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Decreasing trend of *β*-lactam resistance in *Salmonella* isolates from broiler chickens due to the cessation of ceftiofur *in ovo* administration

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ABSTRACT

Ceftiofur, a third-generation cephalosporin antimicrobial, was used in Japanese hatcheries for many years before 2012. We continue to study *Salmonella* in broilers and their antimicrobial resistance. The current study aimed to express how the cessation of ceftiofur affects the antimicrobial resistance of *Salmonella* isolated from broiler chickens in Kagoshima Prefecture, Japan in 2017 and 2018. A total of 274 *Salmonella* isolates were recovered from 1535 cecal content samples obtained from 96 broiler flocks over years 2017 and 2018. Among the *S. enterica* isolates, the predominant serovars were *S.* Manhattan (128/274, 46.7%), *S.* Schwarzengrund (120/274, 43.8%), and *S.* Infantis (26/274, 9.5%). The isolates showed a high proportion of antimicrobial resistance for oxytetracycline, sulfamethoxazole, and streptomycin. However, the β-lactam resistance rates were significantly decreased ($p < 0.01$) in 2017, while no *β*-lactam resistant isolates detected in 2018. The highlight of this study was the complete disappearance of *β*-lactam resistance in *Salmonella* isolates from broiler chicken in Kagoshima, Japan.

1. Introduction

Salmonellae are facultative intracellular Gram-negative bacteria that cause high morbidity and mortality in many hosts including humans, birds, mammals, and insects (Bäumler, Tsolis, Ficht & Adams, 1998). They are among the most problematic, foodborne, and zoonotic pathogens that cause health threats and challenges to general human well-being (Balasubramanian et al., 2019). *Salmonella* spp. are one of the main pathogens causing foodborne bacterial infections in humans and poultry products are the main sources of bacterial contamination (Vieira et al., 2009).

Poultry is one of the most widely consumed food products worldwide. Chicken is the most commonly farmed species, with over 90 billion tons of chicken meat produced per year (Food & Agriculture Organization. FAO Publications Catalogue 2017, 2019). A large diversity of antimicrobials are used to raise poultry in most countries (Boamah, Agyare, Odoi & Dalasgaar, 2016; Landers, Cohen, Wittum & Larson, 2012; Sahoo, Tamhankar, Johansson & Lundborg, 2010). The main reasons for using antimicrobials in food-producing animals include prevention of infections, treatment of infections, promotion of growth, and improvement in production of farm animals (Castanon, 2007; Mathew, Liamthong & Lin, 2009). However, one of the reasons that contribute to emergence of antimicrobial-resistant strains is the frequent administration of antimicrobials in the treatment of poultry diseases.

Antimicrobial resistance is one of the biggest threats to global health, food security, and development. A growing number of salmonellosis infections are becoming harder to treat as the antimicrobials used to treat them become less effective (WHO "Antimicrobial resistance, 2020).

Over the last decade, the high incidence of multidrug resistance in *Enterobacteriaceae* has become a serious public health problem worldwide. Because of their critical importance for human and veterinary

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medicine (Perez, Endimiani, Hujer & Bonomo, 2007), β-lactams are among the most clinically important antimicrobials in both human and veterinary medicine. Until now, more than 700 distinct β-lactams have been described (Ambler et al., 1991).

Ceftiofur (CTF), a third-generation cephalosporin antimicrobial, has been administered *in ovo* to reduce early chick mortality in many countries (Agunos et al., 2017). In Japan CTF was extensively used off-label until March 2012 as a disinfectant for embryonated eggs and newborn chicks, but CTF was not except by officially licensed for therapeutic use in Japanese poultry farm (Hiki et al., 2015). After the voluntary cessation of CTF in 2012, the decreasing trend of CTF resistant *E.coli* and *Salmonella* was found in some Japanese poultry retail meat (Hiki et al., 2015; Shigemura et al., 2018).

Thus, the primary objective of this study was to express how the cessation of ceftiofur affects the antimicrobial resistance of *Salmonella* isolated from broiler chickens in Kagoshima Prefecture, Japan in 2017 and 2018.

2. Materials and methods

2.1. Sample collection

A total of 1535 cecal specimens derived from 96 broiler flocks (approximately 10,000 birds per flock) collected by prefectural officials at an accredited poultry processing plant in Kagoshima Prefecture, Japan, were analyzed over years 2017 and 2018. The poultry processing plant released these samples (which would otherwise have been disposed of as waste material) with the approval of prefectural officials and sent them to our laboratory. Typically, 16 randomly selected samples per flock were collected every two weeks. Samples were collected in sterile bags, and transported in isothermal boxes to maintain the refrigeration temperature until arrival at the laboratory, and same day processing (Chuma et al., 2013; Duc et al., 2019).

2.2. Salmonella isolation and identification

Approximately 1 g of cecal contents was aseptically mixed with 5 mL of sterilized distilled water and homogenized by vortexing. Then, 1 mL of the suspension was pre-enriched in 5 mL of Hajna tetrathionate broth (Eiken Chemical Co., Ltd., Tokyo, Japan) and incubated in a water bath at 42 °C. After 24 h of incubation, a loopful of the culture was streaked onto a selective Rambach agar plate, which was incubated at 37 °C for 24 h. Characteristic colonies were confirmed by biochemical tests (Shahada et al., 2006). After this, they were submitted to serology performed with reliable commercial antisera (Denta Seiken, Niigata, Japan), and the results were interpreted according to the Kaufmann-White scheme (Popoff & Le Minor, 1992).

2.3. Determination of MICs

The antimicrobial susceptibility of *Salmonella* isolates was ascertained by the agar dilution method using Mueller-Hinton agar (Oxoid Ltd., Basingstoke, UK) (Shahada, Amamoto, Chuma, Shirai & Okamoto, 2007, 2010). Strains were tested for sensitivity to ampicillin, cefotaxime, cefoxitin, chloramphenicol, streptomycin, sulfamethoxazole, oxytetracycline, kanamycin, ofloxacin, and ceftiofur. The MIC range was set at 0.25–512 μg/mL for all tested antimicrobial agents. MIC breakpoints were interpreted according to the criteria established by the Clinical and Laboratory Standards Institute (CLSI, 2012), streptomycin, and oxytetracycline were adopted resistance MIC breakpoint as previously recommended (Duc et al., 2019). The MIC breakpoint of ceftiofur was established by NARMS (resistance breakpoint ≥ 8) (Antimicrobials tested by NARMS (National Antimicrobial Resistance Monitoring System for Enteric Bacteria 2020). *Escherichia coli* (America Type Culture Collection (ATCC 25,922), and *Staphylococcus aureus* (ATCC 29,213) were used as reference and quality control strains.

2.4. Statistical analysis

The changes in the percentage of resistant isolates in each antimicrobial agent and the decrease in the percentage of resistant isolates in each *Salmonella* serovars were compared by multiple comparisons. A chi-square test was first performed to detect significant differences for each antimicrobial agent, and each serovar. When the result was significant, a test for multiple proportion comparisons was performed (Ryan, 1960).

3. Results

3.1. Prevalence and serovar switching

The prevalence and distribution of *Salmonella* in broiler chickens in 2017 and 2018 in Kagoshima Prefecture, Japan is shown in Table 1. Overall, the prevalence of positive flocks, and positive samples exhibited a significant decrease over the two years. The incidence of *Salmonella* in the flocks was 81.3% (78/96; 48 flocks per year for two years), and the rate of positive sample was 17.9% (274/1535). The proportion of positive flocks was 89.6% in 2017 and significantly decreased to 72.9% in 2018, while the rate of positive samples was 23.2% in 2017 and significantly decreased to 12.5% in 2018.

Among the 274 *Salmonella* isolates obtained in 2017 and 2018, the most prevalent serovars were *S.* Manhattan (46.7%: 128/274), *S.* Schwarzengrund (43.8%: 120/274), and *S.* Infantis (9.5%: 26/274). The dominant serovars in both years were *S.* Manhattan and *S.* Schwarzengrund: 51.1% (91/178) and 42.3% (77/178) in 2017 and 38.5% (37/96) and 44.8% (43/96) in 2018, respectively.

3.2. Antimicrobial resistance and the β-lactam resistance trend

The distribution of MIC of 264 *Salmonella* strains isolated in two years 2017 and 2018 is showed in Table 2. The difference was 274 *Salmonella* isolated; however, 10 strains were lost during the stock. All 264 strains were susceptible to chloramphenicol. The rates of resistance were the highest for streptomycin, sulfamethoxazole, and oxytetracycline, and over 80% of strains were resistant to these antimicrobials; 249 (94.3%) were resistant to streptomycin (MIC $\geq$16 μg/mL), 217 (82.2%) to oxytetracycline (MIC $\geq$16 μg/mL), 213 (80.7%) to sulfamethoxazole (MIC $\geq$512 μg/mL), and 77 (29.2%) strains had kanamycin resistance. Many isolates have a low resistance to the β-lactam group including ampicillin (7.6%), ceftiofur (7.6%), cefotaxime (5.7%), and cefoxitin (0.8%). The *Salmonella* isolated resistance to ofloxacin at 1.9%.

Table 3 demonstrated the comparison in β-lactam resistance of each *Salmonella* serovars between three periods (2009–2012, 2013–2016, and 2017–2018), and in 2018. In all three periods, we did not compare the *β*-lactam resistance of *S.* Schwarengrund because that was susceptible to all of β-lactam included ampicillin, cefotaxime, cefoxitin, and ceftiofur. A significant decrease trend of *β*-lactam resistance can be seen in the recent periods. The resistant proportion of *S.* Manhattan to ampicillin, cefotaxime, and ceftiofur decreased continuously over three periods. From over ninety percent to around forty-five percent and around ten percent, respectively. The cefoxitin resistance rate of this serovar decreased from more than ten percent in the period (2013–2016) to zero percent in the study period. While the resistance proportion of *S.* Infantis to ampicillin, cefotaxime, and ceftiofur showed a slight decrease according to three periods. The ampicillin-resistant rate decreased from 29.3% to 20.9%, and 7.7%, respectively. Cefotaxime resistance rate went down from 25.7% to 16.5%, and 7.7%, respectively. Ceftiofur resistant rate decreased from 24.3% to 15.8%, and 7.7%, respectively. In the year 2018, all *S.* Manhattan and *S.* Infantis were susceptible to β-lactam.

Table 1
The prevalence and distribution of *Salmonella* serovars isolated from broilers in Japan in 2017 and 2018.

Year	No. of flocks	No. of positive flocks (%)	No. of samples	No. of positive samples (%)	*Salmonella* serovars		
					Infantis isolates	Manhattan isolates	Schwarzengrund isolates
2017	48	43 (89.6)	767	178 (23.2)	10	91	77
2018	48	35 (72.9)	768	96 (12.5) [#]	16	37	43
Total	96	78 (81.3)	1535	274 (17.9)	26	128	120

* Significant decreased from previous year: $p < 0.05$.
[#] Significant decreased from previous year: $p < 0.001$.

Table 2
The distribution of MIC on 264 *Salmonella* isolates in 2017 and 2018.

Antimi-crobial agent	MIC break point (μg/ml)	No. of isolates at the MIC (μg/mL)												No. of resistance (%)
		0.25	0.5	1	2	4	8	16	32	64	128	256	512	
AMP	≥32	1	1	56	169	14	0	0	0	1	7	0	12	20 (7.6)
CTX	≥4	247	2	0	0	1	1	0	1	6	0	0	6	15 (5.7)
CFX	≥32	0	0	9	181	61	11	0	2	0	0	0	0	2 (0.8)
CTF	≥8	41	100	81	21	1	0	3	2	8	6	1	0	20 (7.6)
CP	≥32	0	1	1	51	124	81	2	0	0	0	0	0	0 (0.0)
SM	≥16	0	0	0	0	0	15	5	39	163	37	4	1	249 (94.3)
SUL	≥512	0	0	0	0	0	0	5	33	9	4	0	213	213 (80.7)
OTC	≥16	0	0	6	9	28	4	3	2	0	57	152	3	217 (82.2)
OFLX	≥2	249	5	5	4	0	0	1	0	0	0	0	0	5 (1.9)
KM	≥64	0	5	24	61	89	8	0	0	0	2	1	74	77 (29.2)

*From 274 *Salmonella* isolated, 10 strains were lost during the stock.

Table 3
Comparison in β-lactam resistance of *S.* Manhattan, and *S.* Infatis between three periods (2009–2012, 2013–2016, and 2017–2018), and the year 2018.

Antimicr-obial agent	No. of resistant isolates (%)							
	S. Manhattan				*S.* Infantis			
	(2009–2012) [11] *n* = 98 (%)	(2013–2016) [12] *n* = 263 (%)	(2017–2018) [*] *n* = 120 (%)	2018 [*] *n* = 34 (%)	(2009–2012) [11] *n* = 140 (%)	(2013–2016) [12] *n* = 139 (%)	(2017–2018) [*] *n* = 26 (%)	2018 [*] *n* = 16 (%)
AMP	93 (94.9)	119 (45.2) [↓]	18 (15.0)[#,↓]	0 (0.0)[#,↓]	41 (29.3)	29 (20.9) [↓]	2 (7.7)[#,↓]	0 (0.0)[#,↓]
CTX	92 (93.9)	109 (41.4) [↓]	13 (10.8)[#,↓]	0 (0.0)[#,↓]	36 (25.7)	23 (16.5) [↓]	2 (7.7)[#,↓]	0 (0.0)[#,↓]
CFX	0 (0.0)	27 (10.3)	0 (0.0) [↓]	0 (0.0) [↓]	15 (10.7)	15 (10.8)	2 (7.7)	0 (0.0)[#,↓]
CTF	90 (91.8)	90 (34.2) [↓]	18 (15.0)[#,↓]	0 (0.0)[#,↓]	34 (24.3)	22 (15.8) [↓]	2 (7.7)[#,↓]	0 (0.0)[#,↓]

[11] : Cited from Duc et al. 2019.
[12] : Cited from Duc et al. 2020.
[*] : This study.
[↓] : Significant decrease from the previous period ($p < 0.05$).
[#] : Significant decrease from the period (2009–2012) ($p < 0.01$).

4. Discussion

As shown in Table 1, the prevalence of *Salmonella* at the flock broiler chicken level in the present study was slightly higher than that in our previous study (81.3% and 78.6%). However, in 2018 the positive flock rate (72.9%) was much lower than those from 2014 to 2017 (Duc et al., 2020). The proportion of positive samples decreased two-fold in 2018, and was the lowest rate in the last 5 years. These findings may signal the beginning of a decreasing trend of *Salmonella* colonization among broiler chicken in Kagoshima Prefecture, Japan.

The specific serovars identified in Kagoshima Prefecture, Japan, belonged to *S.* Infantis, *S.* Manhattan, and *S.* Schwarzengrund. The current study results were similar to those of previous studies where all three serovars were investigated. However, the distribution of each serovar changed each year (Duc et al., 2019, 2020). *S.* Schwarzengrund and *S.* Manhattan isolated were the main serovar, while *S.* Infantis was not predominant in recent isolates. We believed that imported original breeder chickens were the source of *S.* Schwarzengrund contamination and spreading throughout Japan, but there was no evidence. The top three serovars *S.* Infantis, *S.* Manhattan, and *S.* Schwarzengrund were also found in other studies in Japan (Sasaki et al., 2012; Shigemura et al., 2018).

However, the distribution of *Salmonella* serovars isolated from broiler chicken was not the same in other countries. In Taiwan between 2000 and 2005 the main serovars were *S.* Albany, *S.* Schwarzengrund, *S.* Istanbul, *S.* Derby, and *S.* Typhimurium (Chen et al., 2010), and the most common serovars in Ecuadorian broilers were *S.* Infantis, *S.* Enteritidis, and *S.* Corvallis (Vinueza-Burgos, Cevallos, Ron-Garrido, Bertrand & De Zutter, 2016).

In the study we used the same MIC methodology as previous studies (Duc et al., 2019, 2020). In comparison with previous studies from 2009 to 2012 (Duc et al., 2019), and from 2013 to 2016 (Duc et al., 2020), the high proportions (more than 80%) of resistance to OTC, SM, and SUL were similar to those reported previously. However, most of the *Salmonella* isolates were susceptible to chloramphenicol and ofloxacin.

In all three periods (2009–2012, 2013–2016, and 2017–2018), *S.* Schwarengrund strains were susceptible to four antimicrobial agents in the β-lactam group. On the other hand, there was were significant differences in the proportion of *S.* Manhattan and *S.* Infantis resistant to the β-lactam group across previous periods and the current study periods. In particular, the β-lactam resistance rate of *S.* Manhattan decreased markedly in three stages [Table 3], and that serovars were the most prevalent in the 2013–2016, and 2017–2018 periods.

Several studies worldwide reported the high resistance to antimicrobials in the β-lactam group: a study in Ecuadorian broiler in 2016 showed that 78.8% and 80.8% of *Salmonella* isolates were resistant to ampicillin and cefotaxime (Vinueza-Burgos et al., 2016). In a study in Taiwan, 228 *S.* Schwarzengrund strains selected from chicken meat were highly resistant to ampicillin but most of them were susceptible to cefoxitin (Chen et al., 2010). Our result of 2017 and 2018 research is consistent with our previous results for cefoxitin resistance when *S.* Schwarzengrund isolates were the most prevalent recently, but not in ampicillin resistance. Some studies in the Japanese poultry industry suggested that the decreasing trend of extended-spectrum cephalosporin-resistant in *Salmonella,* and *E. coli* may be due to not combining ceftiofur in vaccination for broiler chickens after 2012 (Hiki et al., 2015; Shigemura et al., 2018). This survey was performed in the same methodology and under the same conditions as the previous report, so it can be compared with historical data. In the present study, a decreasing tendency of resistance to ceftiofur was observed.

Together, our findings revealed that *S.* Manhattan, and *S.* Schwarzengrund are the main serovar of *Salmonella* isolated from broiler chickens in Kagoshima Prefecture, Japan. In a previous study *S.* Schwarzengrund was susceptible to β-lactams, but *S.* Infantis and *S.* Manhattan were not (Duc et al., 2019, 2020). However, in this study, we saw the disappearance of β-lactams resistance not only in *S.* Schwarzengrund but also in *S.* Infantis and *S.* Manhattan in 2018. These changing profiles indicate the need for continual evaluation and research regarding the molecular characteristics of *Salmonella* in broiler chickens.

Author contribution

Vu Minh Duc: Sampling; MIC assay; Data curation; Investigation; Writing original draft. Rina Kakiuchi: Sampling; Isolation: Identification. Hiroka Muneyasu Sampling; Isolation: Identification. Hajime Toyofuku: Data analysis; Interpretation; Revision. Takeshi Obi: Data analysis; Interpretation; Revision. Takehisa Chuma: Study design; Supervision: Writing-review & editing.

Ethical statement

Specimen collection and carcass handling were conducted in accordance with Japanese regulations on poultry carcass inspection. The specimens were released from the accredited poultry processing plant to the analysis laboratory under Japanese regulations on poultry processing. This study was approved by the institutional animal care and ethics committee of the joint Faculty of Veterinary Medicine, Kagoshima University.

Declaration of Competing 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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