STUDIES ON THE TRANSITION IN SEROVARS AND ANTIMICROBIAL RESISTANCE OF *SALMONELLA* ISOLATED FROM BROILER CHICKENS IN KAGOSHIMA, JAPAN

鹿児島の肉用鶏から分離されたサルモネラの血清型と抗菌剤耐性の推移に関する研究

The United Graduate School of Veterinary Science

Yamaguchi University

VU MINH DUC

March, 2021

STUDIES ON THE TRANSITION IN SEROVARS AND ANTIMICROBIAL RESISTANCE OF *SALMONELLA* ISOLATED FROM BROILER CHICKENS IN KAGOSHIMA, JAPAN

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LIST OF ABBREVIATIONS

AMP	: Ampicillin
AR	: Antimicrobial resistance
ATCC	: American type culture collection
CFX	: Cefoxitin
CP	: Chloramphenicol
CTF	: Ceftiofur
CTX	: Cefortaxime
ESBL	: Extended-Spectrum beta lactamase
KM	: Kanamycin
MDR	: Multidrug Resistance
MIC	: Minimum Inhibitory Concentration
OTC	: Oxytetracycline
OFLX	: Ofloxacin
PCR	: Polymerase Chain Reaction
SM	: Strptomycin
SUL	: Sulfamrthoxazole
VPH	: Veterinary Public Health

GENERAL INTRODUCTION

The importance of *Salmonella* on Veterinary Public Health

The World Health Organization (WHO) defines veterinary public health (VPH) as "the sum of contributions to complete physical, mental and social well-being of humans through an understanding and application of veterinary medical science" **[WHO, 2002].** With definition every veterinary surgeon contributes to public health, whether through provision of health care for pets, protection of animal welfare, biomedical research, or ensuring adequate food animal production and food safety.

Food contamination caused by pathogens has attracted increasing attention worldwide. Although the study of food contaminant detection has made some progress, contamination with pathogenic organisms is still a common health problem worldwide **[Gil *et al.*, 2015].** Emerging and re-emerging infectious diseases are recognized as a global problem, and 75% of these are potentially zoonotic **[Taylor *et al.*, 2001].** According to the report of WHO **[WHO, 2020]**, an estimated 600 million people (almost 1 in 10 people in the world) fall ill after eating contamination food and 420.000 die every year. *Salmonella, Campylobacter,* and Enterohaemorrhagic

Escherichia coli are among the most common foodborne pathogens that affect millions of people annually – sometimes with severe and fatal outcomes. The symptoms are fever, headache, nausea, vomiting, abdominal pain and diarrhea. Besides that, foodborne salmonellosis is the most relevant disease with a high global impact in human health. It was estimated that non-typhoidal *Salmonella* causes around 93.8 million illnesses and 155.000 deaths each year worldwide **[Majowicz *et al.*, 2010].**

In addition, human infections caused by *S. enterica* are commonly considered to be associated with food of animal origins, mainly poultry meat and eggs **[Foley *et al.*, 2008].** Some research reports indicate possible transmission of *S. enterica* from chicken and eggs to humans **[Best *et al.*, 2007; Kim *et al.*, 2008].** A study in South Korea in 2017 also suggested the high potential for transmission of *S. enterica* between humans and chickens, supporting significant risks to public health posed by *S. enterica* **[Kang *et al.*, 2017].** Salmonellosis is an important global public health problem causing substantial morbidity and thus also has a significant economic impact. Although most infections cause mild to moderate self-limited disease, serious infections leading to deaths do occur **[de Jong *et al.*, 2006].** Foodborne diseases still dominate as the most important public health problem in most countries. Globally,

despite the institution of several control measures, *Salmonella* infections continue to be problematic with millions of cases occurring annually, both in humans and animals **[Hoelzer *et al.*, 2011].**

Contamination of *Salmonella* on poultry production

Salmonella is named after an American bacteriologist, D. E. Salmon, who first isolated *Salmonella choleraesuis* from porcine intestine in 1884 **[Smith, 1894].** *Salmonella* is a Gram-negative, facultative intracellular pathogen belonging to the Enterobacteriaceae family. The genus *Salmonella* is composed of two taxonomical species, *S. bongori,* and *S. enterica,* with all medically relevant salmonellae apart of the latter **[CDC, 2018].** *S. enterica* is the type species and it further divided into six subspecies **[Su *et al.*, 2007]** that includes over 2.600 serotypes **[Gal-Mor *et al.*, 2014].** *Salmonella* species are non-spore-forming predominantly motile enterobacteria with cell diameters between about 0.7 and 1.5 μm, lengths from 2 to 5 μm, and peritrichous flagella (all around the cell body) **[Fabrega A, and Vila J., 2013].** The antigenic classification system of various *Salmonella* serovars used today has accumulated for many years of studies on antibody interactions with surface antigens of *Salmonella* organisms established by Kauffman and White more than a century ago. All antigenic

formulae of recognized *Salmonella* serotypes are listed in a document called the Kauffmann-White scheme. **[Popoff, and Le Minor L., 2001].**

The taxonomy and classification of *Salmonella* was **Kingdom:** Bacteria, **Phylum:** Proteobacteria, **Class:** Gamaproteobacteria, **Order:** Enterobacteriales, **Family:** Enterobacteriaceae, **Genus:** *Salmonella,* **Species:** *Salmonella bongori,* and *Salmonella enterica* consists of six subspecies*: S. enterica* subsp. *enterica, S. enterica* subsp. *salamae, S. enterica* subsp. *arizonae, S. enterica* subsp. *diarizone, S. enterica* subsp. *houtenae, and S. enterica* subsp. *indica.*

Salmonella bacteria live in the intestines of people, animals, and birds. Most people are infected with *Salmonella* by eating contaminated foods i.e. poultry meat, eggs, fruits and vegetables. Some of 2,600 *Salmonella* serovars identified were responsible for human illness and diseases in a wide variety of animals **[Gast, 2008]**. In addition to the risks to public health, *Salmonella* spp. infections impose economic losses to both healthcare systems and the poultry industry **[Collard *et al.,* 2008]**.

In spite of significant improvement in technology and hygienic practices at all stages of poultry production accompanied with advanced improvement in public sanitation, salmonellosis and *Salmonella* infection remain a persistent threat to human and animal health. In many countries high incidence of salmonellosis in man appears

to be caused by infection derived from contaminated eggs, poultry meat, and meat-products. The contaminated products cause disease as a results of inadequate cooking or cross contamination of working surfaces in kitchen environment **[Hafez, 2001, Thorns, 2000, and Omwandho *et al.,* 2010].**

The risk factors associated with *Salmonella* infection and contamination in broiler chickens include contaminated chicks, size of the farm and contaminated feed **[Marin *et al.,* 2011, Arsenault *et al.,* 2007].** It also depends on age of the chicken, animal health, survival of organism in the gastric barrier, diet and genetic constitution of the chicken could also affect the colonization ability of *Salmonella* spp. in poultry **[Cosby *et al.,* 2015].**

Many *Salmonella* serovars have been associated with poultry meat and eggs. Because some of serovars can colonize and infect live birds, *Salmonella* contamination has been a continuous problem in the poultry industry. Therefore, many countries have national programs to monitor and control *Salmonella* in poultry. **[Wegener *et al.,* 2003].** In order to manage the risk to public health, it is essential to investigate the prevalence of *Salmonella* infections at the farm level and counteract this problem to reduce the amount of cross contamination which can occur throughout the food chain process.

Prevalence of *Salmonella* species varies considerably depending on countries and types of production as well as the detection methods applied. In developing countries, the wet markets or poultry cottages serve as important supply sources of chickens. At the outlets, prevalence rate for *Salmonella* spp. in raw dressed chicken can vary among countries and between outlets as documented in Korea was 42.7% **[Bae *et al.,* 2013]**, in Vietnam 45.9% **[Ta *et al.,* 2012]**, in India 65.0% **[Badhe *et al.,* 2013]**, and in Malaysia, 100% **[Nidaullah *et al.,* 2017].**

Antimicrobial resistance of *Salmonella enterica*

Antimicrobial resistance (AR) which defined as the ability of an organism to resist the killing effects of an antimicrobial to which it was normally susceptible **[Madigan *et al.,* 2014]** and it has become an issue of global interest **[WHO, 2014].** In recent years, enough evidence highlighting a link between excessive use of antimicrobial agents. AR from animals as a contributing factor to the overall burden of AR has emerged **[Marshall *et al.,* 2011].** The extent of usage is expected to increase markedly over coming years due to intensification of farming practices in most of the developing countries **[Van Boeckel *et al.,* 2015].** The main reasons for the use of antimicrobials in food-producing animals include prevention of infections,

treatment of infections, promotion of growth and improvement in production in the farm animals **[Mathew *et al.,* 2009, and Castanon, *2007*].**

AR is a global phenomenon resulting in the emergence of pathogens with resistance to clinically important antimicrobials **[Bell *et al.,* 2014]**. AR bacteria cause life-threatening illness in humans and pose a significant threat to health and well-being. AR in foodborne pathogens such as *Salmonella* is a major concern for public health. More focus is required to target them in the animals food supply **[CDC, 2013].** *Salmonella* is difficult to eliminate from its reservoir hosts, and food animals often serve as reservoirs of the pathogen. Non-typhoidal *Salmonella* causes the high number of illness, hospitalizations, and deaths associated with foodborne illness **[Scallan *et al.,* 2011].**

Foodborne pathogens such as *Salmonella* enter a farm from different sources, such as water, litter, personnel, equipment, vehicles, rodents, insects, and pets. In addition, the movement of portable equipment and vehicles can act as a vector for carrying the pathogen to the farm environment or slaughterhouses **[Heyndrickx *et al.,* 2002].** Similarly AR bacteria also spread through truck-washing systems, barn floor, barn flush, and holding pens, and potentially and up in animal carcasses during slaughter **[Dorr *et al.,* 2009].**

Poultry is one of the most widespread food industries worldwide. Chicken is the most common farmed species, with over 90 billion tons of chicken meat produced per year **[FAO, 2017]**. A large diversity of antimicrobials are used to raise poultry in many countries **[Landers *et al.,* 2012, Sahoo *et al.,* 2010, and Boamah *et al.,* 2010]**, and a large number of such antimicrobials are considered to be essential in human medicine **[WHO, 2010].** In addition, there are also human health concerns about the presence of antimicrobial residues in meat, eggs, and other animal products **[Aalipour *et al.,* 2013, Darwish *et al.,* 2013, Goetting *et al.,* 2011].**

Recently, *Salmonella* Heidelberg and, *Salmonella* Kentuckey isolated from broiler farms in Brazil was resistant to multiple antimicrobials, including streptomycin, Gentamycin, sulfadimethoxine, tetracycline and trimethoprim-sulfamethoxazole combination. **[Lijiebjelke *et al.,* 2017]**. *Salmonella* Enteritidis were reported to be resistant to multiples drugs including ampicillin, nalidixic acid, and tetracycline **[Al-Zenki *et al.,* 2007]**. The high resistant of *Salmonella* serovars such as: *S.* Enteritidis, *S.* Infatis, *S.* Typhimurium, and *S.* Heidelberg isolated from broiler carcasses towards ceftriaxone (75%) and ceftiofur (44%) **[Medeiros *et al.,* 2011].** Duffy *et al.* revealed a high prevalence of AR *Salmonella* in poultry meat compared to beef and lamb samples **[Duffy *et al.,* 1999]**. And, they observed

Salmonella Bredeney, *S.* Kentuckey, and *S.* Enteritidis as the major serotypes. These serotypes showed high resistance to antimicrobials such as rifampicin, tetracycline, oxytetracycline, and sulphamethoxazole. A study about *Salmonella* isolated from retail meats showed high resistance to common antimicrobials such as: tetracycline, streptomycin, sulfamethoxazole, and ampicillin **[Chen *et al.,* 2004].** Paveen *et al.* reported that *Salmonella* spp. isolated from poultry chiller water and carcasses were resistant to antimicrobials including tetracycline, ampicillin, amoxicillin-clavulanic acid, ceftiofur, streptomycin, and sulfamethoxazole **[Parveen *et al.,* 2007]**.

Generally, when an antimicrobial is used in any setting, it eliminates the susceptible bacterial strains leaving behind those with traits that can resist the drug. These resistant bacteria then multiply and become the dominating population and as such, are able to transfer (both horizontally and vertically) the genes responsible for their resistance to other bacteria. Once these pathogens are in the human system, they could colonize the intestines and the resistant genes could be shared or transferred to the endogenous intestinal flora **[De Leener *et al.,* 2005, Hall *et al.,* 2011, and Jakobsen *et al.,* 2010].** In addition, farm environments are the reservoirs of pathogens, including AR bacteria **[Kelley *et al.,* 1998, and Chen *et al.,* 2014].**

Inappropriate antimicrobial usage in animal production is one of the reasons that makes AR. Some countries have withdrawn the use of some classes of antimicrobials or set up structures that regulate the use of selected antimicrobials in animal production **[Chock, 2001]**. Despite these developments, it is currently estimated that over 60% of all antimicrobials produced are used in livestock production, including poultry **[Van Boeckel *et al.,* 2014].** The use of antimicrobials in poultry and livestock production is favorable to farmers and the economy as well, because it has generally improved poultry performance effectively and economically. But, at the same time, the likely dissemination of AR strains of pathogenic and non-pathogenic organisms into the environment and their further transmission to humans via the food chain could also lead to serious consequences on public health **[Apata, 2009].**

The horizontal transmission of resistance genes plays a vital role in the dissemination of antimicrobial resistance in *Salmonella enterica* species. These resistance genes can be found in the resistant plasmids or within the chromosome of bacteria **[Carattoli, 2003]**. The resistant genes that are acquired by plasmids, integrons, or transposons are capable of transferring resistance to other strains or other species **[Domingues *et al.,* 2012]**.

Salmonella spp. showing resistance to extended-spectrum cephalosporins is a serious concern. One of the major mechanisms of developing resistance against β-lactam antimicrobials in bacteria is the direct inactivation of antimicrobials by enzymes hydrolysis **[Blair *et al.,* 2015]**. The production of extended spectrum β-lactamases (ESBLs) is a major mechanism conferring resistance in most of the *Enterobacteriaceae*. Many types of ESBLs are present based on the substrates and inhibitor mechanisms **[Shaikh *et al.,* 2015]**.

Aminoglycoside-modifying enzymes (aminoglycoside acetyltransferases) mainly mediate resistance to aminoglycoside antimicrobials. The genes encoding aminoglycoside resistance are named as *aac*, and are the typically located in *Salmonella* genomic islands, integrons, and plasmids. The acetyltransferases provide resistance to major antimicrobials such as gentamycin and kanamycin. In addition, aminoglycoside hydroxyl group phosphorylating enzymes, namely aminoglycoside phosphotransferases, are involved in resistance against aminoglycoside antimicrobials in *Salmonella*. These enzymes are encoded by the genes *strA, strB, aph3-Ib,* and *aph6-Id* providing resistance to streptomycin. Some of the aminoglycoside phosphotransferases also provide resistance to kanamycin and neomycin. Among the different varieties of aminoglycoside adenylyltranferases

encoding genes, *aadA* provides resistance to streptomycin whereas *aadB* provides resistance to gentamicin and tobramycin in *Salmonella* **[Alcaine *et al.,* 2007]**.

Tetracycline resistance is mainly developed in *Salmonella* due to the acquisition of genes that code for energy-dependent efflux mechanisms **[Alcaine *et al.,* 2007]**. Mainly *tet* genes are involved in efflux mechanisms, and confer resistance to chlortetracycline, doxycycline, oxytetracycline, and tetracycline **[Roberts *et al.,* 2016]**. Among these, *tetA* is common. However, others such as *tetB, tetC, tetD, tetG,* and *tetH* have been reported in *Salmonella enterica* from clinical or retail meat isolates **[Alcaine *et al.,* 2007, McDermott *et al.,* 2016].** The *tetA* genes have been found in plasmids, integrons, and genomic island 1.

The resistance development in microorganisms against phenicol antimicrobials including chloramphenicol and florfenicol is mainly by two mechanisms involving efflux pumps or enzymatic inactivation of antimicrobials by chloramphenicol O-acetyltrasferase. The genes encoding chloramphenicol O-acetyltrasferase are referred to as *cat* genes and are often associated with plasmids **[Schwarz *et al.,* 2004]**. The *floR* genes are widely distributed among the *Salmonella* serovars and are found to be associated with transferable plasmids and *Salmonella* genomic islands **[Alcaine *et al.,* 2007].**

The sulfonamide resistance in *Salmonella* is due to the presence of *sul* gene, which causes the expression of an insensitive from dihydropteroate synthetase that cannot be inhibited by sulfonamides. The *sul* genes like *sul1, sul2,* and *sul3* have been identified frequently from major *Salmonella* serovars, including *S.* Enteritidis, *S.* Typhimurium, *S.* Heidelberg, and *S.* Hadar **[Alcaine *et al.,* 2007, Huovinen *et al.,* 1995]**. These genes are present in integrons, *Salmonella* genomic islands, or transferrable plasmids.

Objectives

The objectives of these studies were to determine the prevalence of *Salmonella* amongst broiler chickens to establish the actual status of *Salmonella* infections, to investigate the change of serovars and antimicrobial resistance profiles, and to provide the relationship between them in eight years from 2009 to 2016.

In Chapter 1, the prevalence, serovars, and antimicrobial resistance of *Salmonella* isolated from broiler chickens in the period from 2009 to 2012 were investigated. In particular, the multidrug resistance, and the third-generation cephalosporin resistance were focused.

In chapter 2, *Salmonella* serovars and antimicrobial resistance were investigated in the period from 2013 to 2016 and compared to the previous period. In particular, the increase of kanamycin resistance and the decrease of the third-generation cephalosporin resistance were focused on.

CHAPTER 1

PREVALENCE OF *SALMONELLA* IN BROILER CHICKENS IN KAGOSHIMA, JAPAN IN 2009 TO 2012 AND THE RELATIONSHIP BETWEEN SEROVARS CHANGING AND ANTIMICROBIAL RESISTANCE

Introduction

Salmonella is a major foodborne pathogen that causes an estimated 153 million enteric infections and 56,969 diarrheal deaths each year worldwide **[Kirk *et al.,* 2015]**. Chicken meat and eggs have been reported as a major source of *Salmonella* contamination. Therefore, it is important to control *Salmonella* in chicken- and egg-containing food products **[Hope *et al.*, 2002, Hedican *et al.,* 2009]**.

Despite significant improvements in technology and hygienic practices at all stages of chicken production, salmonellosis and *Salmonella* infections remain an intransigent threat to human and animal health. In many countries the high incidence of salmonellosis in humans appears to be caused by infection derived from contaminated eggs, poultry meat and meat-containing products. The contaminated products cause disease as a result of inadequate cooking or cross contamination of working surfaces in the kitchen environment **[Hafez 2001, Omwandho *et al.,* 2010].**

According to food poisoning statistics from the Infectious Disease Surveillance Center in Japan, there were 93,444 bacterial foodborne illnesses between 1999 and 2002, and 32% of these cases were salmonellosis **[http://idsc.nih.go.jp/iasr/index.html]**. According to another survey in Japan, *Salmonella* is the second most common (after *Campylobacter* infection) cause of bacterial foodborne outbreaks **[Ministry of Health, Labour and Welfare of Japan. 2015].**

Poultry, especially broiler chickens, are well known reservoirs of various *Salmonella* serovars, many of which are able to infect humans and *Salmonella* Infantis has been the most prevalent serovar isolated from fresh poultry meat and broiler flocks all over in Europe **[Nógrády *et al.,* 2012].** Nine serovars of *Salmonella* were detected in Japan from retail chicken meat in 2012, *S.* Infantis (33%), *S.* Schwarzengrund (12%), and *S.* Manhattan (9%) were the most frequent **[Furukawa *et al.,* 2017]**. Beside, *Salmonella* Schwarengrund is one of the *Salmonella* serovars responsible for human and poultry infections in some countries, for sample, the United States, Denmark and Thailand **[Aarestrup *et al.,* 2007; Silva *et al.,* 2013]**.

Antimicrobial resistance is becoming an increasingly important issue in salmonellosis in both animals and human **[Su *et al.,* 2004]**. In poultry production,

antimicrobial agents are widely used for growth promotion, or treatment purposes **[Gyles, 2008]**. As a consequence, chicken and chicken meat can harbor antimicrobial resistant strains and function as a vehicle for dissemination of these to human. Today, antimicrobial resistant of *Salmonella* strains are frequently encountered in most of the world and the proportion of antimicrobial resistant dramatically increased over the past decade **[WHO, 2018]**.

Research on the epidemiology of *Salmonella* throughout the food chain is important for determining the specific distribution patterns of antimicrobial resistance for this pathogen. In this study, we analyzed the prevalence, serovars, and antimicrobial resistance profiles of *Salmonella* isolates from broiler chickens in Kagoshima, Japan. This knowledge will help to understand the relationship between changes in the serovar and antimicrobial resistance patterns of *Salmonella*, and define guidelines for improved salmonellosis control which in turn might lead to fever human foodborne salmonellosis cases.

Materials and Methods

Sampling

We analyzed a total of 3071 cecal specimens derived from 192 broiler flocks (ca. 10.000 birds per flock) collected by prefectural officials at an accredited poultry

processing plant during the period of 2009-2012. The processing plant released these samples (which would otherwise have been disposed of a waste material) with the approval of prefectural officials and sent them to our laboratory. Typically, 16 randomly selected samples per flocks were collected fortnightly **[Chuma *et al.*, 2013].**

Salmonella Isolation and identification

Samples were collected using sterile techniques, placed in sterile plastic sampling bags, and chilled with ice blocks during transport. Samples were delivered to the Laboratory of Veterinary Public Health, Kagoshima University, and cultured on the day of arrival. 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 incubation, a loopful of the culture was streaked onto a selective Rambach agar plate, which was incubated at 37°C for 24 h **[Shahada *et al.*, 2006]**.

Suspected pink colonies were selected from each plate and streaked on nutrient agar slants. Salmonella identification was confirmed by biochemical tests, including fermentation of glucose, lactose and sucrose, hydrogen sulfide production, citrate utilization, lysine decarboxylation, methyl red and indole tests. Serotyping of isolated

Salmonella strains was performed with reliable commercial antisera (Difco, Detroit, MI, USA), and the results were interpreted according to the Kaufmann-White scheme **[Popoff *et al.*, 1992**].

Determination of minimum inhibitory concentrations (MICs)

The antimicrobial susceptibility of the *Salmonella* isolates was assessed by the agar dilution method on Mueller-Hinton agar (Oxoid Ltd., Basingstoke, UK) plates according to the guidelines of the Clinical and Laboratory Standards Institute (formerly the National Committee for Clinical Laboratory Standards [NCCLS]) **[Shahada *et al.*, 2007]**. Strains were tested for sensitivity to ampicillin, chloramphenicol, streptomycin, sulfamethoxazole, oxytetracycline, kanamycin, ofloxacin, cefotaxime, cefoxitin, ceftiofur. The MIC range was set at 0.25-512 µg/mL for all tested antimicrobial agents. MIC breakpoints were interpreted according to the new criteria established by the **Clinical and Laboratory Standards Institute (2012) and Clinical and Laboratory Standards Institute (2013)**. *Escherichia coli (E. coli)* ATCC 25922 and *Staphylococcus aureus* ATCC 29213 were used as quality control strains.

Statistical analysis

The prevalence of antimicrobial-resistant isolates across three study periods was compared by using multiple comparisons. A chi-square test was first performed to detect significant differences for each antimicrobial agent. When the result was significant, a test for multiple comparisons of proportions **[Ryan, 1960]** was then performed.

Results

Prevalence and serovars of Salmonella isolated from broiler chickens in Kagoshima, Japan, in 2009-2012

The prevalence of *Salmonella* in broiler chickens in 2009-2012 in Kagoshima, Japan is presented in Table 1.1. The prevalence of *Salmonella*-positive flocks varied slightly from year to year during the study period, and the overall percentage of positive flocks was 49.0% (94/192). The same number of flocks (48 flocks) was collected each year. The percentage of positive flocks was 50.0% in 2009, which decreased dramatically to 39.6% in 2010. However, the trend then changed, and the percentage increased in the next two years to 45.8% in 2011 and 60.4% in 2012. However, there was no significant difference year by year from 2010 to 2012.

The prevalence of *Salmonella* among all tested samples was 7.9% (243/3071), and is was highest in 2012, at 8.6%, followed by 8.3% in 2011, 7.8% in 2010, and 6.9% in 2009. These differences were not significant.

The year-to-year changes in the serovars of the *Salmonella* isolates were investigated, and the results are presented in Figure 1.1. The strains of *Salmonella* isolated from broiler chickens in Kagoshima, Japan (n = 243) in the four year period in 2009-2012 belonged to three serovars, *S.* Infantis 57.6% (140/243), *S.* Manhattan 40.3% (98/243), and *S.* Schwarzengrund 2.1% (5/243).

Figure 1.1 shows a contrasting trend in the number of *S.* Manhattan and *S.* Infantis isolates detected from 2009 to 2012. The percentage of *S.* Infantis isolates decreased gradually, from 66.0% in 2009 to 50.0% in 2011, but increased to 57.6% in 2012, whereas the percentage of *S.* Manhattan isolates significantly increased from 26.4% in 2009 to 50.0% in 2011 after decreased moderately to 40.9% in 2012.

The highest percentage of *S.* Schwarzengrund isolates was observed in 2009 (7.6%; 4/53). This serovar was not detected in 2010 or 2011; however 1 isolate (1/66; 1.5%) was detected in 2012.

Antimicrobial resistance phenotypes

The results of the MIC analysis of 243 *Salmonella* isolates are summarized in Table 1.2. All 243 strains were susceptible to chloramphenicol, with a MIC $\geq$ 32 μg/mL. The rates of resistance were the highest for streptomycin, sulfamethoxazole, and oxytetracycline, and >90% of strains were resistant to these antimicrobials; 231 (95.1%) were resistant to streptomycin (MIC $\geq$16 μg/mL), 221 (91.0%) were resistant to sulfamethoxazole (MIC $\geq$512 μg/mL), and 222 (91.4%) were resistant to oxytetracycline (MIC $\geq$16 μg/mL). Many isolates were also resistant to ampicillin (55.1%) and cefotaxime (52.7%). The three antimicrobials with the lowest resistance rates were kanamycin (6.6%), cefoxitin (6.2%), and ofloxacin (1.6%).

Each serovar of *Salmonella* showed a different resistance prevalence to antimicrobial used in the study (Table 1.3). Serovar *S.* Infantis and *S.* Manhattan exhibited resistance to streptomycin, sulfamethoxazole and oxytetracycline, ranging from 84.6% (resistance of *S.* Infantis to sulfamethoxazole) to 96.9% (resistance of *S.* Manhattan to streptomycin and oxytetracycline), while they were sensitive ofloxacin with a resistance rate of 1.0% and 2.1% in *S.* Manhattan and *S.* Infantis, respectively. We also found a significant difference in the antimicrobial resistance rates of the two serovars to four other antimicrobials. Resistance to cefoxitin, ceftiofur, cefotaxime

and ampicillin was 0.0%, 92.9%, 93.9% and 94.9%, respectively, for *S.* Manhattan, and 10.7%, 25.0%, 25.7% and 29.3%, respectively, for *S*. Infantis. *S.* Schwarzengrund showed high sensitivity to seven antimicrobials with 0% resistance, but exhibited a high resistance (100%) to streptomycin, sulfamethoxazole and oxytetracycline (Table 1.3).

Table 1.4 shows the prevalence and distribution of different multidrug resistance on each serovar: On overall, 231 *Salmonella* strains investigated were resistant or intermediately resistant to three or more of the 10 antimicrobial agents tested. Resistance to three antimicrobials was detected in 91 (37.4%) of the isolates. Resistance to four, five and six antimicrobials were detected 18 (7.4%), 9 (3.7%) and 104 (42.8%), respectively.

S. Infantis has a variety of resistance to antimicrobials agent examined from sensitive to resistance to eight antimicrobials: 5 strains were sensitive, resistance to three antimicrobials was highest 78 (55.7%), followed by resistance to six, four and five antimicrobials agent at 19 (13.6%); 17 (12.1%) and 8 (5.7%) strains, respectively. Multidrug resistance to more than six antimicrobials was detected in 113 (46.5%) of the isolates, and most of them were *S.* Manhattan (88/113), all 5 strains of *S.* Schwarzengrund have three resistance pattern (SSuT).

In Table 1.5, we have described the resistance proportion of *S.* Infantis and *S.* Manhattan to ampicillin, cefotaxime, ceftiofur and cefoxitin for each year during the period 2009 - 2012. In the course of the four years of study, resistance proportion of *S.* Manhattan to ampicillin, cefotaxime and ceftiofur (from 76.0% for ceftiofur in 2009 to 100% for three different antimicrobials in 2012) was much higher than in *S.* Infantis (7.9% for ceftiofur in 2012 to 52.3% for ampicillin in 2010). On the other hand, all the strains of *S.* Manhattan (98) were sensitive to cefoxitin, while 10.7% (15/140) of *S.* Infantis were resistant to cefoxitin in the period of study.

To understand the changes in the antimicrobial resistance of the *Salmonella* isolate over time, we compared our results to data obtained in two previous studies **[Chuma *et al.,* 2013, Shahada *et al.,* 2010]**. The comparison is shown in Table 1.6. In all three studies, all *Salmonella* strains were susceptible to chloramphenicol, with a MIC ≥32 μg/mL. The percentage of antimicrobial-resistant strains was also high for three other antimicrobials, streptomycin, sulfamethoxazole, and oxytetracycline, although rates of resistance decreased significantly over time ($p < 0.05$).

In all three studies, we observed a significant increase in the rates of antimicrobial resistance for two antimicrobials, ampicillin and cefotaxime ($p < 0.05$). For ampicillin, the resistance rate was 22.4% in 2004-2006, 36.5% in 2007, and

55.1% in the present study period (2009-2012). A similar trend was observed for cefotaxime resistance, with 9.1% in the first study period, 35.5% in the second study period, and 52.7% in 2009-2012.

In contrast, the percentage of ofloxacin-resistant strains decreased dramatically (and significantly) with time across the three study periods, from 20.8% in 2004-2006 to 11.8% in 2007 and 2008, and 1.6% in the present study ($p < 0.05$).

Discussion

The overall percentages of *Salmonella*-positive flocks (70.6%) and samples (14%) detected in 1998-2003 **[Shahada *et al.,* 2006]** were higher than the corresponding percentages in 2004-2012, which is clearly demonstrated in the studies by **[Shahada *et al.,* 2010]** and **[Chuma *et al.,* 2013]** as well as the present study (Table 1.1). In 2004-2006, the percentage of positive flocks was 39.2% and the percentage of positive samples was 5.2% **[Shahada *et al.,* 2010]**. In 2007 and 2008, the respective positive rates were 58.7% and 6.3% **[Chuma *et al.,* 2013]**. This decline in the percentages of positive flocks and samples described above may be due to the use of certain antimicrobials in chicken production in Kagoshima, Japan in 2004-2012.

The prevalence of *Salmonella* in Kagoshima, Japan observed in the present study (shown in Table 1.1) was similar to that reported in the Kyushu region of Japan, where 88.0% of cecal samples from broiler flocks were positive for *Salmonella* **[Yamazaki *et al.,* 2016]**. This same rate of positivity for *Salmonella* infection in the flock in the two studies may be because the studies were conducted in the regions with the same climatic conditions.

The serovar changes among *Salmonella* isolates were very clear when the present serovars were compared to those in previous studies. In 1998-2003, 93.4% (526/563) of isolates originating from 135 flocks were *S.* Infantis **[Shahada *et al.,* 2006]**. In 2004-2006, 100% (193/193) of *Salmonella* isolates were *S.* Infantis, whereas in 2007 and 2008, 97.4% (113/116) of *Salmonella* isolates were *S.* Infantis, and 2.6% (3/116) were *S.* Manhattan **[Chuma *et al.,* 2013]**.

Our result demonstrated the same predominant *Salmonella* serovars (as shown in Figure 1.1) as in the other previous studies conducted in Kyushu, Japan. However, there were some differences; for instance, of the 184 *Salmonella* strains isolated from broiler chickens, 123 were *S.* Schwarzengrund (O4:d:1,7), 41 were *S.* Infantis (O7:r:1,5), 9 were *S.* Manhattan (O6,8:d:1,5), 3 were *S.* Yovokome (O8:d:1,5), 5 were OUT:d:1,7, and 4 were OUT:r:1,5 **[Yamazaki *et al.,* 2016].**

To our knowledge, this is the first study examining serovar changes in *Salmonella* isolates from broiler chickens over time in Japan. In this study, only three serovars, *S.* Manhattan, *S.* Infantis, and *S.* Schwarzengrund (Figure 1.1), were detected. This result is similar to those reported in other countries in Asia **[Park *et al.,* 2015, Rahmani *et al.,* 2013]**, with *S.* Infantis as the main serovar. Although, the main serovar in some European countries was *S.* Typhimurium **[Terentjeva *et al.,* 2017; El-Sharkawy *et al*., 2017 and Wierup *et al.*, 2017]**. The different *Salmonella* serovars present in broilers may depend on the region or country due to variations in climate, geographical regions and chicken husbandry practices among countries.

This is also first study to report the relationship between serovar and antimicrobial resistance in *Salmonella* isolates from broiler chickens in Kagoshima, Japan. In the previous studies, these results were not clearly presented. In China, 457 *Salmonella* isolates from chickens, pigs, and dairy cows were most commonly resistant to nalidixic acid (39.17%), sulfamethoxazole-trimethoprim (39.61%), doxycycline (28.22%), and tetracycline (27.58%) **[Kuang *et al.,* 2015]**. A study in Serbia showed that 100% of *S.* Infantis isolates were resistant to ciprofloxacin and anlidixic acid **[Velhner *et al.,* 2014]**. In Kagoshima (2007-2008) most *Salmonella* isolates were *S.* Infantis; however, the rate of resistance to ofloxacin was only 11.8%

[Chuma *et al.,* 2013]. In addition, a study in Iran reported high fluoroquinolone resistance in both *S.* Infantis and *S.* Enteritidis isolates **[Rahmani *et al.,* 2013]**. The low fluoroquinolone resistance rates in our study may be explained by the differences in the serovars of *Salmonella* isolates. There appeared to be a relationship between the serovars of the *Salmonella* isolates and antimicrobial resistance, especially for ampicillin, cefotaxime, and ofloxacin.

Increased multidrug resistant (MDR) has been reported in *Salmonella* isolates in many countries. In the study the high level of MDR observed among *Salmonella* serovars (table 1.4) were in agreement with several studies from different countries **[Rahmani *et al.,* 2013; Kuang *et al.,* 2015; Nógrády *et al.,* 2008]**. Special *S.* Infantis has wide resistance pattern from zero to eight in total 10 antimicrobials agent were tested.

From Table 1.3, Table 1.5 and Fugure 1.1. It can be inferred that the resistance rate of *Salmonella* to certain antimicrobials (streptomycin, sulfamethozaxole, oxytetracycline, chloramphenicol and ofloxacin) remained unchanged each year during the period 2009-2012. However, the proportion of serovars isolated each year varied. All three isolated serovars were sensitive to chloramphenicol and ofloxacin but resistance to streptomycin, sulfamethozaxole, and oxytetracycline. Since, the

number isolated strains of *S.* Schwarzengrund was very small (5 strains isolated during 2009-2012), it might not be expected to show changes in antimicrobial resistance rate with each year.

In the present study, the percentage of resistance to ampicillin, cefotaxime and ceftiofur showed a huge difference between *S.* Manhattan and *S.* Infantis. The resistance of *S.* Manhattan to the three antimicrobials mentioned above was more than 3 times higher than in *S.* Infantis in the hold period. In particular, during 2010-2012, the resistance rate of *S.* Infantis decreased while an opposite trend was observed for *S.* Manhattan; the resistance rate of *S.* Manhattan increased eventually to 100%. This indicates a great influence of serovar change on the resistance to the three antimicrobials (ampicillin, cefotaxime and ceftiofur).

In Kagoshima, Japan, certain antimicrobials, such as: ampicillin, enrofloxacin, amoxicillin and doxycycline are used in the treatment of broiler ascites caused by *E.coli* infection in broiler chicken. That might have affected to the rate of antimicrobial resistance to similar antimicrobials, such as β-lactam, tetracycline, in case of *Salmonella*.

In the previous reports from 2007-2008, the prevalence rates of *S.* Infantis and *S.* Manhattan were 97.4% and 2.6%, respectively, and the rates of penicillin,

cefotaxime, and ofloxacin resistance were 36.5%, 35.5%, and 11.8%, respectively. In 2004-2006, when all isolates were *S.* Infantis (100%), the rates of penicillin, cefotaxime, and ofloxacin resistance were 22.4%, 9.1%, and 20.8%, respectively **[Chuma *et al.*, 2013]**. In our survey, the percentage of *S.* Manhattan isolates resistant to ampicillin and cefotaxime was higher than in *S.* Infantis. From these results, it seems that the increase in the proportion of the *S.* Manhattan serovar leads to the increase in resistance to ampicillin, cefotaxime and ceftiofur with each passing year; this observation is supported by data from the present study and from previous studies.

Conclusion

Taken together, our data show that there was no significant change in the prevalence of *Salmonella* in broiler chickens in Kagoshima, Japan in 2009-2012 when compared to the prevalence in previously surveyed time periods. We have not yet found a definitive pattern in the prevalence of *Salmonella* and the rates of resistance to some antimicrobials. However, when the proportion of *S.* Manhattan isolates increased, the percentage of penicillin-, cefotaxime- and ceftifour-resistant isolates showed a similar increasing trend. Furthermore, the percentage of ofloxacin-resistant strains decreased when the percentage of *S.* Infantis isolated decreased.

Continuous research on the relationship between *Salmonella* isolates serovars and the antimicrobial resistance profiles of each serovar will help reduce the risk of antimicrobial resistant organisms.

Table 1.1. Prevalence of *Salmonella* in broilers in Kagoshima, Japan in 2009-2012

Year	No. of flocks	No. of positive flocks (%)	No. of samples	No. of positive samples (%)
2009	48	24 (50.0)	768	53 (6.9)
2010	48	19 (39.6)	768	60 (7.8)
2011	48	22 (45.8)	767	64 (8.3)
2012	48	29 (60.4)	768	66 (8.6)
Total	192	94 (49.0)	3071	243 (7.9)

Table 1.2. Antimicrobial susceptibility profiles of 243 *Salmonella* isolates from 2009 to 2012

Antimicrobial agent	No. of isolates at the MIC (μg/mL)												MIC break-point (μg/ml)	Resistance no. (%)
	0.25	0.5	1	2	4	8	16	32	64	128	256	512		
SM	0	0	0	0	3	9	7	124	100	0	0	0	≥16	231 (95.1)
OTC	0	2	12	7	0	0	0	0	2	114	106	0	≥16	222 (91.4)
SUL	0	0	2	1	3	9	6	1	0	0	0	221	≥512	221 (91.0)
AMP	0	33	70	6	1	0	0	0	5	8	1	120	≥32	134 (55.1)
CTX	112	0	1	2	9	13	87	17	1	1	0	0	≥4	128 (52.7)
CTF	2	50	62	1	3	11	25	8	64	10	7	0	≥8	125 (51.4)
KM	15	0	17	144	53	0	0	0	0	0	0	16	≥64	16 (6.6)
CFX	0	2	45	114	60	2	5	10	5	0	0	0	≥32	15 (6.2)
OFLX	228	2	7	4	0	0	0	0	0	0	0	0	≥2	4 (1.6)
CP	0	32	95	110	4	0	0	0	0	0	0	0	≥32	0 (0.0)

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

Table 1.3. Distribution of antimicrobial resistance of *Salmonella* isolated from 2009 to 2012 according to the serovar

***Salmonella* serovars** (No. Isolates)	**SM (%)**	**OTC (%)**	**SUL (%)**	**AMP (%)**	**CTX (%)**	**CTF (%)**	**KM (%)**	**CFX (%)**	**OFLX (%)**	**CP (%)**
Infantis (n = 140)	132 (94.3)	122 (87.9)	121 (86.4)	41 (29.3)	36 (25.7)	34 (25.0)	13 (8.6)	15 (10.7)	3 (2.1)	0 (0.0)
Manhattan (n = 98)	94 (95.9)	95 (96.9)	95 (96.9)	93 (94.9)	92 (93.9)	91 (92.9)	3 (2.0)	0 (0.0)	1 (1.0)	0 (0.0)
Schwarzengrund (n = 5)	5 (100)	5 (100)	5 (100)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Total (n = 243)	231 (95.1)	222 (91.4)	221 (91.0)	134 (55.1)	128 (52.7)	125 (51.4)	16 (6.6)	15 (6.2)	4 (1.6)	0 (0.0)

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

Table 1.4. Prevalence and distribution of different multidrug resistance phenotypes among three serovars isolated

Serovars	Resistance pattern									Total
	0	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	
S. Infantis	5	3	4	78	17	8	19	5	1	140
S. Manhattan	-	-	-	8	1	1	85	3	-	98
S. Schwarzengrund	-	-	-	5	-	-	-	-	-	5
Total	5	3	4	91	18	9	104	8	1	243

Resistance pattern of 3: SSuT.

Resistance pattern of 4: ASSuT.

Resistance pattern of 6: ASSuT-CT, CF and ASSuT-CT, CX

S: Streptomycin, Su: Sulfamethoxazole, T: Oxytetracycline, A: Ampicillin, CT: cefotaxime, CF: Ceftiofur, CX: Cefoxitin.

Table 1.5. The proportion of *S.* Infantis and *S.* Manhattan resistance to ampicillin, cefotaxime and ceftiofur in each year from 2009 to 2012

Year	***S.* Infantis**				Year	***S.* Manhattan**			
(No. isolated)	AMP (%)	CTX (%)	CTF (%)	CFX (%)	(No. isolated)	AMP (%)	CTX (%)	CTF (%)	CFX (%)
2009 (35)	13 (37.1)	12 (34.3)	12 (34.3)	4 (11.4)	2009 (14)	14 (100)	13 (92.9)	13 (92.9)	0(0.0)
2010 (35)	19 (52.3)	16 (45.7)	15 (42.9)	6 (17.1)	2010 (25)	20 (80.0)	20 (80.0)	19 (76.0)	0(0.0)
2011 (32)	5 (15.6)	5 (15.6)	5 (15.6)	3 (9.4)	2011 (32)	32 (100)	32 (100)	32 (100)	0(0.0)
2012 (38)	4 (10.5)	3 (7.9)	3 (7.9)	2 (5.3)	2012 (27)	27 (100)	27 (100)	27 (100)	0 (0.0)
Total (140)	**41 (29.3)**	**36 (25.7)**	**35 (25.0)**	**15(10.7)**	**Total (98)**	**93 (94.9)**	**92 (93.9)**	**91 (92.9)**	**0 (0.0)**

AMP, ampicillin; CTX, cefotaxime; CTF, ceftiofur; CFX, Cefoxitin

Table 1.6. Antimicrobial susceptibility profiles in this study and previous studies of *Salmonella* isolates from broiler chickens in Kagoshima, Japan

Antimicrobial agent	MIC break-point (μg/mL)	No. of resistant isolates (%)		
		Previous studies		This study
		2004 – 2006 (n = 120)[a]	2007 – 2008 (n = 93)[b]	2009 – 2012 (n = 243)[c]
SM	≥16	120 (100)	86 (92.5)	231 (95.1)
OTC	≥16	120 (100)	86 (92.5)	222 (91.4)
SUL	≥512	120 (100)	86 (92.5)	221 (91.0)
AMP	≥32	29 (22.4) *	34 (36.5) #	134 (55.1)
CTX	≥4	11 (9.1)	33 (35.5)	128 (52.7)
KM	≥64	9 (7.5)	12 (12.9)	16 (6.6)
CFX	≥32	0 (0.0)	8 (8.6)	15 (6.2)
OFLX	≥2	25 (20.8) $	11 (11.8)	4 (1.6)
CP	≥32	0 (0.0)	0 (0.0)	0 (0.0)

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

[a] *Cited from Shahada et al., 2010*

[b] *Cited from Chuma et al., 2013*

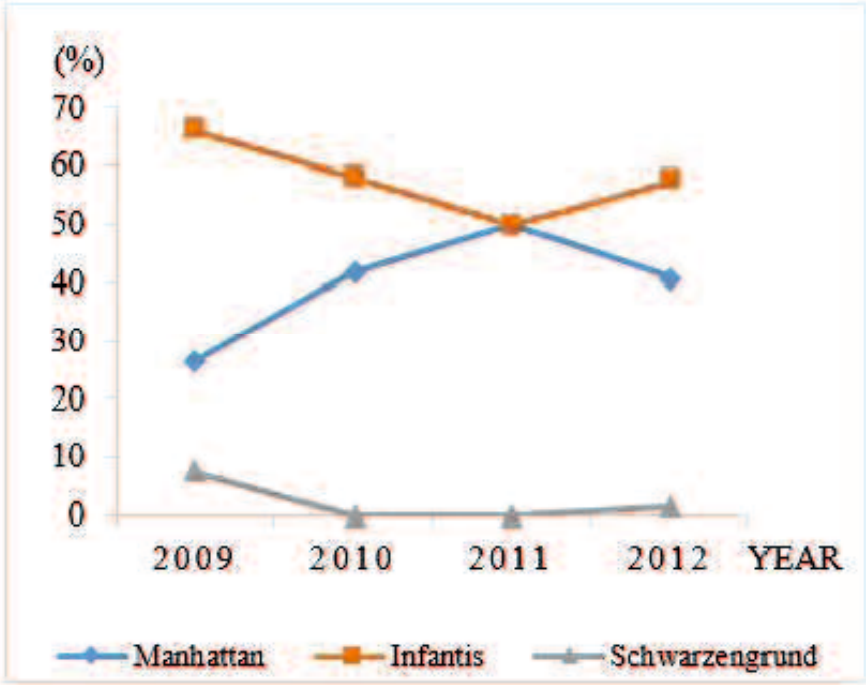
[c] *This study.*

** Significantly increased from the period of 2004–2006 ($p < 0.05$)*

 # Significantly increased from the period of 2007–2008 ($p < 0.05$)

[$] *Significantly decreased from the period of 2004–2006 ($p < 0.05$)*

Figure 1.1. Change of distribution of *Salmonella* serovars isolates from broiler in Kagoshima, Japan in the period from 2009 to 2012



CHAPTER 2

INCREASED *SALMONELLA* SCHWARZENGRUND PREVALENCE AND ANTIMICROBIAL SUSCEPTIBILITY OF *SALMONELLA ENTERICA* ISOLATED FROM BROILER CHICKENS IN KAGOSHIMA PREFECTURE IN JAPAN BETWEEN 2013 AND 2016

Introduction

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 **[Gast, 2008].** *Salmonella* is one of the four key global causes of diarrheal diseases, with 2579 serovars identified till date **[Patrick *et al.,* 2007]**. Antimicrobial agents are widely used during poultry production for growth promotion, or treatment purposes **[Gyles *et al.,* 2008]**. 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 **[Barbosa *et al.,* 2000, and Schwarz *et al.,* 2001]**. 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 **[Blair *et al.,* 2015]**.

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 **[Chapter 1]**. *S.* Schwarzengrund has been reported as an emerging pathogen in Asia, Denmark, the United States of America and Brazil **[Aarestrup *et al.,* 2007, Asai *et al.,* 2009, and Luisa *et al.,* 2019]**. 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.

Materials and methods

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 delivered to the Laboratory of Veterinary Public Health, Kagoshima University, and

cultured on the day of arrival. **[Chuma *et al.,* 2013, and chapter 1]**. The antimicrobial susceptibility of the *Salmonella* isolates was ascertained by the agar dilution method using Mueller Hinton agar (Oxoid Ltd.; Basingstoke, UK) **[Shahara *et al.,* 2007, Shahara *et al.,* 2010, and NARMS, 2009].** Two kanamycin resistance genes *aphA1,* and *kn* were detected by using PCR **[Chen *et al.,* 2004, Frana *et al.,* 2001, and Gebreyes *et al.,* 2005]**.

Results and discussion

Salmonella prevalence in broiler chickens from 2013 to 2016 in Kagoshima Prefecture, Japan is shown in Table 2.1. 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 2.1, *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%) **[Chapter 1]**. Our report was similar to the *Salmonella* prevalence in Japan reported by Yamazaki *et al*.

[Yamazaki *et al.,* 2016], and Sasaki *et al.* **[Sasaki *et al.,* 2012]**. 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% **[Wierup *et al.,* 2017]**. A study in Egypt reported that 41.0% of tested broiler flocks were positive for *Salmonella* along with 1.09% of tested samples **[El-Sharkawy *et al.,* 2017]**.

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 chapter 1, although the relative proportions differed as shown in Table 2.2. 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 **[Barbosa *et al.,* 2000]**; 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 **[Yamazaki *et al.*, 2016]**. Moreover, a study conducted in Taiwan from 2004 to 2006 indicated *S.* Schwarzengrund contamination prevalence in raw chicken meat samples as 30.5% **[Chen *et al.*, 2010]**. In our present study, the number of *S.* Schwarzengrund strains isolated increased dramatically from 5 to 116 (Table 2.2). Together, these studies support that *S.* Schwarzengrund has become one of the most prevalent serovars in broiler chickens in East Asia.

Table 2.3 describes that the proportion of *Salmonella* antimicrobial resistance slightly changed across the previous (2009-2012) **[Chapter 1]** 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 2013-3016. The majority of *S.* Schwarzengrund were sensitive to ampicillin, cefotaxime, cefoxitin, and ceftiofur (zero percent resistance).

As shown in table 2.4, 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 in chapter 1, the increased proportion of the *S*. Manhattan serovar led to an annual increase in resistance to ampicillin, cefotaxime, and ceftiofur. In the present study, although *S.* Manhattan percentage was 51.8% (Table 2.2) the resistance rate of all *Salmonella* serovars decreased compared to that in chapter 1 from 2009 to 2012. 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 2.4). The β-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 β-lactam resistance proportion in our study may be the same as reported by Mauro et al **[Mauro *et al.,* 2018]**, 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 **[Shigemura *et al.,* 2018]**.

Figure 2.1 shows a comparison of the specific antimicrobial resistance rates for *S.* Infantis (Fig. 2.1a) and *S.* Manhattan (Fig. 2.1b) between the current (2013-2016) and in chapter 1 (2009-2012) study periods. However, as only five strains of *S.* Schwarzengrund were isolated in chapter 1, 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. 2.1a). No change was observed in cefoxitin, 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 2.5). All the 13 *S.* Infantis isolates (MIC: 512 μg/m*l*) carried *aphA1*. Of the three *S.* Manhattan isolates, one (MIC: 512 μg/m*l*) carried *aphA1* whereas two others (MIC: 256 and 128 μg/m*l*) did not. The 51 *S.* Schwarzengrund isolates with MIC of 512 μg/m*l* carried *aphA1*; that with MIC of 256 μg/m*l* 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 **[Chen *et al.,* 2010]**. A study in the United States of America and China **[Chen *et al.,* 2004]** 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.

Conclusion

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 β-lactams in this study slightly decreased compared to that in chapter 1, which also affected the overall rate of resistance to β-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.

Table 2.1. 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)**	**3069**	**546 (17.8)**

Table 2.2. Incidence of *Salmonella* serovars in broiler chickens in Kagoshima, Japan during two periods (2009-2012 and 2013-2016)

Serovar	Survey period	
	2009– 2012[a]	**2013-2016[b]**
No. of *S.* Infantis	140	147
(%)	(57.6)	(26.9)
No. of *S.* Manhattan	98	283
(%)	(40.3)	(51.8)
No. of *S.* Schwarzengrund	5	116
(%)	(2.1)	(21.3)
Total	**243**	**546**

[a] Cited from chapter 1

[b] This study

Table 2.3. Antimicrobial susceptibility profiles from the current study and study in chapter 1 of *Salmonella* isolates from broiler chickens in Kagoshima, Japan

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

[a] Cited from chapter 1

[b] This study

* The number of strains (511) differs from the total given in Table 2.2 (546) because at the time of MIC testing, some strains were dried and not suitable for use.

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

Table 2.4. Comparison of antimicrobial resistance of *Salmonella* Schwarzengrund, *S.* Manhattan and *S.* Infantis during 2013 to 2016

Antimicrobial agent	No. of resistant isolates (%)		
	***S.* Schwarzengrund**	***S.* Manhattan**	***S.* Infantis**
	n = 109	**n = 263**	**n = 139**
AMP	0 (0.0)	119 (45.2)	29 (20.9)
CTX	0 (0.0)	116 (44.1)	21 (15.1)
CFX	0 (0.0)	0 (0.0)	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)	121 (87.1)
KM	52 (47.7)	3 (1.1)	15 (10.8)
OFLX	0 (0.0)	0 (0.0)	3 (2.2)
CTF	0 (0.0)	116 (44.1)	22 (15.8)

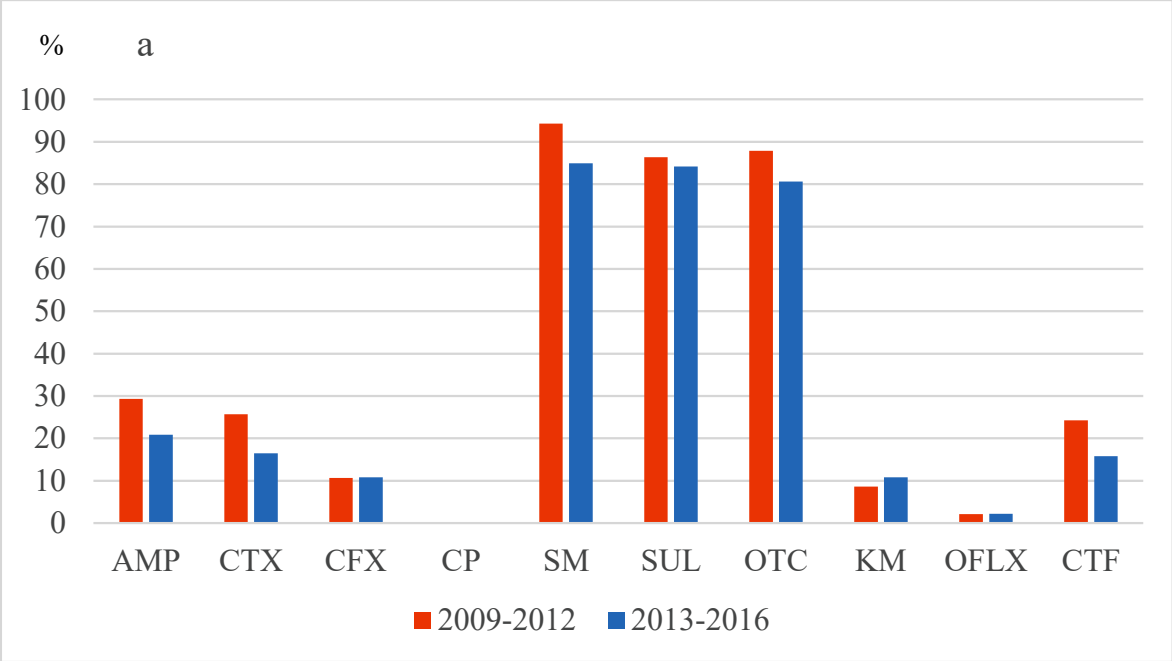
AMP, ampicillin; CTX, cefotaxime; CFX, cefoxitin; CP, chloramphenicol; SM, streptomycin; SUL, sulfamethoxazole; OTC, oxytetracycline; KM, kanamycin; OFLX, ofloxacin; CTF, ceftiofur.

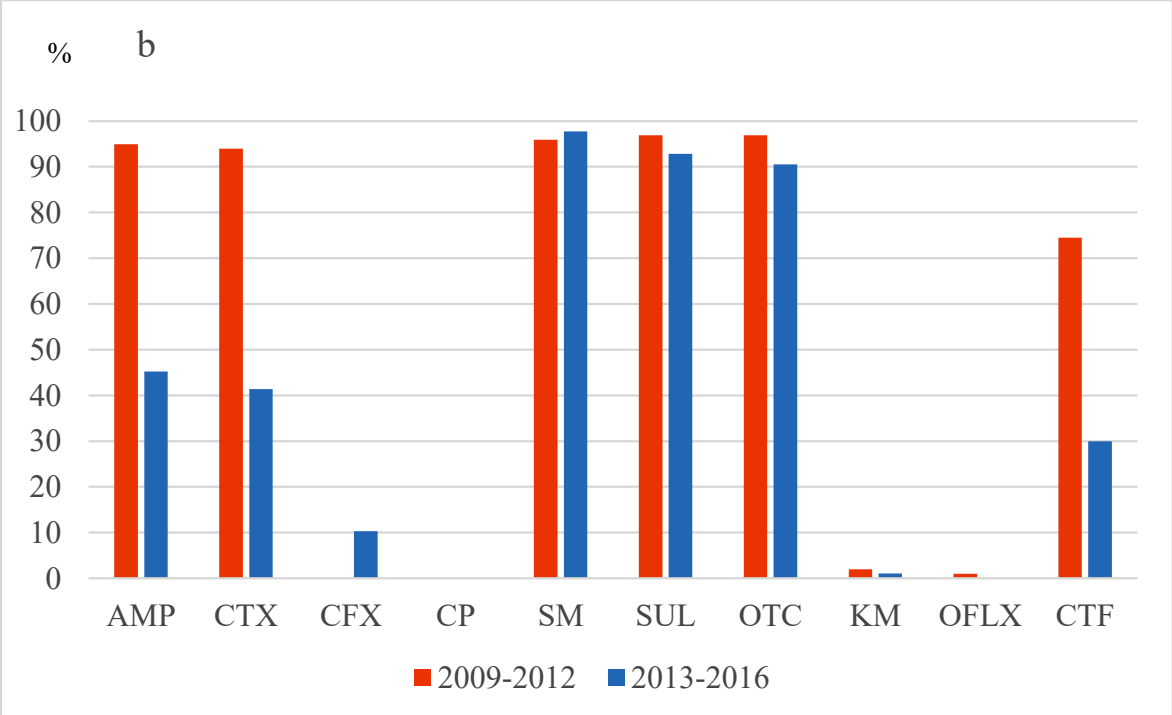
Table 2.5. Distribution of the *aphA1* kanamycin resistance gene from *Salmonella* serovars isolated from broiler chickens during the study period from 2013 to 2016

Serovar (no. of isolates)	MIC of kanamycin *(µg/ml)*	No. of isolates tested	No. of isolates positive for *aphA1* resistant gene (%)
***S.* Infantis (13)**	512	13	13 (100)
	256	-	-
	128	-	-
***S.* Manhattan (3)**	512	1	1 (100)
	256	1	0 (0.0)
	128	1	0 (0.0)
***S.* Schwazengrund (52)**	512	51	51 (100)
	256	1	0 (0.0)
	128	-	-
Total		68	65 (95.6)

MIC, minimal inhibitory concentration

Fig. 2.1. Change of antimicrobial resistance from 2009–2012 to 2013–2016 among (a) *Salmonella.* Infantis and (b) *S.* Manhattan.





GENERAL CONCLUSION

Salmonella serovars during the phase of our research from 2009 to 2016 belonged to three serovars: *S.* Infantis *S.* Manhattan, and *S.* Schwarzengrund. But, from 2009 to 2012, the main serovars isolated were *S.* Manhattan and *S.* Infantis. However, from 2013 to 2016 *S.* Schwarzengrund increased drastically.

In all the period of the study, most of the isolates were resistant to streptomycin, sulfamethoxazole, and oxytetracycline. In contrast, most of isolates were sensitive to chloramphenicol and ofloxacin. Antimicrobial resistance proportion of beta-lactam group (ampicillin, cefotaxime, cefoxitin and ceftiofur) was high in the period from 2009 to 2012, but decresed in the period from 2013 to 2016. In opposite, kanamycin-resistant percentage was very low in the first study (2009 - 2012), but increase in the next period (2013 – 2016). Among the kanamycin-resistant *Salmonella* isolates, *aphA1* constituted the main resistance gene detected.

It was revealed that around 2012, there was a significant change in the serovar and antimicrobial resistance of *Salmonella* isolated from broiler chickens in Kagoshima, Japan.

ABSTRACT

Salmonella is an enteric pathogen that can infect almost all animals and humans. Foodborne salmonellosis is the most relevant source with a high global impact on human health. It was estimated that non-typhoidal *Salmonella* causes around 93.8 million illnesses and 155.000 deaths each year all over the world. There are two species in this genus, *enterica,* and *bongori*. *Salmonella enterica* is the main serovars implicated in *Salmonella* infections in both humans and poultry worldwide.

Nowadays, *Salmonella* contamination has been a continuous problem in the poultry industry. Therefore, many countries have a national program to monitor and control *Salmonella* on poultry. In this thesis we present studies on prevalence, serovars changing, and antimicrobial resistance to continue research on *Salmonella* in broiler chickens in Kagoshima Prefecture, Japan.

In the period from 2009 to 2012, we conducted research about the prevalence, serovars, and antimicrobial resistance of *Salmonella* isolates from 192 broiler flocks and 3071 cecal samples. Among the tested farms, 49.0% of flocks were positive for *Salmonella*, and 243 isolates were obtained from samples 7.9%. All the *Salmonella* isolates were one of three serovars: *S.* Infantis (57.6%); (140/243), *S.* Manhattan

(40.3%; 98/243 and *S.* Schwarzengrund (2.1%; 5/243). The proportion of *S.* Infantis isolates decreased from 66.0% in 2009 to 50.0% in 2011 but increased to 57.6% in 2012, while the proportion of *S.* Manhattan isolates significantly increased from 26.4% to 50% from 2009 to 2011, and decreased moderately to 40.9% in 2012. Most of the recovered *Salmonella* isolates were resistant to three antimicrobials, i.e., streptomycin (95.1%), sulfamethoxazole (91.0%) and oxytetracycline (91.4%). In contrast, all *Salmonella* strains were susceptible to chloramphenicol. Comparison of this study to previous studies of the antimicrobial susceptibility of *Salmonella* isolates showed that: the percentage of antibiotic-resistance isolates increased dramatically for two antibiotics, ampicillin (from 22.4% to 55.1%) and cefotaxime (from 9.1% to 52.7%). In contrast, the percentage of ofloxacin-resistant isolates decreased across the three survey periods, from 20.8% in 2004-2006 to 1.6% in the present study period (2009-2012). In addition, *S.* Infantis exhibited a variety of resistance to antimicrobials examined from sensitivity to resistance to eight antimicrobials. Multidrug resistance to more than 6 six antimicrobials was detected in 113 (46.5%) of the isolates, and most of them were *S.* Manhattan.

In the period between 2013 and 2016, we analyze the *Salmonella* serovars, measure the minimum inhibitory concentration of antimicrobials, and examine the

antimicrobial resistance genes of *Salmonella* isolated from 192 broiler flocks and 3069 cecal samples. The result showed that *S.* Schwarzengrund prevalence had increased annually from 2.1% in (2009-2012) to 21.3% in (2013 – 2016). Compared to the result of chapter 1, ampicillin-, cefotaxime-, and ceftiofur-resistance showed a dramatic decrease trend. But the proportion of kanamycin-resistance increased sharply.

Most of the recovered *Salmonella* isolates in this study were resistant highly to three antimicrobials, i.e., streptomycin, sulfamethoxazole, and oxytetracycline. In contrast, all *Salmonella* strains were susceptible to chloramphenicol and ofloxacin. In detail, different resistance characteristics were observed for each serovar. *S.* Schwarzengrund were sensitive to ampicillin, cefotaxime, and cefoxitin but resistant to kanamycin at 47.7%. On the other hand, *S.* Manhattan and *S.* Infantis, were resistant to the first three kinds of antibiotics listed above (from 10.3% to 45.2%), and sensitive to kanamycin at 1.1% and 10.8%, respectively. Among a total of 68 *Salmonella* strains were resistant to kanamycin, 65 strains which showed MIC value higher than 512μg/ml carried *aphA1* gene, with the. However, 3 strains that showed MIC value at 256μg/ml and 128μg/ml did not carry the *aphA1* gene.

In conclusion, *Salmonella* serovars during the phase of our research from 2009 to 2016 belonged to three serovars: *S.* Infantis *S.* Manhattan, and *S.* Schwarzengrund. But, from 2009 to 2012, the main serovars isolated were *S.* Manhattan and *S.* Infantis. However, from 2013 to 2016 *S.* Schwarzengrund increased drastically.

In all the period of the study, most of the isolates were resistant to streptomycin, sulfamethoxazole, and oxytetracycline. In contrast, most of the isolates were sensitive to chloramphenicol and ofloxacin. Antimicrobial resistance proportion of beta-lactam group (ampicillin, cefotaxime, cefoxitin, and ceftiofur) was high in the period from 2009 to 2012 but decreased in the period from 2013 to 2016. In opposite, the kanamycin-resistant percentage was very low in the first study (2009 - 2012) but increased in the next period (2013 – 2016). Among the kanamycin-resistant *Salmonella* isolates, *aphA1* constituted the main resistance gene detected. It was revealed that the serovar and antimicrobial resistance changed significantly around 2012 in *Salmonella* isolated from broiler chickens in Kagoshima, Japan.

Keywords: *Salmonella,* antimicrobial resistance, serovar, prevalence, broiler, beta-lactam, cephalosporin, fluoroquinolone, kanamycin resistance gene, *Salmonella, S.* Schwarzengrund.

PUBLICATIONS AND CONFERENCES

Publications

1. **Vu Minh Duc**, Yuko Nakamoto, Ayaka Fujiwara, Hajime Toyofuku, Takeshi Obi, and Takehisa Chuma. 2019. Prevalence of *Salmonella* in broiler chickens in Kagoshima, Japan in 2009 to 2012 and the relationship between serovars changing and antimicrobial resistance. *BMC Vet. Res.* **15**: 108. https://doi.org/10.1186/s12917-019-1836-6.

2. **Vu Minh Duc**, Jiye Shin, Yamato Nagamatsu, Ayaka Fujiwara, Hajime Toyofuku, Takeshi Obi, and Takehisa Chuma. 2020. Increased *Salmonella* Schwarzengrund prevalence and antimicrobial susceptibility of *Salmonella enterica* isolated from broiler chickens in Kagoshima Prefecture in Japan between 2013 and 2016. *J Vet Med Sci.* 82(5): 585-589.

Conferences

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2. **Vu Minh Duc**, Hajime Toyofuku, Takeshi Obi, and Takehisa Chuma. Emergence of Salmonella Schwarzengrund and antimicrobial succestibility of *Salmonella* from broiler chicken in Kagoshima, Japan during 2013-2016. **The 161st meeting of the Japanese Society of Veterinary science.** Tsukuba, Japan. 11 to 13 of September 2018.

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4. **Vu Minh Duc** and Takehisa Chuma. Genetic Characterization of Beta - lactam resistence in *Salmonella* isolated from broiler chickens in Kagoshima, Japan. **The 162nd meeting of the Japanese Society of Veterinary Science.** Tsukuba, Japan. 10 to 12 of September 2019.

5. **Vu Minh Duc,** Hajime Toyofuku, Takeshi Obi and Takehisa Chuma. Multi-drug resistance of *Salmonella* isolates from broiler chickens in Kagoshima, Japan. **The 7th Sapporo Summer Symposium for One Health.** Sapporo, Hokkaido, Japan. 19-20 of September 2019.

6. **Vu Minh Duc,** Hiroka Muneyasu, Hajime Toyofuku, Takeshi Obi, and Takehisa Chuma. The disappearance of β-lactam resistance in *Salmonella* isolates from broiler chickens in Kagoshima Prefecture in 2018. **The 163rd Annual Meeting of the Japanese Society of Veterinary Science.** Yamaguchi, September 2020.

ACKNOWLEDGMENT

After 4 years of studying to achieve the Ph.D. I would like to express my deep gratitude to all of the people for helping and accompanying me throughout this journey.

First, I am greatly indebted to my main supervisor Prof. Dr. Takehisa Chuma, Laboratory of Veterinary Public Health at Kagoshima University for his unconditional support in all the study periods and guidance towards my Ph.D.'s research and thesis write-up, and also for my life in Japan.

I would like to thank my vice-supervisor Prof. Dr. Hajime Toyofuku, and Prof. Dr. Takeshi Obi. Those two have accompanied me during my Ph.D. study period and played a key role.

I would like to thank the Ministry of Education and Training of Vietnam, for funding me to study in an advanced country to improve my knowledge and research techniques.

Last but not least, I gratefully appreciate the cooperation accorded by all students at the Laboratory of Veterinary Public Health in Kagoshima University, all members of my family, all my dear friends, and relatives.

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