and sleep there at night. During their stay, manufactured foods such as wheat are provided to them, and sardines also are fed to the cranes before they return to the North.

Our laboratory has continued to research *Salmonella* infection in this group of migrating cranes since 1996, and the results of our research for 1996 to 2001 have already been reported [3, 4]. Beginning after 2002, *Salmonella* has also been isolated every winter. Here, the relevance among *Salmonella* strains isolated from November 2002 until February 2008 is examined and discussed based on isolation conditions, antibiotic sensitivity and pulsed-field gel electrophoresis (PFGE).

Every winter from November 2002 to February 2008, samples (2,251 in total, 20–141 samples/time) of fresh crane feces were collected at Izumi Plain into a sterile dish at a frequency of one time each month. Compared with other wild birds, crane feces are easy to distinguish by their large size. Although it was possible to distinguish and collect feces portions from Hooded Cranes and White-naped Cranes, the results were compiled together as a mixture of cranes (no classification) due to the fact that it was impossible to distinguish them among a majority of the collected samples.

Feces (1 g) was transferred into a test tube with 10 m*l* of Hajna Tetrathionate (HTT) Broth (Eiken, Tokyo, Japan) and cultured at 40°C for 24–48 hr. Afterward, one inoculation loop was taken from the test tube, inoculated to the selective medium of desoxycholate-hydrogen sulfide-lactose (DHL) agar and cultured at 37°C for 24 hr. The black-colored colonies grown on the DHL agar were then selected and purely cultured in a standard agar medium. The purely cultured bacteria was then placed through a differential medium for *Salmonella* and was identified serologically in accordance with a previous report [3, 4].

From the tested crane feces, *Salmonella enterica* was isolated at relatively high rates (6.4–43.5%), resulting in 359 isolation-positive samples (Table 1). Furthermore, the monthly isolation rate of *Salmonella* showed a higher trend prior to the cranes returning North than the period directly after their arrival at the Izumi Plain. The serotypes of isolates were determined; there were 332 isolates of ST (92.5%), 9 isolates of *S*. Hvittingfoss/II, 4 isolates of *S*. Abaetetuba, 3 isolates of *S*. Enteritidis, 2 isolates of *S*. Konstanz, 1 isolate of *S*. Pakistan and 8 isolates of unclassifiable types (Table 2).

Out of the 359 isolates, 92 isolates (67 ST, 9 *S*. Hvittingfoss/II, 4 *S*. Abaetetuba, 1 *S*. Enteritidis, 2 *S*. Konstanz, 1 *S*. Pakistan and 8 unclassifiable type strains) were used for the antimicrobial sensitivity test. The test was carried out according to the Japanese Society of Chemotherapy Standard Method [5] using the following 12 antimicrobial agents (antibiotics) purchased commercially: ampicillin (ABPC),

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Table 1. Isolation of *Salmonella* from wild crane feces from Nov. 2002 to Feb. 2008

Year (Fiscal)	Oct.	Nov.	Dec.	Jan.	Feb.	Mar.	Total (%)
2002		2/20[a)]	1/20	12/21	22/24		37/85 (43.5)
2003		0/84	0/62	0/107	60/141	35/72	95/466 (20.4)
2004	9/34	40/120	14/110	5/100	36/140		104/504 (20.6)
2005		1/99	0/106	16/112	52/94		69/411 (16.8)
2006			10/96	0/114	14/106		24/316 (7.6)
2007		4/128	3/120	1/113	22/108		30/469 (6.4)
Total (%)	9/34 (26.5)	47/451 (10.4)	28/514 (5.4)	34/567 (6.0)	206/613 (33.6)	35/72 (48.6)	359/2,251 (15.9)

a) No. of *Salmonella* isolates / No. of feces samples tested.

Table 2. Serotypes of *Salmonella* strains isolated from Nov. 2002 to Feb. 2008

Serotype	No. of isolates	Fiscal years isolated
S. Typhimurium	332	2002–2007
S. Hvittingfoss/II	9	2006
S. Abaetetuba	4	2007
S. Enteritidis	3	2003, 2004
S. Konstanz	2	2006
S. Pakistan	1	2005
Untyped	8	2006, 2007
Total	359	

cefazolin (CEZ), dihydrostreptomycin (DSM), kanamycin (KM), gentamicin (GM), colistin (CL), oxytetracycline (OTC), chloramphenicol (CP), fosfomycin (FOM), nalidixic acid (NA), oxolinic acid (OA) and norfloxacin (NFLX). The starting concentration as 1 mg (titer)/m*l* of each agent was made using a solvent appropriate for each of these agents. The minimum inhibitory concentration (MIC) value was determined by the minimum concentration of the agents that completely prevented any inoculum growth.

As a result, except for one isolate for ampicillin, the MIC distribution displays a single phase in correspondence to any 12 agents (Table 3). The isolate showing an MIC to ampicillin of > 1,000 μg/m*l* was *S*. Enteritidis.

DNA analysis based on PFGE was performed using 68 ST isolates (12 isolates in 2002, 8 in 2003, 13 in 2004, 22 in 2005 and 13 in 2006). After these strains were shock cultured at 37°C for 18 hr in 5 m*l* of LB medium, 50 μ*l* was transferred to a microtube, boiled for 30 sec and then immediately frozen. Next, the supernatant was removed after centrifugation, and a plug was created by adding the same volume of 1.6% chromosomal agarose (Bio-Rad Laboratories, Hercules, CA, U.S.A.) after suspension in 100 μ*l* of EET (100 mM EDTA, 10 mM EGTA, 10 mM Tris, pH 8.0). Afterward, the plug was processed with lysozyme at 37°C for 3 hr and then processed with proteinase K at 50°C for one night. Furthermore, after washing with 40 m*l* of TE (10 mM Tris, 1 m*l* EDTA, pH 8.0), the DNA in the plug was left at 37°C for 18 hr, cut off with Xbal (25 U) and electrophoretized (14°C, 6V/cm, 5–50 sec, 22 hr) with a PFGE device (Bio-Rad Laboratories, Hercules, CA, U.S.A.) utilizing 1% agarose gel. It was then stained with ethidium bromide after the completion of electrophoresis, and banding patterns were observed after taking pictures using a Polaroid camera on a transilluminator.

Among the tested 68 ST isolates, 2 of the strains could not be determined because of an inability to form banding in 1 case and lack of clarity in the other. Examples of the gel patterns are shown in Fig. 1. Among the 66 clear bandings formed, 65 displayed nearly the same gel pattern (pattern I:

Table 3. Distribution of the MICs of antimicrobial agents to 92 *Salmonella* strains isolated from Nov. 2002 to Feb. 2008

Anti-microbial agents	MIC (μg/m*l*)/No. of isolates												
	<0.1	0.1	0.2	0.39	0.78	1.56	3.13	6.25	12.5	25	50	100	>100
ABPC				1	16	21	24	29					1
CEZ					18	59	10	1					
OTC					9	25	31	27					
CL				5	41	33	13						
CP					8	11	59	11	3				
OA		1	13	40	31	7							
NA						4	30	57	1				
NRFX	5	12	44	30	1								
GM				17	55	18	2						
KM					1	26	35	20	10				
DSM								4	21	52	15		
FOM								6	43	39	4		

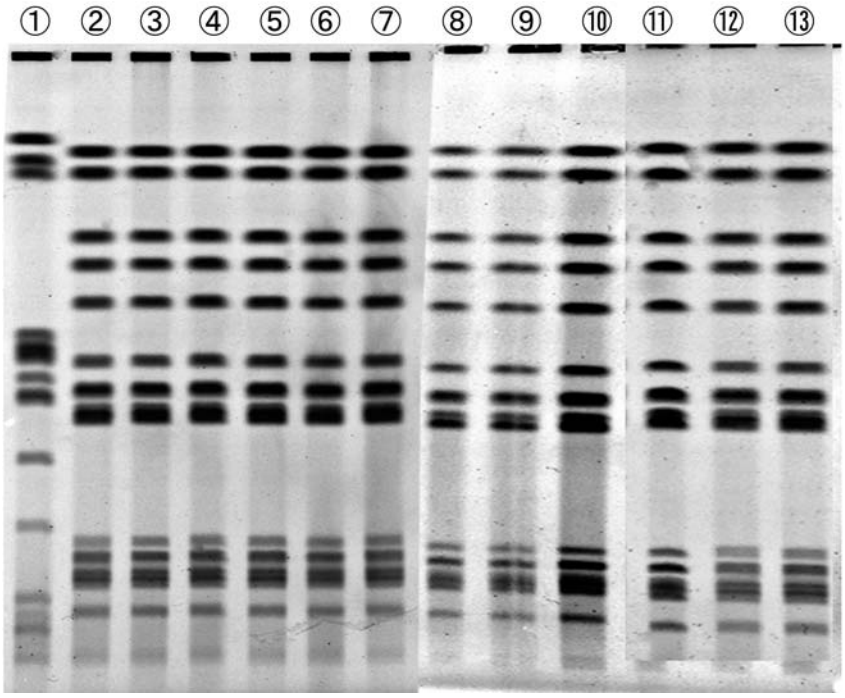

Fig. 1. PFGE pattern of *S.* Typhimurium isolated from wild crane feces.
①–⑦: Isolated in 2004. ⑧–⑩: Isolated in 2002. ⑪–⑬: Isolated in 2006. Pattern ① is clearly different from patterns ②–⑬.

②–⑬), and only 1 isolate, from 2004, displayed a different gel pattern (pattern II: ①).

For approximately 5 months, over 10,000 cranes live together in flocks in the Izumi Plain. When a pathogen invades a flock of cranes, cross infection of the pathogen among cranes will occur extremely easily. Besides cranes, other migratory birds such as ducks and crows come to Izumi Plain in large numbers and contend for the same food. There is also a large number of broiler and layer poultry farms in the area. In such an environment, the transmission of a pathogen from the cranes to chickens or other migratory birds is a great source of concern.

From the present and past [3, 4] studies, which cover a period of 10 years, ST was isolated at a high rate from crane feces every winter, and infrequent isolation of *Salmonella* serotypes other than ST was also found.

In an antibiotic sensitivity test for 159 *Salmonella* isolates, there were no discernable antibiotics with a clearly biphasic MIC value distribution; however, only 2 isolates (ST isolated in 2000 [3] and SE isolated in 2003) were considered to be resistant to gentamicin or ampicillin. Since these resistant bacteria were not isolated afterwards, they seemed to be transient. Thereafter, the crane flocks have been considered to not contain antibiotic-resistant *Salmonella*.

In previous reports [3, 4], ST isolates were reported as displaying the same attributes according to PFGE or a plasmid profile test. In the present study, the hereditary relevance among ST isolates was analyzed with PFGE using 68 ST isolates. The results showed that, excluding one ST isolate (isolated in 2004), all the isolates displayed the same gel pattern. Based on the PFGE gel pattern interpretation of Tenover *et al.* [6], it is highly possible that ST of the same origin is infecting crane flocks in the Izumi Plain.

Over a period of 10 years, the fact that the ST strains showing the same hereditary attributes were isolated each year from the crane flocks migrating to the Izumi Plane is of great interest. Considering the resting grounds are used as paddy fields from summer to spring, it is hard to imagine that the excreted ST would be preserved in the cultivated soil for about 8 months or more and then become a source of infection for cranes in the following winter.

To discuss the route of ST infection further, it is necessary to examine the existence of *Salmonella* in the soil of the resting grounds before and after crane arrival, in foods provided for cranes or in wild animals that gather and mingle there with the cranes.

In the case of chickens infected with *Salmonella* persistently, after a period in which bacteria are not detectable in feces, the bacteria become detectable again when the birds are subjected to stress, such as diseases, molt and so on [1, 2]. Considering the case of the cranes in the same light, if some of the cranes infected with ST in the Izumi Plain return to their breeding grounds in Siberia or Northern China while still carrying ST latently and then return to Izumi Plain with their chicks the following year, fatigue from such aspects as

the long journey and increased stress may cause them to excrete the bacteria again. Afterwards, the excreted ST infects the new sensitive chicks easily, propagates and is excreted in their feces on a large scale, which results in expanding contamination in the crane flocks. It is inferred that such an infection may well be repeated annually. Such conjecture does not conflict with the results showing that the isolation rate becomes higher before the cranes return to the North than soon after their arrival at the Izumi Plain.

Although serotypes other than ST have been infrequently isolated, the isolation rate is extremely low, and there have not been any cases in which they continue to be isolated. No data have been reported that explain this phenomenon. Differences in susceptibility of cranes to *Salmonella* serotypes, if they exist, might be a possible explanation.

It is of great interest whether or not the same PFGE pattern found in the ST isolated from cranes could be detected in the ST isolated from other wild birds flying to the Izumi Plain or from chickens reared in the surrounding area.

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