NOTE *Public Health*

Antimicrobial Resistance in *Salmonella* Isolates from Apparently Healthy Food-Producing Animal from 2000 to 2003: the First Stage of Japanese Veterinary Antimicrobial Resistance Monitoring (JVARM)

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ABSTRACT. Antimicrobial susceptibility of 183 *Salmonella* isolates from apparently healthy food-producing animals obtained during the period from 2000 to 2003 throughout Japan was examined. Of 29 serovars identified, *Salmonella* Infantis (37.7%) was the most prevalent, followed by *S.* Typhimurium (19.7%). *Salmonella* bacteria resistant to dihydrostreptomycin (77.6%) were about 10% more prevalent than those resistant to oxytetracycline (67.8%), though the nation-level veterinary use of tetracycline antibiotics is much greater than that of streptomycin in Japan. In seventeen isolates (9.3%) resistant to nalidixic acid, single point mutations were detected at 84 or 87 in the quinolone resistance-determining region of the gyrA gene.

KEY WORDS: antimicrobial resistance, food-producing animal, *Salmonella*.

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Salmonella, recognized as a causative agent of foodborne diseases, may cause gastrointestinal disease in humans and animals. It is sometimes present in the intestinal tract in food-producing animals. The use of antimicrobial agents in humans and animals can lead to the emergence of antimicrobial-resistant bacteria [1, 17]. In Japan, the occurrence of antimicrobial resistance in bacteria in food-producing animals has been monitored in the Japanese Veterinary Antimicrobial Resistance Monitoring (JVARM) system since 1999 [16]. Since the first stage of the JVARM program was completed in 2003, we show here data on the antimicrobial susceptibility of *Salmonella* isolates obtained from cattle, pigs, and broiler and layer chickens throughout Japan from 2000 to 2003.

In the JVARM program, the 47 prefectures of Japan were divided into four groups selected evenly on the basis of geographical difference from northern to southern areas (11 or 12 prefectures per year). Sampling and bacterial isolation were carried out at livestock hygiene service centers. In brief, freshly voided fecal samples were taken from healthy beef cattle, pigs and broiler and layer chickens at the farm. In most cases, six samples per animal species were collected from different farms in each prefecture. One gram of fecal sample was inoculated into 10 m*l* of Hajna tetrathionate broth, followed by incubation at 42°C for 18 hr or an additional 5–7 days at room temperature as delayed secondary enrichment culture. After incubation, each culture was streaked onto DHL and brilliant green agar plates each containing 20 μg/m*l* of novobiocin. Candidate colonies were identified biochemically. Identification of isolates for serovar was then performed by slide and tube agglutination according to the latest versions of the Kauffmann-White scheme.

Salmonella was isolated from 16 of 650 cattle (2.5%, 25 isolates), 20 of 527 pigs (3.8%, 39 isolates), 57 of 283 broiler chickens (20.1%, 91 isolates) and 15 of 444 layer chickens (3.4%, 25 isolates). A total of 183 isolates were obtained between 2000 and 2003. Twenty-nine serovars were identified as shown in Table 1. The major serovars of isolates were *S.* Typhimurium in cattle (76.0%, 19/25) and pigs (43.6%, 17/39) and *S.* Infantis in broiler chickens (71.4%, 65/91). A wide variety of serovars was found in the isolates from layer chickens.

The minimum inhibitory concentrations (MICs) of 20 different antimicrobials were determined by the agar dilution method according to the guidelines of National Committee for Clinical Laboratory Standards (NCCLS) [11]. *Staphylococcus aureus* ATCC 29213, *Enterococcus faecalis* ATCC29212, *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 were used as quality control strains. As antimicrobial susceptibility of the following isolates had been determined in our previous study: 22 *Salmonella* isolates in 2001 [6], 18 *S.* Typhimurium DT104 isolates in 2000 [5] and 39 *S.* Infantis isolates between 2002 and 2003 [2], 104 isolates were subjected to antimicrobial susceptibility testing. The MICs of each antimicrobial were interpreted using the NCCLS criteria [12]. The resistant breakpoints of dihydrostreptomycin (DSM), oxytetracycline (OTC), bicozamycin (BCM), colistin (CL), oxalic acid (OA), enrofloxacin (ERFX) and ofloxacin (OFLX) were determined according to our previous report [6].

Resistance was found for 9 of 20 antimicrobials tested, 77.6% for DSM and 67.8% for OTC (Table 2). *Salmonella* resistant to DSM was about 10% more prevalent than that resistant to OTC, though the nation-level veterinary use of tetracycline antibiotics is much greater than that of streptomycin [17]. In Japan, the frequency of OTC resistance was

Table 1. *Salmonella* serovars isolated from food-producing animals between 2000 and 2003

Serovars	Cattle	Pigs	Broiler	Layer	Total
Infantis		2	65	2	69
Typhimurium	19	17			36
Agona		4	4	2	10
Thompson			2	4	6
Enteritidis			3	2	5
Virchow			4	1	5
Dublin	4				4
Brandenburg		3			3
Hader			3		3
Anatum		4			2
Bareilly				2	2
Blockley			2		2
Corvallis				2	2
Derby		2			2
Haifa			2		2
Havana				2	2
Istanbul			2		2
Mbandaka	2				2
Minesota		2			2
Mons				2	2
Montevideo				2	2
Newport			2		2
Othmarschen				2	2
Tennessee				2	2
Albany				1	1
Isangi				1	1
Pakistan		1			1
Zanzibar		1			1
Untypable		3	2	1	3
Total	25	39	91	28	183

higher than that of DSM resistance in *Escherichia coli* isolates from apparently healthy food-producing animals [9]. The antimicrobial resistance rates of *E. coli* isolates were significantly correlated to the usage of antimicrobial agents in food-producing animals [3]. However, the overall usage of veterinary antimicrobials did not necessarily contribute to appearance of antimicrobial resistance in *Salmonella* isolates. Someya *et al.* [16] have shown the persistence of antimicrobial-resistant *Salmonella* in layer chicken flocks without antimicrobial selective pressure.

There were no significant differences in resistance rates to the antimicrobials tested except for ABPC among the 4 years. The resistance rate to ABPC decreased from 29.7% in 2000 to 0% in 2003 (Table 2). However, no isolates originating from cattle were obtained in 2003, in which resistance to ABPC was frequently found. A small number of isolates were used for antimicrobial susceptibility tests in 2001 and 2003. Thus, it is difficult to appraise the annual data of antimicrobial resistance in *Salmonella* isolates from food-producing animals in JVARM program.

The present study showed that 131 (71.6%) isolates were resistant to two or more of the antimicrobials tested (Table 3). Resistance rates of layer chicken isolates to two or more antimicrobials (10.7%, 3/28) were the lowest among those of the 4 animal species. The majority of multiantimicrobial-resistant (MAR) isolates were derived from cattle, pigs, and broiler chickens. *S.* Typhimurium accounted for 70.0% of the MAR isolates from cattle (18/22) and pigs (17/28), and *S.* Infantis accounted for 78.2% (61/78) of the MAR isolates from broiler chickens. OTC and DSM resistances of *Salmonella* have been shown to be strongly associated with the

Table 2. Antimicrobial susceptibility of *Salmonella* isolates[a] (n=183) from food-producing animals

Antimicrobial agents	Break point (μg/m*l*)	MIC range (μg/m*l*)	No. resistance (%) 2000 (n=91)	2001[b] (n=22)	2002 (n=50)	2003 (n=20)	No. resistance (%) Cattle (n=25)	Pig (n=39)	Broiler (n=91)	Layer (n=28)	Total
Ampicillin	32	0.5–512<	27 (29.7)	4 (18.2)	6 (12.0)	0 (0)	18 (72.0)	15 (38.5)	4 (4.4)	0 (0)	37 (20.2)
Cefazolin	32	1–16	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0(0)
Cefuroxime		2–16									
Ceftiofur		0.5–2									
Apramycin		0.5–8									
Destomycin A		8–64									
Dihydrostreptomycin	32	8–512<	68 (74.7)	17 (77.3)	43 (86.0)	14 (70.0)	22 (88.0)	32 (82.1)	79 (86.8)	9 (32.1)	142 (77.6)
Gentamicin	16	≤0.125–1	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0(0)
Kanamycin	64	0.5–512<	31 (34.1)	6 (27.3)	8 (40.0)	12 (40.0)	6 (24.0)	5 (12.8)	54 (59.3)	0 (0)	65 (35.5)
Oxytetracycline	16	1–512	57 (62.6)	15 (68.2)	38 (76.0)	14 (70.0)	18 (72.0)	26 (66.7)	76 (83.5)	4 (14.3)	124 (67.8)
Bicozamycin	128	16–512<	2 (2.2)	2 (9.1)	3 (6.0)	0 (0)	0 (0)	2 (5.1)	1 (1.1)	4 (14.3)	7 (3.8)
Chloramphenicol	32	1–512<	22 (24.2)	4 (18.2)	6 (12.0)	0 (0)	17 (68.0)	13 (33.3)	2 (2.2)	0 (0)	32 (17.5)
Colistin	16	0.5–64	1 (1.1)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (3.6)	1 (0.5)
Nalidixic acid	32	2–512	7 (7.7)	2 (9.1)	4 (8.0)	4 (20.0)	4 (16.0)	0 (0)	13 (14.3)	0 (0)	17 (9.3)
Oxolinic acid	2	≤0.125–16	7 (7.7)	2 (9.1)	4 (8.0)	4 (20.0)	4 (16.0)	0 (0)	13 (14.3)	0 (0)	17 (9.3)
Enrofloxacin	2	≤0.125–1	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0(0)
Ofloxacin	2	≤0.125–2	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0(0)
Olaquindox		8–128									
Trimethoprim	16	≤0.125–512<	19 (20.9)	4 (18.2)	17 (34.0)	6 (30.0)	1 (4.0)	4 (10.3)	40 (44.0)	1 (3.6)	46 (25.1)
Sulphadimethoxine		256–512<									

a) Isolates includes 22 *Salmonella* isolates in 2001 [6], 18 *S.* Typhimurium DT104 isolates in 2000 [5] and 39 *S.* Infantis isolates between 2002 and 2003 [2].

b) Data from our previous report [6] were interrupted according to resistant breakpoint in this study.

Table 3. Antimicrobial resistance patterns of *Salmonella* isolates by each animal origin

No. of antimicrobials	Antimicrobial resistance pattern	Cattle	Pigs	Broiler	Layer
0	Susceptible	3(1/0)[a)]	6	7	13(0/2)
1	ABPC[b)]	0	1	0	0
	BCM	0	0	0	4
	CL	0	0	0	1
	DSM	0	4	3(0/2)	6
	KM	0	0	2(0/2)	0
	OTC	0	0	1	1
2	ABPC–DSM	0	1	0	0
	DSM-KM	0	1	0	0
	DSM-OTC	0	9(6/0)	10(0/10)	2
	NA-OA	0	0	2	0
3	ABPC -DSM-OTC	0	0	2	0
	DSM-KM-OTC	0	0	13(0/10)	0
	DSM-KM-TMP	0	0	1(0/1)	0
	DSM-OTC-TMP	0	2(0/2)	4(0/4)	1
4	ABPC -DSM-KM-OTC	1(1/0)	0	0	0
	ABPC -DSM-OTC-CP	16(16/0)	11(9/0)	0	0
	DSM-KM-NA-OA	4	0	0	0
	DSM-KM-OTC-BCM	0	2	0	0
	DSM-KM-OTC-CP	0	0	2	0
	DSM-KM-OTC-TMP	0	0	30(0/28)	0
	DSM-OTC-NA-OA	0	0	8(0/4)	0
5	ABPC -DSM-KM-OTC-TMP	0	0	2	0
	DSM-KM-OTC-BCM-TMP	0	0	1(0/1)	0
	DSM-KM-OTC-NA-OA	0	0	1(0/1)	0
6	ABPC -DSM-KM-OTC-TMP-CP	1(1/0)	2(2/0)	0	0
	DSM-KM-OTC-TMP-NA-OA	0	0	2(0/2)	0
	Total	25(19/0)	39(17/2)	91(0/65)	28(0/2)

a) No. of *Salmonella* isolates (No. of *S.* Typhimurium isolates / Infantis isolates.
b) ABPC: Ampicillin, DSM: Dihydrostreptomycin, KM: Kanamycin, OTC: Oxytetracycline, BCM: Bicozamycin, CP: Chloramphenicol, NA: Nalidixic acid, OA: Oxolinic acid, TMP: Trimethoprim.

dissemination of *S.* Typhimurium in cattle/pigs and *S.* Infantis in poultry [6]. In our previous study carried out between 1999 and 2001, DT104 was found in 53.1% of *S.* Typhimurium isolates from food-producing animals [5]. In Japan, *S.* Typhimurium DT104 was first isolated in the late 1980s and has spread widely among domestic animals in Japan in the past decade [15]. *S.* Infantis from retail broiler meats increased in 1997 [10], and the isolates from broiler meats harbored antimicrobial-resistant types similar to those of broiler isolates [2]. The prevalence of MAR *S.* Infantis is much higher in broilers in Japan than in other countries [8, 14]. Both serovars have been prevalent on farms in Japan for a long time. Therefore, eradication of *Salmonella* from herds is essential for resolving the problem of antimicrobial resistance as well as for maintenance of public and animal health.

Fluoroquinolones have been used for veterinary medicine in Japan since approval in 1991, and the use of fluoroquinolones has been increasing since around 1992 [18]. Fluoroquinolone resistance in *E. coli* has increased in food-producing animals in Japan [9]. All of the isolates were susceptible to fluoroquinolones (ERFX and OFLX), though 17 isolates exhibited resistance to quinolones (nalidixic acid (NA) and OA). Resistance to quinolones in *Salmonella* can be caused by alterations in the target enzymes (DNA gyrase and topoisomerase IV), decreased permeability of drugs, or an active efflux mechanism [13]. Point mutations in the quinolone resistance-determining region (QRDR) of the *gyrA* gene in the GyrA subunit of DNA gyrase have frequently been detected in *Salmonella* [7, 13]. Mutations in the QRDRs of the *gyrA, gyrB* and *parC* genes were detected by direct DNA sequencing. Amplification of the genes and purification of amplicons were performed as described previously [7]. Nucleotide sequence was determined by using a Dye Terminator Cycle Sequencing Ready Kit with a 310 genetic analyzer (Applied Biosystems). In all of the quinolone-resistant isolates, single point mutations in the QRDR were detected in the *gyrA* gene but not in the *gyrB* and *parC* genes (Table 4). Recently, we reported that fluoroquinolone-resistant *S.* Choleraesuis from a diseased pig in 2001 had two point mutations in the *gyrA* gene and a mutation in the *parC* gene [4]. Levels of resistance of *Salmonella* to quinolones were markedly higher in broiler isolates. The rate of resistance of *E. coli* to ERFX increased in broiler iso-

Table 4. Mutations in the *gyrA* gene in quinolone-resistant *Salmonella* isolates from apparently healthy animals in Japan

Serovar	No. of isolates	MIC range (μg/ml) of			*gyrA* 83 Ser	*gyrA* 87 Asp	Isolation year
		NA	OA	ERFX	TCC	GAC	
Infantis	4[a)]	128–256	2	≤0.125	– – –	A– –	2002
	2	128	2	≤0.125	– – –	–G–	2000
	1	256	4	0.25	–T–	– – –	2000
Dublin	4	256–512	4–8	0.25–0.5	– – –	T– –	2000
Istanbul	2	256	2–4	0.25	– – –	T– –	2001
Haifa	2	512	8	0.5	– – –	A– –	2003
O-Untypable	2	512	8–16	1	– – –	A– –	2003

a) Data of mutation in the *gyrA* gene from our previous study [2].

lates from 0% in 1992 to 9.9% in 1999 [9] and to 3.1% in 2001 [3]. Fluoroquinolones are one of the most valuable antimicrobial classes available for treating human infection. We will continue to monitor fluoroquinolone resistance and its use in food-producing animals in order to prevent the spread of fluoroquinolone-resistant *Salmonella*.

In the 1st stage of JVARM, antimicrobial susceptibility of *Salmonella* isolates to 20 antimicrobials was examined. MIC of cefuroxime was found to be similar to that of cefazolin even though the generation levels of cephalosporin are different. Both destomycin A and olaquindox were ingredients of antimicrobial growth promoter for gram-positive bacteria, and MIC distributions of them were not bimodal. Developments of resistance to OA and OFLX were similar to those of NA and ERFX, respectively. In prior consultation for the 2nd stage, the following antimicrobials were excluded from the targeted agents on the basis of the results obtained in the1st stage of JVARM: cefuroxime, destomycin A, OA, OFLX, and olaquindox.

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