



## NOTE

Public Health

# Increased *Salmonella* Schwarzengrund prevalence and antimicrobial susceptibility of *Salmonella enterica* isolated from broiler chickens in Kagoshima Prefecture in Japan between 2013 and 2016

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**ABSTRACT.** This study aimed to analyze the *Salmonella* serovars, measure the minimum inhibitory concentration of antimicrobials, and examine the antimicrobial resistance genes of *Salmonella* isolated from 192 broiler flocks in Kagoshima Prefecture in Japan, from 2013 to 2016. We found that all *Salmonella* isolates belonged to three serovars: *Salmonella* Manhattan, *S. infantis*, and *S. Schwarzengrund*. Among them, *S. Schwarzengrund* prevalence has recently increased annually making the main serovar. Most recovered isolates were highly resistant to streptomycin, sulfamethoxazole, and oxytetracycline. We saw the reduction of third-generation cephalosporin resistance and identified the reason of increased kanamycin resistance to be the increased number of *S. Schwarzengrund* isolates. Among the kanamycin-resistant *Salmonella* isolates, *aphA1* constituted the main resistance gene detected.

**KEY WORDS:** antimicrobial susceptibility, broiler chicken, kanamycin resistance gene, *Salmonella*, *Salmonella* Schwarzengrund

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Salmonellosis, one of the most important diseases in both humans and animals, has been described as the second most commonly caused foodborne bacterial disease worldwide [12]. *Salmonella* is one of the four key global causes of diarrheal diseases, with 2579 serovars identified till date [17]. Antimicrobial agents are widely used during poultry production for growth promotion, or treatment purposes [14]. Resistance to antimicrobial agents in bacteria is mediated by several mechanisms including changes in bacterial cell wall permeability, energy-dependent removal of antimicrobials via membrane-bound efflux pumps, modification of the site of drug action, and destruction or inactivation of the drug [3, 19]. Bacteria can acquire resistance genes through mobile elements, such as plasmids, which provide flexibility to the host bacteria and promote the spread and distribution of these genes across the diverse bacterial population [4].

Notably, we recently reported an increase in the prevalence of *Salmonella* in broiler chickens in Japan including the first report of *Salmonella* Schwarzengrund detection in 2012, which is the main serovar detected in Kagoshima Prefecture, Japan presently [9]. *S. Schwarzengrund* has been reported as an emerging pathogen in Asia, Denmark, the United States of America and Brazil [1, 2, 15]. In this study, we analyze the *Salmonella* serovars, measure the minimal inhibitory concentration (MIC) of antimicrobials, and examine the resistance genes in order to describe the recent fluctuations of antimicrobial susceptibility of *Salmonella* in broiler chickens and investigate its mechanism.

During 2013 to 2016, we analyzed 3069 cecal specimens from 192 broiler flocks (approximately 10,000 birds per flock) collected by the prefectural officials at an accredited poultry processing plant in Kagoshima Prefecture, Japan. Samples were

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**Table 1.** Prevalence of *Salmonella* in broiler chickens during 2013–2016 in Kagoshima, Japan

Year	No. of flocks	No. of positive flocks (%)	No. of samples	No. of positive samples (%)
2013	48	31 (64.6)	767	82 (10.7)
2014	48	41 (85.4)	767	153 (19.9)
2015	48	40 (83.3)	768	157 (20.4)
2016	48	39 (81.3)	767	154 (20.1)
Total	192	151 (78.6)	3,069	546 (17.8)

**Table 2.** Incidence of *Salmonella* serovars in broiler chickens in Kagoshima, Japan during two periods (2009–2012 and 2013–2016)

Serovar	Survey period	
	2009–2012 <sup>a</sup> (%)	2013–2016 <sup>b</sup> (%)
No. of <i>S. Infantis</i>	140 (57.6)	147 (26.9)
No. of <i>S. Manhattan</i>	98 (40.3)	283 (51.8)
No. of <i>S. Schwarzengrund</i>	5 (2.1)	116 (21.3)
Total	243	546

a) Cited from our previous study [9]. b) This study.

delivered to the Laboratory of Veterinary Public Health, Kagoshima University, and cultured on the day of arrival. [8, 9]. The antimicrobial susceptibility of the *Salmonella* isolates was ascertained by the agar dilution method using Mueller Hinton agar (Oxoid Ltd., Basingstoke, UK) [20, 21, 25]. Two kanamycin resistance genes *aphA1*, and *kn* were detected by using PCR [7, 11, 13].

*Salmonella* prevalence in broiler chickens from 2013 to 2016 in Kagoshima Prefecture, Japan is shown in Table 1. Overall, the prevalence of *Salmonella*-positive flocks exhibited a dramatic increase during the last three years in the study period compared to that during the first year. In general, the incidence of *Salmonella* in the flocks was 78.6% (151/192; 48 flocks per year for four years) and the proportion of *Salmonella*-positive samples in the total number of samples from broiler chickens was 17.8% (546/3069). As shown in Table 1, *Salmonella* prevalence at both the flock and individual broiler chicken levels in the present study (78.6%) is much higher than that in our previous study (49.0%) [9]. Our report was similar to the *Salmonella* prevalence in Japan reported by Yamazaki *et al.* [24], and Sasaki *et al.* [18]. Alternatively, *Salmonella* prevalence was reported to vary considerably across different geographic regions worldwide. In Sweden, a study from 2007 to 2015 on housed broilers and laying hens reported that the percentage of *Salmonella*-positive broiler flocks was 2.0% [23]. A study in Egypt reported that 41.0% of tested broiler flocks were positive for *Salmonella* along with 1.09% of tested samples [10].

The *Salmonella* isolates from broiler chickens in Kagoshima Prefecture, Japan belongs to three serovars: *Infantis*, *Manhattan*, and *Schwarzengrund* across the four years of the present study, as also reported in our previous study [9], although the relative proportions differed as shown in Table 2. The largest differences were observed in *Infantis* and *Schwarzengrund* serovars. Across both studies, *S. Infantis* proportion exhibited a dramatic decrease. In contrast, *S. Schwarzengrund* and *S. Manhattan* percentage steadily increased from 2.1 and 40.3%, respectively, in 2009–2012 to 21.3 and 51.8%, respectively, in 2013–2016.

In Japan, *S. Schwarzengrund* proportion of broiler chicken origin increased from 0% in 2000–2003 to 28.1% in 2005–2007 and was resistant to streptomycin, oxytetracyclin and kanamycin [2]; a high incidence of *S. Schwarzengrund* was also detected in Kyushu region, Japan with 123 positive samples from 184 *Salmonella* strains (66.8%) isolated from broiler chickens [24]. Moreover, a study conducted in Taiwan from 2004 to 2006 indicated *S. Schwarzengrund* contamination prevalence in raw chicken meat samples as 30.5% [6]. In our present study, the number of *S. Schwarzengrund* strains isolated increased dramatically from 5 to 116 (Table 2). Together, these studies support that *S. Schwarzengrund* has become one of the most prevalent serovars in broiler chickens in East Asia.

Table 3 describes that the proportion of *Salmonella* antimicrobial resistance slightly changed across the previous (2009–2012) [9] and current (2013–2016) study periods. Ampicillin, cefotaxime, and ceftiofur resistance was concurrently and markedly decreases. Conversely, kanamycin-resistant *Salmonella* proportion increased from 6.6% in 2009–2012 to 13.7% in 2013–2016. The majority of *S. Schwarzengrund* were sensitive to ampicillin, cefotaxime, cefoxitin, and ceftiofur (zero percent resistance).

As shown in Table 4, almost all *Salmonella* strains of the three serovars were sensitive to chloramphenicol and ofloxacin, whereas over 80% of each serovar exhibited resistance to streptomycin, sulfamethoxazole, and oxytetracycline. In our survey from 2009 to 2012, the increased proportion of the *S. Manhattan* serovar led to an annual increase in resistance to ampicillin, cefotaxime, and ceftiofur [9]. In the present study, although *S. Manhattan* percentage was 51.8% (Table 2) the resistance rate of all *Salmonella* serovars decreased compared to that from 2009 to 2012 [9]. This may be due to the decrease in the number of *S. Infantis* and increase in *S. Schwarzengrund* from 2013 to 2016, as all isolated *S. Schwarzengrund* (109 isolates) were sensitive to ampicillin, cefotaxime, and ceftiofur (Table 4). The  $\beta$ -lactam antimicrobial resistance rate of *S. Manhattan* was higher than those of *S. Infantis* and *S. Schwarzengrund*. In addition, considerable differences in kanamycin resistance were detected among the three serovars. While the majority of *S. Manhattan* was susceptible to kanamycin, *S. Infantis* exhibited a resistance rate at 10.8% and *S. Schwarzengrund* showed the maximum rate, with 47.7% resistance to kanamycin. The reduction of  $\beta$ -lactam resistance proportion in our study may be the same as reported by Mauro *et al.* [16], where the authors indicated that the off-label use of ceftiofur with Marek's vaccine is associated with the short-term increase in ESBL-producing *Escherichia coli* in the gut of broiler chickens. In Japan, the same situation appeared following the cessation of ceftiofur use by the Japanese poultry industry [22].

Figure 1 shows a comparison of the specific antimicrobial resistance rates for *S. Infantis* (Fig. 1a) and *S. Manhattan* (Fig. 1b) between the current (2013–2016) and previous (2009–2012) [9] study periods. However, as only five strains of *S. Schwarzengrund* were isolated in the previous period [9], we did not perform the comparison for this serovar. *S. Infantis* proportion exhibiting antimicrobial resistance to ampicillin, cefotaxime, streptomycin, sulfamethoxazole, and oxytetracycline slightly decreased in the current study period compared to that in the previous study period (Fig. 1a). No change was observed in cefoxitin,

**Table 3.** Antimicrobial susceptibility profiles from the current study and our previous study [9] of *Salmonella* isolates from broiler chickens in Kagoshima, Japan

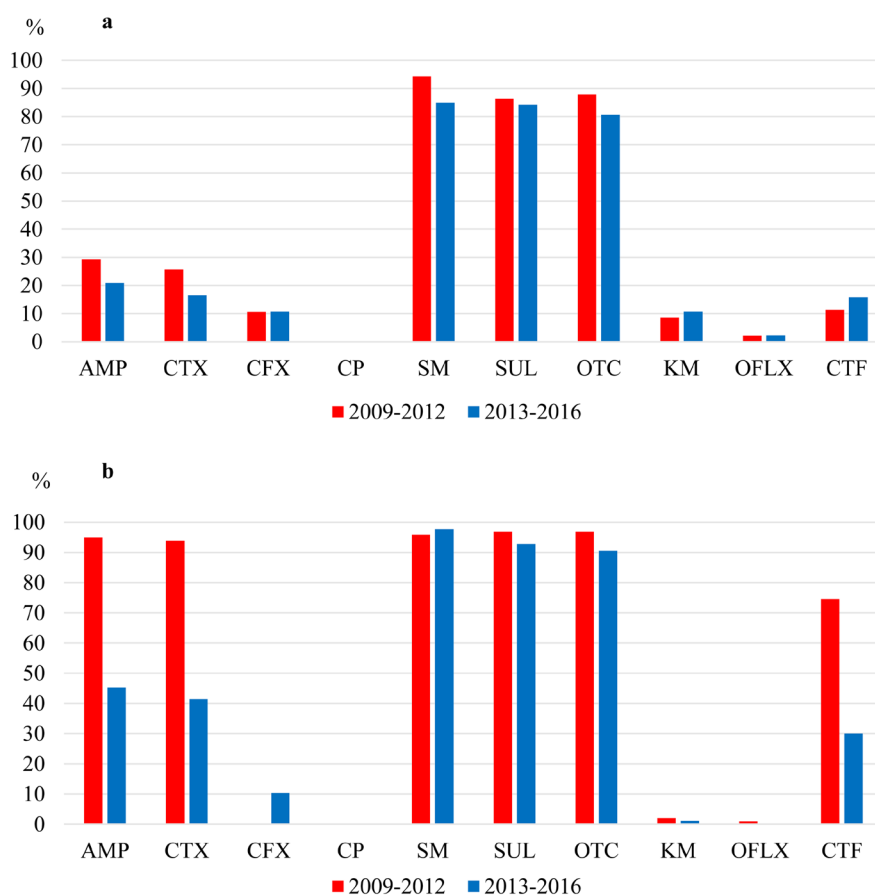
Antimicrobial agent	MIC Break-point ( $\mu\text{g/ml}$ )	No. of resistant isolates (%)	
		Previous study <sup>a)</sup>	Current study <sup>b)</sup>
		2009–2012 n=243 <sup>a)</sup>	2013–2016 n=511 <sup>*b)</sup>
AMP	$\geq 32$	134 (55.1)	148 (29.0)
CTX	$\geq 4$	128 (52.7)	132 (25.8)
CFX	$\geq 32$	15 (6.2)	42 (8.2)
CP	$\geq 32$	0 (0.0)	0 (0.0)
SM	$\geq 16$	231 (95.1)	484 (94.7)
SUL	$\geq 512$	221 (91.0)	463 (90.6)
OTC	$\geq 16$	222 (91.4)	451 (88.3)
KM	$\geq 64$	16 (6.6)	70 (13.7)
OFLX	$\geq 2$	4 (1.6)	3 (0.59)
CTF	$\geq 8$	124 (51.0) <sup>b)</sup>	112 (22.0)

a) Cited from our previous study [9]. b) This study. \*The number of strains (511) differs from the total given in Table 2 (546) because at the time of MIC testing, some strains were dried and not suitable for use. AMP, ampicillin; CTX, cefotaxime; CFX, ceftiofur; CP, chloramphenicol; SM, streptomycin; SUL, sulfamethoxazole; OTC, oxytetracycline; KM, kanamycin; OFLX, ofloxacin; CTF, ceftiofur.

**Table 4.** Comparison of antimicrobial resistance of *Salmonella* Schwarzengrund, *S. Manhattan* and *S. Infantis* during the 2013–2016 study period

Antimicrobial agent	No. of resistant isolates (%)		
	<i>S. Schwarzengrund</i> n=109	<i>S. Manhattan</i> n=263	<i>S. Infantis</i> n=139
	AMP	0 (0.0)	119 (45.2)
CTX	0 (0.0)	109 (41.4)	23 (16.5)
CFX	0 (0.0)	27 (10.3)	15 (10.8)
CP	0 (0.0)	0 (0.0)	0 (0.0)
SM	109 (100)	257 (97.7)	118 (84.9)
SUL	102 (93.6)	244 (92.8)	117 (84.2)
OTC	101 (92.7)	238 (90.5)	112 (80.6)
KM	52 (47.7)	3 (1.1)	15 (10.8)
OFLX	0 (0.0)	0 (0.0)	3 (2.2)
CTF	0 (0.0)	90 (34.2)	22 (15.8)

AMP, ampicillin; CTX, cefotaxime; CFX, ceftiofur; CP, chloramphenicol; SM, streptomycin; SUL, sulfamethoxazole; OTC, oxytetracycline; KM, kanamycin; OFLX, ofloxacin; CTF, ceftiofur.



**Fig. 1.** Change of antimicrobial resistance from 2009–2012 to 2013–2016 among (a) *Salmonella Infantis* and (b) *S. Manhattan*. AMP, ampicillin; CTX, cefotaxime; CFX, ceftiofur; CP, chloramphenicol; SM, streptomycin; SUL, sulfamethoxazole; OTC, oxytetracycline; KM, kanamycin; OFLX, ofloxacin; CTF, ceftiofur.

**Table 5.** Distribution of the *aphA1* kanamycin resistance gene from *Salmonella* serovars isolated from broiler chickens during the 2013–2016 study period

Serovar (no. of isolates)	MIC of kanamycin ( $\mu\text{g/ml}$ )	No. of isolates tested	No. of isolates positive for <i>aphA1</i> resistant gene (%)
<i>S. Infantis</i> (13)	512	13	13 (100)
	256	-	-
	128	-	-
<i>S. Manhattan</i> (3)	512	1	1 (100)
	256	1	0 (0.0)
	128	1	0 (0.0)
<i>S. Schwarzengrund</i> (52)	512	51	51 (100)
	256	1	0 (0.0)
	128	-	-
Total		68	65 (95.6)

MIC, minimal inhibitory concentration.

chloramphenicol, and ofloxacin resistance, whereas kanamycin and ceftiofur resistance was slightly increased. In comparison, the resistance rate of *S. Manhattan* to streptomycin, sulfamethoxazole, oxytetracycline, chloramphenicol, kanamycin, and ofloxacin minimally fluctuated between the two periods. The percentage of resistance to three antimicrobials decreased in the present period: ampicillin (from 94.9% to 45.2%), cefotaxime (from 93.9% to 41.4%), and ceftiofur (from 74.5% to 30.0%). In contrast, cefoxitin-resistant *S. Manhattan* resistance increased from 0 to 10.3% between the previous and current study periods.

We further evaluated 68 kanamycin-resistant *S. enterica* isolates from Kagoshima Prefecture, Japan during the present study period (2013–2016) (13 *S. Infantis*, 3 *S. Manhattan*, and 52 *S. Schwarzengrund*) for kanamycin resistance genes (*kn* and *aphA1*) by PCR. None of the 68 isolates carried *kn*, whereas 65/68 (95.6%) carried *aphA1* (Table 5). All the 13 *S. Infantis* isolates (MIC: 512  $\mu\text{g/ml}$ ) carried *aphA1*. Of the three *S. Manhattan* isolates, one (MIC: 512  $\mu\text{g/ml}$ ) carried *aphA1* whereas two others (MIC: 256 and 128  $\mu\text{g/ml}$ ) did not. The 51 *S. Schwarzengrund* isolates with MIC of 512  $\mu\text{g/ml}$  carried *aphA1*; that with MIC of 256  $\mu\text{g/ml}$  did not.

*aph* gene was found in almost kanamycin-resistant of *S. enterica* serovars isolated in some regions of the United States of America in 2005 [5]. A study in the United States of America and China [7] found *aph* in *S. Enteritidis*, *S. Haardt*, and an unidentified serovar from chicken meat and *S. Derby* from pork. In comparison, we found the *aph* gene (but not *kn*) in three serovars: *S. Infantis*, *S. Manhattan*, and *S. Schwarzengrund*. This may suggest that this gene commonly serves to provide kanamycin resistance in numerous *Salmonella* serovars.

Together, our findings revealed that there has been a recent increase in the population of the *S. Schwarzengrund*-strain, making it the main serovar of *Salmonella* isolated from broiler chickens in Kagoshima Prefecture in Japan, followed by *S. Manhattan*. In turn, the increase of *S. Schwarzengrund*, which exhibited a high level of kanamycin resistance, led to a decrease in the rate of antimicrobial resistance to ampicillin, cefotaxime, and ceftiofur among all *Salmonella* isolates and affected the increase in the percentage of kanamycin-resistant isolates. In addition, the resistance rate of *S. Manhattan* to  $\beta$ -lactams in this study slightly decreased compared to that in our previous study [9], which also affected the overall rate of resistance to  $\beta$ -lactams. Moreover, we demonstrated that *aphA1* is the main antimicrobial resistance gene in *Salmonella* isolates. These changing profiles indicate the need for continual evaluation and research regarding the molecular characteristics of *Salmonella* in broiler chickens.

**CONFLICT OF INTEREST.** The authors declare that this research was conducted in the absence of any commercial or financial relationship that could be construed as a potential conflict of interest.

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