แบบจำลองความน่าจะเป็นและลักษณะทางพันธุกรรมของ SALMONELLA ในกระบวนการผลิตไก่เนื้อ

นางสาวนิอร บุญประเสริฐ

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PROBABILISTIC MODEL AND GENETIC CHARACTERISTICS OF SALMONELLA IN BROILER PRODUCTION

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การศึกษานี้มีวัตถุประสงค์เพื่อประยุกต์ใช้แบบจำลองความน่าจะเป็นในการอธิบายการเปลี่ยนแปลงการปนเปื้อนของเชื้อแซลโมเนลลาที่แยกได้จากกระบวนการผลิตไก่เนื้อ และการหาลักษณะทางพันธุกรรมเพื่อระบุแหล่งที่มาและรูปแบบการดื้อยาของเชื้อแซลโมเนลลา โดยเก็บตัวอย่างจากฟาร์มในระบบการผลิตไก่เนื้อแบบครบวงจรในเขตภาคตะวันออกเฉียงเหนือของประเทศไทย ระหว่างปี 2010-2012 ตัวอย่างที่เก็บมาทั้งหมดเป็นตัวอย่างที่มาจากตัวไก่ 1,449 ตัวอย่างและตัวอย่างจากสิ่งแวดล้อม 935 ตัวอย่าง จากทั้งหมด 3 วงรอบของการผลิตไก่เนื้อ ตั้งแต่ระดับฟาร์มพ่อแม่พันธุ์ โรงฟัก ฟาร์มไก่เนื้อ และโรงเชือด จากนั้นได้ทำการวิเคราะห์เพื่อหาลักษณะทางพันธุกรรมของเชื้อที่แยกได้ด้วยวิธี pulsed-field gel electrophoresis (PFGE) และรูปแบบการดื้อต่อยาปฏิชีวนะด้วยวิธี disk diffusion ทั้งนี้ค่าความน่าจะเป็นของความชุกของการปนเปื้อนเชื้อแซลโมเนลลาในตัวอย่างชนิดต่างๆอธิบายด้วยการแจกแจง beta และค่าความเข้มข้นของการปนเปื้อนเชื้อแซลโมเนลลาอธิบายด้วยการแจกแจง lognormal โดยค่าความน่าจะเป็นของการปนเปื้อนเชื้อแซลโมแนลลาในแต่ละแหล่งหรือแต่ละหน่วยการผลิตกำหนดเป็นค่าตัวแปรต้นของแบบจำลอง สำหรับรูปแบบทางพันธุกรรม (PFGE pattern) ของเชื้อแซลโมเนลลาที่แยกได้ พบว่าในทุกหน่วยการผลิต มีเชื้อที่แยกได้จากตัวอย่างที่มาจากตัวไก่และสิ่งแวดล้อมที่มี PFGE pattern เหมือนกัน แสดงให้เห็นว่ามีการปนเปื้อนหมุนเวียนของเชื้อแซลโมเนลลาระหว่างตัวไก่และสิ่งแวดล้อม ผลการศึกษานี้สรุปได้ว่าแหล่งของเชื้อแซลโมเนลลาที่สำคัญในกระบวนการผลิตไก่เนื้อคืออุปกรณ์และสิ่งแวดล้อมที่มีการปนเปื้อนในโรงฟัก การนำพาเชื้อจากลูกไก่วันแรกที่มีการปนเปื้อนจากโรงฟักเข้าสู่ฟาร์มไก่เนื้อ อาหาร น้ำ และสัตว์พาหะ โดยเฉพาะอย่างยิ่งจิ้งจก ที่พบได้บ่อยในฟาร์มไก่เนื้อ นอกจากนี้การปนเปื้อนข้ามในระหว่างกระบวนการเชือดพบว่าเป็นปัจจัยสำคัญของการแพร่กระจายเชื้อแซลโมเนลลาไปยังซากไก่ ผลการวิเคราะห์ความไวจากแบบจำลองความน่าจะเป็นพบว่าสถานะของลูกไก่วันแรกและสัตว์พาหะเป็นแหล่งสำคัญของการปนเปื้อนเชื้อแซลโมเนลลาในขั้นตอนการผลิตในฟาร์มไก่เนื้อ โดยขั้นตอนก่อนเข้าโรงเชือดและโรงเชือดเป็นหน่วยการผลิตสำคัญที่ทำให้เกิดการปนเปื้อนเชื้อแซลโมเนลลาไปยังซากไก่ นอกจากนี้ยังพบว่าเชื้อแซลโมเนลลาที่แยกได้มีความสามารถในการดื้อต่อยาปฏิชีวนะหลายชนิด (multidrug-resistant) ในระดับสูง โดยมีสัดส่วนการดื้อยาของเชื้อที่แยกได้จากฟาร์มพ่อแม่พันธุ์ โรงฟัก ฟาร์มไก่เนื้อและโรงเชือด ร้อยละ 92.9, 71.4, 36.6 และ 78.0 ตามลำดับ ชี้ให้เห็นว่าการใช้สารต้านจุลชีพในกระบวนการผลิตไก่เนื้ออย่างรอบคอบเป็นสิ่งที่จะต้องตระหนักและให้ความสำคัญ การศึกษาครั้งนี้ทำให้ทราบถึงการเปลี่ยนแปลงของเชื้อแซลโมเนลลาที่ปนเปื้อนในกระบวนการผลิตไก่เนื้อทั้งในเชิงคุณภาพและเชิงปริมาณ ซึ่งจะเป็นข้อมูลสำคัญให้ผู้ประเมินความเสี่ยงและผู้จัดการความเสี่ยงทั้งในหน่วยงานภาครัฐบาลและเอกชนดำเนินมาตรการในการควบคุมเชื้อแซลโมเนลลาอย่างเป็นองค์รวมและสามารถนำไปปฏิบัติได้จริงต่อไป

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NION BOONPRASERT: PROBABILISTIC MODEL AND GENETIC CHARACTERISTICS OF SALMONELLA IN BROILER PRODUCTION. ADVISOR: ASSOC. PROF. SUPHACHAI NUANUALSUWAN, D.V.M., M.P.V.M., Ph.D., CO-ADVISOR: NIPA CHOKESAJJAWATEE, Ph.D., ASST. PROF. PRAPAS PATCHANEE, D.V.M., 134 pp.

The objectives of this study were to apply probabilistic models to describe the dynamics of Salmonella contamination in an integrated broiler production and to investigate genetic characteristics and antibiotic resistance patterns of Salmonella isolated throughout the broiler production. The samples were chronologically collected in an integrated broiler production located in the Northeastern Thailand during 2010-2012. A total of 1,449 chicken-related samples and 935 environmental samples from three broiler production cycles were collected in a series of production units i.e., “breeding farm”, “hatchery”, “broiler farm” and “slaughterhouse”. The Salmonella isolates were tested for their genetic characteristics and antimicrobial resistance patterns by using pulsed-field gel electrophoresis (PFGE) and disk diffusion method, respectively. For the probabilistic models, beta and lognormal distributions were used to describe the uncertainty of contamination in terms of prevalence and concentration, respectively. The probability of Salmonella contamination in various sample types as well as in different production units were used as input variables of the probabilistic model. From PFGE pattern analysis, identical PFGE patterns of Salmonella isolates between chicken-related and environmental samples were found in all production units. This finding indicated Salmonella transfer between the chicken and its environment. This study suggested that contaminated equipment and environment in the hatchery, contaminated day-old chick, feed, water and pest especially house lizard were among the importance sources of Salmonella during the broiler production. In addition, the cross contamination during the slaughter process was the main element for Salmonella dissemination to the chicken carcasses. The sensitivity analysis indicated that the highly significant sources of contamination during rearing in the broiler farm were day-old chicks and pest. The most significant units contributing to the broiler carcasses contamination were pre-slaughter and slaughterhouse. The alarming rates of multidrug-resistant Salmonella were found among the isolates collected from the breeding farm (92.9%), hatchery (71.4%), broiler farm (36.6%), and slaughterhouse (78.0%). This result emphasized the importance of prudent use of antimicrobial agents and related chemicals in the broiler production. In conclusion, this study provided both qualitative and quantitative information on dynamics of Salmonella contamination and important sources of the contamination in broiler production. This scientific evidence is essential for risk assessors and risk managers in both government and private sectors to readily implement the holistic and realistic Salmonella control measures.

Department:	Veterinary Public Health	Student's Signature	.....
Field of Study:	Veterinary Public Health	Advisor's Signature	.....
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BPW	Buffered Peptone Water
°C	degree Celsius
CDC	The Center for Disease Control and Prevention, USA
CLSI	The Clinical and Laboratory Standards Institute
cm^2	square centimeter
EDTA	Ethylenediaminetetraacetic acid
EFSA	European Food Safety Authority
e.g.	exempli gratia and for example
et al.	et alibi and others
EUCAST	European Committee on Antimicrobial Susceptibility Testing
g	gram (s)
h	hour (s)
ISO	International Organization for Standardization
kb	kilo base pair
LIA	Lysine iron agar
m^2	square meter
MDR	multidrug resistance
MHA	Muller Hinton agar
ml	milliliter (s)
µl	micro liter (s)
MKTTn	Muller-Kauffmann Tetrathionate Novobiocin broth
MPN	The most probable number
MSRV	Modified semi-solid Rappaport-Vassiliadis medium

NA	Nutrient agar
NARMS	The National Antimicrobial Resistance Monitoring System
NIH	National Institutes of Heath
NSSC	National *Salmonella* and *Shigella* Center, Thailand
PFGE	Pulsed-Field Gel Electrophoresis
RVS	Rappaport Vassiliadis with soya broth
S.	*Salmonella*
SIM	Sulfide-Indole-Motility medium
spp.	species
TBE	Tris-Borate EDTA
TE	Tris-EDTA
TSI	Triple sugar iron agar
UPGMA	Unweighted Pair Group Method with Arithmetic Mean
WHO	World Health Organization
XLD	Xylose lysine deoxycholate agar

CHAPTER I

INTRODUCTION

Salmonella is one of the most common causes of human foodborne disease worldwide (646/2007, 2007; WHO, 2007; Scallan et al., 2011b). The human infected with *Salmonella* can develop the clinical sign within 12-14 hours after ingesting contaminated foods. The clinical sign consists of nausea, vomiting, abdominal pain, headache, chills and diarrhea. In most cases, the illness can be recovered without any antimicrobial treatments. However, the severe symptom can be even fatal to the elderly, infants and immunocompromised patients.

In the United States of America, it has been estimated that *Salmonella* caused 1.02 million cases of illness, 19,336 hospitalizations and 378 deaths annually (CDC, 2011a). In European Union, the number of human cases suffering from salmonellosis were 99,020 in 2010 (EFSA, 2012a). In Thailand, *Salmonella* is one of the most common pathogen causing diarrheal disease during the last decade (Bangtrakulnonth et al., 1995). In 2008, The National *Salmonella* and *Shigella* Center, Ministry of Public Health, Thailand reported that the number of human *Salmonella*-related cases were 3,083 in a total of 3,485 isolated from human, animals, foods, environment and water (NSSC, 2008).

Among the food of animal origins, poultry meat or poultry products were the major source of human salmonellosis (EFSA, 2012a). In Thailand, *Salmonella* can be frequently found in poultry meat and eggs at the retail market (Jerngklinchan et al., 1994; Saitanu et al., 1994). Moreover, many strains of the *Salmonella* isolated from chicken meat acquired antimicrobial resistance (Boonmar et al., 1998). The

widespread of antimicrobial resistance in *Salmonella* can cause the severe illness and the complication for *Salmonella* treatment in the hospital (Travers and Barza, 2002).

In Thailand, chicken meat production is among the significant imported revenues. In 2012, chicken meat and products export brought in more than 67,000 million baht of Thailand export's revenue (OAE, 2013). However, from poultry export point of view, *Salmonella* seems to be a trade restriction. Some countries have required that no *Salmonella* is detected in 25 grams of broiler meat or its products. So, zero tolerance of *Salmonella* in broiler meat and its products is a vital and obligatory criterion to maintain or even enhance the export volume for the poultry business in Thailand.

In order to control *Salmonella* contamination on chicken meat and chicken products, the whole chain of the broiler meat production need to be considered. In European Union, Regulation 2160/2003/EC had been launched to establish the mandatory investigation for *Salmonella* in breeder flocks, broilers, laying hens and turkeys in all EU-member countries. Moreover, the regulation (EC) No. 1003/2005 and the regulation (EC) No. 646/2007 are enforced in EU-member countries to minimize the prevalence of *Salmonella* in the breeder flocks and broiler flocks, respectively. Currently, the *Salmonella* control programs implemented throughout the poultry production chain has already been applied in some countries such as Sweden. Continuous monitoring and then eradication of *Salmonella*-positive flocks are currently employed (Lewerin et al., 2005). However, the eradication measure is almost impossible to operate and afford to implement in some countries including

Thailand, where the prevalence of *Salmonella* is high (Boonprasert, 2009; Chaengprachak, 2009).

In order to establish some effective *Salmonella* control measures, understand the pathways and dynamics of *Salmonella* contamination in a whole broiler production process is inevitable. Modeling is another tool to simplify and describe the dynamics of infection or contamination of *Salmonella* in the broiler flocks. However, the models such as Susceptible-Infected-Recovered (SIR) model which is commonly used to describe the spread of infectious diseases in animal flocks, has a limitation in explaining the dynamic of *Salmonella* contamination in the poultry environment (Nielsen et al., 2007). Even though the probabilistic model has been developed to describe transmission of *Salmonella* in poultry production chain, a large variability of model can be found in previous study (Nauta et al., 2000)

In Thailand, studies on probabilistic models are still obscure. Hence, it will be valuable to apply the probabilistic model to describe the existence and dynamic of *Salmonella* infection in the chicken and contamination in the environment in the entire broiler production chain. Moreover, to achieve the holistic *Salmonella* control measure, understanding the genetic characteristics of *Salmonella* throughout the broiler production chain is also essential. However, little data have been published about the source tracking and antibiotic resistance patterns of *Salmonella* throughout the broiler production in Thailand. Therefore, the aims of this study were to apply the probabilistic models to describe the dynamics of *Salmonella* contamination and to investigate genetic characteristics in terms of source tracking and antibiotic resistance patterns of *Salmonella* isolated throughout the whole chain of broiler production. The knowledge derived from this study will be helpful for risk

managers in both government and private sectors to implement the holistic and realistic *Salmonella* control measures throughout the broiler production.

CHAPTER II

LITERATURE REVIEW

2.1 Microbiology of *Salmonella* spp.

Salmonellae are facultative anaerobic bacteria in the family *Enterobacteriaceae*. They are gram-negative, rod-shaped, small sizes with 0.7 to1.5 μm in width and 2.0 to 5.0 μm in length. Most of them can be motile by peritrichous flagella except *Salmonella enterica* serovar Pullorum and *Salmonella enterica* serovar Gallinarum as a result of dysfunctional flagellae. *Salmonellae* can grow at temperature range between 5°C and 45°C and pH range between 4.0 and 9.0. The optimum condition for *Salmonellae* proliferation is 37°C with pH 7.0 (D'Aoust et al., 2001; Gast, 2003). The *Salmonella* genus consists of two main species that are *Salmonella enterica* and *Salmonella bongori*. *S. enterica* can be further divided into six subspecies as the following;

1. *S. enterica* subsp. *enterica* (I),
2. *S. enterica* subsp. *salamae* (II)
3. *S. enterica* subsp. *arizonae* (IIIa)
4. *S. enterica* subsp. *diarizonae* (IIIb)
5. *S. enterica* subsp. *houtenae* (IV)
6. *S. enterica* subsp. *indica* (VI).

Salmonella can be differentiated in terms of "serotype" by slide agglutination test between *Salmonella*-specific antibody and *Salmonella* surface antigen. These antigens consist of three main antigens : somatic (O) or lipopolysaccharide (LPS) antigen on the external surface of *Salmonella* membrane

flagella (H) antigen on the peritrichous flagella and capsular (Vi) antigen on the surface capsule which found only in *S.* Typhi, *S.* Paratyphi C and *S.* Dublin (D'Aoust et al., 2001). In 2007, up to 2,579 serotypes of *Salmonella* have been identified based on the Kauffman-White-Le Minor scheme, as shown in Table 1 (Grimont and Weill, 2007)

Table 1. *Salmonella* species and subspecies

***Salmonella* species and subspecies**	**Number of serotypes**
Salmonella enterica	**2,557**
S. enterica subspecies *enterica*	1,531
S. enterica subspecies *salamae*	505
S. enterica subspecies *arizonae*	99
S. enterica subspecies *diarizonae*	336
S. enterica subspecies *houtenae*	73
S. enterica subspecies *indica*	13
Salmonella bongori	**22**
Total	**2,579**

(Modified from Grimont and Weill, 2007)

Furthermore, *Salmonella* serotypes can be categorized by epidemiological purpose into three groups as the following (Jay et al., 2005).

1. Human-infected group is human-specific pathogens such as *S.* Typhi, *S.* Paratyphi A and *S.* Paratyphi C. *S*. Typhi causes typhoid fever with the longest incubation period and the highest mortality rate.
2. Host-adapted group is single host-specific pathogens such as *S.* Gallinarum, *S.* Pullorum in poultry, *S.* Dublin in cattle, *S*. Abortus-equi in hourse, *S*. Choleraesuis in swine. However, some of these serotypes can be pathogenic to human by ingestion of *Salmonella*-contaminated food.

3. Un-adapted group is non-specific pathogens to human and animals. In terms of the public health, the last group is widely known as "non-typhoidal *Salmonella*" such as *S.* Typhimurium, *S*. Enteritidis that are the most common cause of foodborne illness in human.

2.2 *Salmonellosis* in humans and animals

Salmonellosis is the foodborne disease that adversely affects the public health worldwide (WHO, 2007; Scallan et al., 2011a). It is implicated to the consumption of contaminated foods or food products from animal origins such as poultry meat, pork meat, eggs, milk and seafood (Foley et al., 2008). Especially, poultry meat and poultry products were identified as the major source of human salmonellosis (EFSA, 2012a). In the United States of America, the Center for Disease Control and Prevention (CDC) estimated that salmonellosis causes approximately 1.02 million cases of illness, 19,336 hospitalizations and 378 deaths annually. Among the foodborne pathogens non-typhoidal *Salmonella* is the most common cause of death and hospitalization (CDC, 2011a). European Union reported that the number of human salmonellosis was up to 99,020 in 2010 (EFSA, 2012a). In Thailand, among total 3,497 *Salmonella* isolates from human, animals, foods, environment and water, 3,089 *Salmonella* isolates were obtained from human cases (Table 2). Non-typhoid *Salmonella* was the most frequently reported in human cases (NSSC, 2008).

Table 2. Total number of *Salmonella* from various sources

Organisms	Source	Isolates
Typhoidal *Salmonella*	Human	6
Non-typhoidal *Salmonella*	Human	3,083
	Foods raw material	45
	Ready-to-eat foods	47
	Frozen sea foods	26
	Animals	94
	Environment	44
	Water	86
	Other	66
	Total	**3,497**

(Modified from NSSC, 2008)

The clinical signs of human salmonellosis are nausea, vomiting, abdominal pain, headache, chills and diarrhea. The onset of symptoms can be within 12-14 hours after eating contaminated food and usually persist for 2-3 days. However, the susceptible groups such as the elderly, infants and immunocompromised patients, could encounter the severe illness and even death. Among a variety of *Salmonella* serotypes, *S.* Enteritidis and *S*. Typhimurium were the predominant global foodborne disease serotypes during 2001 and 2007 (Hendriksen et al., 2011). In European Union, *S.* Enteritidis and *S.* Typhimurium were the most frequently isolated from human cases from 2009 to 2010 (EFSA, 2012a). This evidence was compatible with the report in the USA, the top two serotypes isolated from human sources between 1999 and 2009 were *S.* Enteritidis and *S.* Typhimurium (CDC, 2011b). However, in Thailand, the most common serotype that caused human salmonellosis differed from that in other countries. *S.* Weltevreden was the most prevalent serotype isolated from human

between 1993 and 2002 followed by *S.* Enteritidis, *S.* Anatum and *S.* Derby (Bangtrakulnonth et al., 2004).

In animals, *Salmonella* can infect many groups of animals such as mammals, birds and reptiles. The clinical signs in animals are varied depending on the infected dose, the virulence of infected serotype and the host susceptibility. In poultry, *S.* Pullorum and *S.* Gallinarum can cause the septicemia with high mortality whereas the poultry infected with *S.* Enteritidis and *S.* Typhimurium may not cause severe disease (Quinn et al., 2002). The most common serotypes isolated from infected animals are varied among animal species. The previous study in the USA reported that *S.* Typhimurium and *S.* Choleraesuis were the most pathogenic serotypes in swine. Whereas, *S.* Pullorum and *S.* Gallinarum caused the severe diseases in chicken. However, the most common serotypes isolated from clinical chickens were *S.* Enteritidis, *S.* Kentucky and *S.* Typhimurium (Foley et al., 2008).

2.3 Source of *Salmonella* contamination in poultry production

There are many possible sources of *Salmonella* that can be introduced and disseminated in the poultry production unit such as litter, water, feed, human, contaminated equipment, dust, and vector such as rodent, insect, files and darkling beetle (Davies et al., 1997; Murray, 2000; Marin et al., 2011). In the hatchery, the contaminated egg trolleys and trays were the main source of *Salmonella* dissemination within the integrated poultry organization (Davies et al., 1997). In the broiler farm, improper cleaning and disinfection procedures can cause the persistence of *Salmonella* and are the significant risk factor related to the flock contamination at the end of rearing period (Marin et al., 2011). Moreover, the stress

during the transportation resulted in the *Salmonella* excretion from the latent infection of chick and spreading throughout the flock before slaughtering (Humphrey, 2000). In the slaughterhouse, the cross-contamination is the important route of *Salmonella* spread in almost every slaughtering process (Van Immerseel et al., 2009).

2.4 Molecular typing technique for *Salmonella*

Molecular typing technique is an epidemiological investigation tool based on the basis of discriminatory mechanisms (Riley, 2004). Foley and colleagues (2009) have classified the molecular typing techniques into three main categories which are restriction-based methods, amplification-based methods and sequencing-based method. Pulse-field gel electrophoresis (PFGE) which is a restriction-based method providing higher reproducibility and discriminatory power for *Salmonella* typing than other molecular typing methods (Foley et al., 2006).

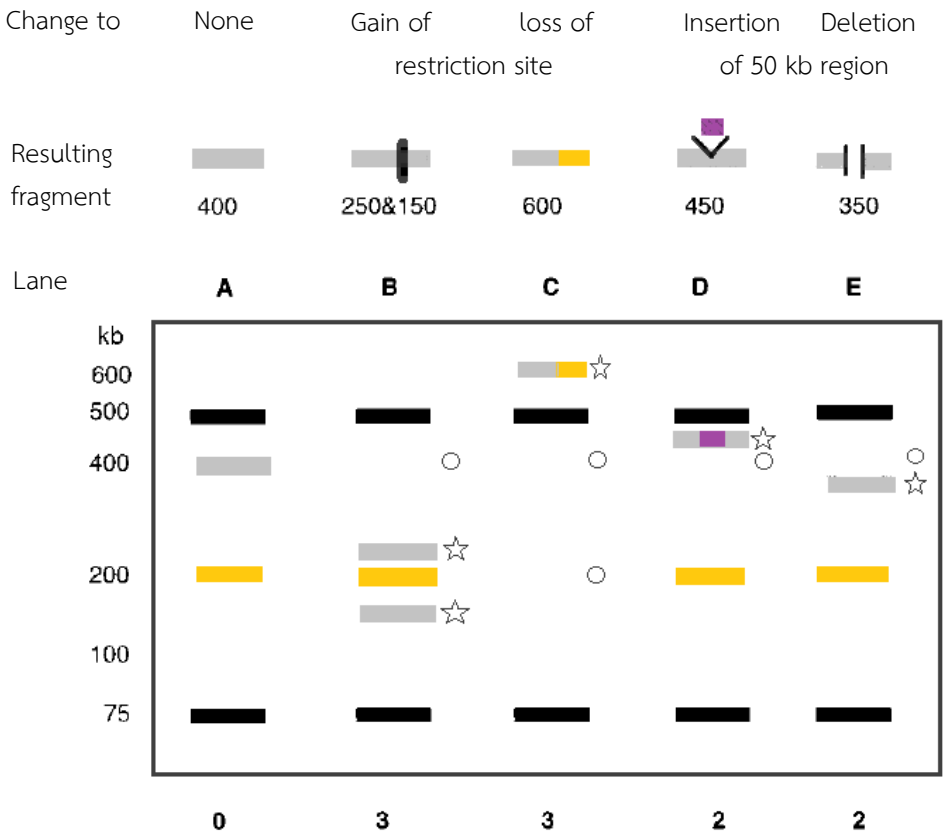
The basis of PFGE is the digestion of bacterial DNA with rare cutter restriction enzyme at the specified recognition sites and then the separation of the large pieces of DNA fragments by pulse field electrophoresis (Gebreyes, 2003; Riley, 2004). Then, the electrophoresis patterns are used for strain comparison. Because of the characteristics of PFGE which can monitor more than 90% of the whole genome, it is highly effective to detect and compare genes within the different strains (Goering, 2010; Shi, 2010). Currently, PFGE is considered the "gold standard" of molecular typing method for *Salmonella* and other bacterial foodborne pathogens (Foley et al., 2009; Boxrud, 2010). Moreover, CDC has already provided the PFGE standard protocols for *Salmonella* in PulseNet. PFGE patterns can also be compared among the different laboratories or with PulseNet database network (Swaminathan et al.,

2001). So, PFGE is one of the popular and practical methods for investigating the source and the genetic characteristics of *Salmonella*.

In terms of epidemiological study, PFGE banding patterns can be divided into 4 categories depending on their genetic relatedness (Tenover et al., 1995).

1. Indistinguishable: When the PFGE banding patterns of isolates show the same numbers of bands and the same apparent sizes, the isolates are designated genetically indistinguishable. The interpretation of the isolates is considered the same strain.
2. Closely related: When the PFGE banding patterns of isolates differ from other patterns for two or three bands, the isolates are considered to be closely related. The change of a single genetic event such as point mutation, insertion or deletion of DNA fragment can create a new chromosomal restriction site, thus generating the closely related PFGE patterns.
3. Possibly related: When the PFGE banding patterns of isolates differ from other patterns up to 4-6 bands, the isolates are considered to be possibly related. The change of two independent genetic events from simple insertion or deletion of DNA fragment or the gain or loss of restriction sites can create the different chromosomal restriction site thus causing the possibly related PFGE patterns.
4. Unrelated: When the PFGE banding patterns of isolates differ from other patterns up to 7 bands or more, the isolates are considered to be unrelated. The change of three or more independent genetic events from simple insertion or deletion of DNA fragment or the gain or loss of restriction sites

can create a variety of chromosomal restriction sites thus causing the unrelated PFGE patterns.



(Modified from Tenover et al., 1995)

Figure 1. Diagram of PFGE banding patterns

Lane A, PFGE pattern of reference isolate; Lane B, PFGE pattern after gain of restriction site; Lane C, PFGE pattern after loss of restriction site; Lane D, PFGE pattern after insert of 50 kb DNA fragment; Lane E, PFGE pattern after delete of 50 kb DNA fragment.

2.5 Antibiotic resistance of *Salmonella* in poultry production

Currently, antimicrobials are widely used for various purposes in poultry such as therapy, prophylaxis, metaphylaxis and growth promoter (McEwen and Fedorka-Cray, 2002). However, these applications promote certain conditions for development, selection and spread of antimicrobial resistant micro-organisms especially *Salmonella* that is predominantly infected and contaminated in poultry and environment. Finally, it could possibly transfer to human via the consumption of contaminated meat.

During the last decade, the awareness of antimicrobial resistance problem among food animals, having human adverse health effect, has been concerned in many countries. In the EU member states and USA, monitoring programs of antimicrobial resistance in *Salmonella* isolates from food animals were set up in many antimicrobial agents in order to assess and report the trend, sources of *Salmonella* in food animals, and antimicrobial resistance pattern (Table 3) (EFSA, 2008, 2012c). In 2012, an EFSA report showed that 24% of *Salmonella* isolates from fowl (*Gallus gallus*) and broiler meat were resistant to ciprofloxacin, whereas *Salmonella* isolates from human were highly resistant to ampicillin, tetracyclines and sulfonamides (EFSA, 2012b). In USA, a CDC report indicated that 3% of non-typhoidal *Salmonella* were resistant to both ciprofloxacin and ceftriaxone evenly. Moreover, approximately 5% of non-typhoidal *Salmonella* were resistance to 5 types of drugs or more (CDC, 2013).

In Thailand, the multidrug-resistant *Salmonella* was the most commonly found in human and poultry isolates (Boonmar et al., 1998; Sirichote et al., 2010). In addition, Chuanchuen and colleagues (2008) reported that the percentage of

multidrug-resistant *Salmonella* isolated from the poultry was up to 26.4%. Among the *Salmonella* isolates, ampicillin and tetracycline were resistant the most at 32% and 31.2%, respectively. Moreover, the highest percentage of multidrug-resistant was found in *Salmonella* isolated from chickens on farm at 100%, followed by those on market and slaughterhouse at 35% and 16%, respectively. The *Salmonella* isolated from such three production units were resistant to nalidixic acid and tetracycline (Padungtod and Kaneene, 2006). However, in the hatchery, as the primary source of *Salmonella* contamination, *Salmonella* was highly resistant to nalidixic acid, ampicillin, streptomycin, tetracycline and doxycycline at the rates of 62.9%, 27.7%, 27.7%, 24.3%, and 22.6%, respectively (Mulika and Yuwapanichsampan, 2008).

Table 3. Monitoring programs of antimicrobial resistance in *Salmonella*

Antimicrobial class	Antimicrobial agent	Monitoring programme	
		EFSA	NARMS*
Aminoglycosides	Amikacin		X
	Gentamicin	X	X
	Kanamycin		X
	Streptomycin	X	X
Aminopenicillins	Ampicillin	X	X
	Amoxycillin		
β-Lactamase inhibitor combinations	Amoxycillin/clavulanic acid		X
Cephalosporin (1st generation)	Cephalothin		X
Cephalosporin (3rd generation)	Cefazolin		
	Ceftiofur		X
	Cefotaxime	X	
	Ceftriaxone		X
	Ceftazidime	X	
Cephamycins	Cefoxitin		X
Carbapanems	Meropenem	X	
Folate pathway inhibitors	Sulphamethoxazole	X	X
	Sulphisoxazole		X
	Trimethoprim	X	
	Trimethoprim/sulphonamides		X
Macrolides	Azithromycin		X
	Erythromycin		X
Phenicols	Chloramphenicol	X	X
Polypeptides	Colistin	X	
Quinolones	Ciprofloxacin	X	X
	Enrofloxacin		
	Nalidixic acid	X	X
Tetracyclines	Tetracyclines	X	
	Oxytetracycline		X
	Total	**13**	**19**

NARMS, National Antimicrobial Resistance Monitoring System (USA)
(Modified from (EFSA, 2008, 2012c))

2.6 Probabilistic models for *Salmonella* in primary broiler production

In order to mathematically describe some model variables, the probability distribution (PD) has been used. The (horizontal) x axis represents the range of possible values in which the variable could possibly take and the (vertical) y axis

indicates the probability of the corresponding variable values (Vose, 2000). PD could be either a discrete or continuous (Dawson and Trapp, 2004). The discrete PD is associated with the random variable that takes only integer values such as binomial and Poisson distributions. On the other hand, the continuous PD is associated with the random variable that takes any value within the continuous scale such as normal and beta distributions (Dawson and Trapp, 2004; OIE, 2004).

Currently, statistical models have been used to describe and predict the foodborne pathogens infected or contaminated in the broiler production chain (Kelly, 2005). In order to construct the statistical model, either prevalence or concentration of foodborne pathogens as a model variable play a significant role in each production unit (Black and Davidson, 2008). By means of the point estimate or deterministic approach, the prevalence is expressed as a percentage. Whereas, in the probabilistic approach, prevalence and its uncertainty (Vose, 2000) is commonly expressed as beta distribution (Black and Davidson, 2008). Since, the prior information about the prevalence was not required. Furthermore, only the small infinite number of the sample size was enough to define this distribution (OIE, 2004).

Previously reported model for the primary production chain, Susceptible-Infected-Recovered (SIR) model has been used to describe the spread of infectious diseases in animal flocks (Nielsen et al., 2007; Gordo et al., 2009). The SIR model focused only on the disease transmission by direct contact across susceptible, infected and recovered groups in an animal population. Since, *Salmonella*'s habitat covers both animal and environment (e.g. insect, human, water, feed and fomite) so SIR model might not cover how the pathogen contaminating the environment of the animal production. However, the alternative model has been developed by Nauta

and colleagues (2000) to describe the *Salmonella* transmission along the poultry meat production chain. One drawback of this model is the larger variability in terms of model predictions. Moreover, this model seems to be too complicated. So that, the probabilistic model that could describe the dynamics of *Salmonella* among and/or between animal and environment in the broiler production is needed.

CHAPTER III
MATERIALS AND METHODS

The longitudinal study was conducted to evaluate the prevalence and concentration of *Salmonella* and possible sources of *Salmonella* contamination and transmission in an integrated broiler chicken production. To identify the source of contamination, *Salmonella* isolated from the chicken-related samples as well as the environment samples were characterized by Pulsed Field Gel Electrophoresis (PFGE) technique. To gain insight into properties of the *Salmonella* and possible control strategies, antimicrobial susceptibility patterns of the isolates were also analyzed. Finally, the probabilistic model was applied to describe the dynamics of *Salmonella* contamination throughout the broiler production.

3.1 Collection and detail of samples

From June 2010 to March 2012, the samples were chronologically collected throughout an integrated broiler production which was located in the Northeastern Thailand. Samples were collected across three cycles of the broiler production in a series of production units i.e., “breeder farm”, “hatchery”, “broiler farm” and “slaughterhouse”. The samples drawn from the aforementioned series of production units were categorized, where applicable, as “chicken-related” and “environmental” samples. Since, the samples from chicken had a variety of sample types in various “production unit” e.g. feces in the breeder flock or eggs from the hatchery. So, in this study such chicken samples were referred as “chicken-related samples” instead.

In the breeder farm production unit, the evaporative cooling system was used for temperature control in the breeder house. The house is 12 meters in width and 120 meters in length accommodating approximately 7,500 birds. The chicken breed was either Ross for the 1st and 3rd sampling or Cobb for the 2nd sampling. All eggs, produced from breeder flocks, were sent to the hatchery production unit about 1 km in distance.

In the broiler farm production unit, the house is 10 meters in width and 100 meters in length rearing approximately 10,000 birds. At the age of 42 days, the broiler chickens were sent to the slaughterhouse, which was located 70 kilometers away from the broiler farm.

Sample size determination

In order to determine the prevalence of *Salmonella* throughout an integrated broiler production, the sample size was calculated using the statistical package Win Episcope program version 2.0. An expected prevalence of 50% with a confidence interval of 95% and an accepted error of 5% were used in this study. The total sample size was 385. However, due to the limitation of sample collection in the 1st sampling, only 294 of pooled samples were collected. For the 2nd sampling and the 3rd sampling, the individual samples were collected at 1,085 and 1,005, respectively.

In this study, a total of 2,384 samples which consist of 1,449 chicken-related samples and 935 environmental samples were collected from 7 breeder flocks (3 flocks from the farm A in the 1st sampling; 2 flocks from farm B in the 2nd sampling and 2 flocks from farm C in the 3rd sampling), 1 hatchery, 3 broiler flocks (reared from the same house in the broiler farm) and 1 slaughterhouse. The age of the breeder flocks were 55 weeks, 35 weeks and 42 weeks for the first, second and third cycle of

sampling, respectively. The sample description in each production unit was shown in Table 4 while sample types and sample size in each production unit were shown in Table 5. Detail of the sample collection procedure was shown in Appendix A.

Table 4. The sample description in a series of production units

Production unit	Category	Sample type	Purpose
Breeding farm	Chicken-related	Cloacal swab or feces, egg	To monitor *Salmonella* infection of breeder flock
	Environmental	Boot swab, egg tray, basket and plate, hand swabs, egg transferring belt	To evaluate and investigate sources of *Salmonella* contamination in eggs
Hatchery	Chicken-related	Eggs (before incubating, 18 days in the incubator), meconium	To monitor *Salmonella* contamination in eggs and baby chicks
	Environmental	Egg storage room, incubating room, hatching room, hook, stand, hand swabs, egg trolley, egg illuminating plate, egg transferring plate, water, hatching tray, belt, chick box, truck	To evaluate and investigate sources of *Salmonella* contamination in the hatchery
Broiler farm • After C&D	Environmental	Floor, wall, pan feeder, litter, watering system, water, pest	To assess *Salmonella* persistence after cleaning and disinfection (C&D)
• Chick arrival	Chicken-related	Meconium (box liner)	To monitor *Salmonella* status of new chicks
	Environmental	Floor and litter, wall, pan feeder, feed, watering system, water, pest	To Assess *Salmonella* contamination in the broiler house before placing new chicks
• Rearing period (weekly)	Chicken-related	Cloacal swab or feces	To monitor *Salmonella* status of broiler flock
	Environmental	Boot swab, feed, water, pest,	To evaluate and investigate the sources of *Salmonella* contamination in the broiler flock
• Slaughter day	Environmental	Water, cage, truck, hand swabs	To evaluate and investigate the sources of *Salmonella* contamination before slaughtering
Slaughterhouse	Chicken-related	Cloacal swab or feather around cloaca, whole carcass (rinse)	To monitor *Salmonella* status in chicken and whole carcass at slaughterhouse

Table 5. Sample types and sample size in a series of production units

Production unit	Sample category	Sample type	Sample size*		
			1st	2nd	3rd
Breeding farm	Chicken-related	Cloacal swab or feces	9	120	120
		Egg	15	50	40
	Environmental	Boot swab	-	10	10
		Egg tray	5	10	10
		Basket and plate	5	10	10
		Hand swabs before working	-	4	2
		Hand swabs after working	-	4	2
		Egg transferring belt	-	4	4
Hatchery	Chicken-related	Egg (before incubating)	10	10	20
		Egg (18 days in the incubator)	10	50	-
		Meconium	12	12	12
	Environmental	Egg storage room	1	1	1
		Egg incubating room	2	-	-
		Egg hatching room	1	-	-
		Hook	1	1	1
		Stand	1	2	2
		Hand swabs before working	16	13	15
		Hand swabs after working	-	13	15
		Egg trolley	4	-	-
		Egg illuminating plate	2	-	-
		Egg transferring plate	2	-	-
		Water	2	1	2
		Hatching tray	12	12	12
		Belt before working	2	2	2
		Belt after working	-	2	2
		Chick box	1	-	-
		Truck	1	-	-

*sample size for 3 sampling cycles

Table 5: Sample types and sample size in a series of production units (continued)

Production unit	Sample category	Sample type	Sample size*		
			1st	2nd	3rd
Broiler farm					
• After C&D	Environmental	Floor	3	3	3
		Wall	2	6	6
		Pan feeder	5	20	5
		Litter before disinfecting	5	10	10
		Litter after disinfecting	5	10	10
		Water	1	2	1
		Water nipple and cub swab	5	20	5
		Pest	1	5	4
• Chick arrival	Chicken-related	Meconium (box liner)	10	10	10
	Environmental	Boot swab	-	5	5
		Wall	-	6	6
		Pan feeder	-	20	5
		Feed	3	2	2
		Water	1	2	2
		Water nipple and cup swab	-	20	5
		Pest	-	3	5
• Rearing period (weekly)	Chicken-related	Cloacal swab or feces	5	60	60
	Environmental	Boot swab	5	5	5
		New feed	5	2	2
		Feed from pan feeder	1	5	5
		Water	1	6	6
		Pest	4-5	4-5	5-11
• Slaughter day	Environmental	Water before spraying	-	1	1
		Water after spraying	-	1	3
		Cages	-	10**	15***
		Truck	-	1	1
		Hand swab before working	-	10	10
		Hand swab after working	-	10	10
Slaughterhouse	Chicken-related	Cloacal swab or cloaca feather	10	60	60
		Whole carcass (rinse)	10	20	20
Total			**294**	**1,085**	**1,005**

* sample size for 3 sampling cycles **cage swab before use ***cage swab before and after use

3.2 Detection and enumeration of *Salmonella*

All samples were analyzed for *Salmonella* contamination according to the ISO 6579:2002/ Amendment 1:2007 (Annex D) "Detection of *Salmonella* spp. in animal feces and in environmental samples from the primary production stage" standard method (ISO, 2002) and serotypes of the isolates were identified by slide agglutination test following the Kauffmann-White Scheme (Grimont and Weill, 2007) at the WHO National *Salmonella* and *Shigella* Center, National Institute of Health (NIH), Ministry of Public Health, Thailand. Particularly samples collected from the first sampling cycle were also enumerated by the most probable number (MPN) method (Appendix B).

3.3 Antimicrobial susceptibility testing of *Salmonella*

At least one isolate per serotype from all positive samples were randomly selected (*n*=220) for antimicrobial susceptibility testing. The disk diffusion method on Mueller Hinton Agar (Difco Laboratories, Detroit, MI, USA) was used in this study. Sixteen antimicrobial drugs that were commonly used in broiler chicken production or human to treat the salmonellosis were selected. Disk potencies of such antimicrobial drugs were 109 µg of lincomycin-spectinomycin, 10 µg of ampicillin, 10 µg of amoxicillin, 5 µg of cefotaxime, 30 µg of ceftazidime, 10 µg of meropenem, 10 µg of gentamicin, 30 µg of doxycycline, 30 µg of tetracycline, 10 µg of colistin sulphate, 30 µg of chloramphenicol, 30 µg of nalidixic acid, 5 µg of ciprofloxacin, 10 µg of norfloxacin, 5 µg of enrofloxacin and 25 µg of Trimethoprim-sulfamethoxazole (Oxoid, UK). The strain of *Escherichia coli* ATCC®25922 was used as the quality control for this study. The interpretation of antimicrobial susceptibility (or resistance)

was referred to the breakpoints recommended by the Clinical and Laboratory Standards Institute guidelines (CLSI, 2008, 2013) and European Committee on Antimicrobial Susceptibility Testing (EUCAST, 2013). Since there was no universal guideline set for colistin sulphate, a guideline set by a previous study was used (Gales et al., 2001). The criterion for multidrug resistance (MDR) was defined as isolates being resistant to at least one antimicrobial drug in three or more antimicrobial categories (Magiorakos et al., 2012). The interpretation based on inhibition zone of each antimicrobial agent was shown in Table 6 (Appendix C).

Table 6. Disk diffusion zone diameter interpretation

Antimicrobial agents	Diameter of zone of inhibition (mm)			CLSI or other references
	Resistant	Intermediate	Susceptible	
Lincomycin-spectinomycin (109 µg)*	≤ 14	15-17	≥ 18	M100-S23
Ampicillin (10 µg)	≤ 13	14-16	≥ 17	M31-A3
Amoxicillin (10 µg)	≤ 13	14-16	≥ 17	M31-A3
Ceftazidime (30 µg)	≤17	18-20	≥ 21	M100-S23
Cefotaxime (5 µg)	<17	17-19	≥ 20	EUCAST, 2013
Meropenem (10 µg)	≤ 19	20-22	≥ 23	M100-S23
Gentamicin (10 µg)	≤ 12	13-14	≥ 15	M31-A3
Doxycycline (10 µg)	≤ 10	11-13	≥ 14	M100-S23
Tetracycline (30 µg)	≤ 14	15-18	≥ 19	M31-A3
Colistin sulphate (10 µg)	≤ 11	12-13	≥ 14	Gales et al., 2001
Chloramphenicol (30 µg)	≤ 12	13-17	≥ 18	M31-A3
Nalidixic acid (30 µg)	≤ 13	14-18	≥ 19	M100-S23
Ciprofloxacin (5 µg)	≤ 20	21-30	≥ 31	M100-S23
Norfloxacin (10 µg)	≤ 12	13-16	≥ 17	M100-S23
Enrofloxacin (5 µg)	≤ 16	17-22	≥ 23	M31-A3
Trimethoprim-sulfamethoxazole (25 µg)	≤ 10	11-15	≥ 16	M31-A3

*Use diameter of zone of inhibition of spectinomycin for interpretation

3.4 PFGE genotyping of *Salmonella*

The isolates that either shared the common serotype between chicken-related and environmental samples or shared across a series of production units were selected to investigate their genetic characteristics by the pulsed-field gel electrophoresis (PFGE) technique at the National Center for Genetic Engineering and Biotechnology (BIOTEC), Food Biotechnology laboratory, Thailand Science Park. PFGE was performed using a CHEF Mapper (Bio-Rad Laboratories, USA) according to the CDC PulseNet "One-Day (24-28 h) Standardized Laboratory Protocol for Molecular Subtyping of *Escherichia coli* O157:H7, *Salmonella* serotypes, *Shigella sonnei* and *Shigella flexneri* (PulseNet, 2009) with a slight modification (Appendix D). Twenty units/µl of *Xba*I (New England BioLabs, USA) was used as the primary restriction enzyme. In the case where PFGE patterns obtained from the isolates were indistinguishable, the isolates were confirmed using five units/µl of *Avr*II (New England Biolabs, USA) as the secondary restriction enzyme. The standard strain "*Salmonella* ser. Braenderup H9812" recommended by PulseNet was used as the molecular weight standard (Appendix D). The gel images were captured using Gel Doc (Synoptics, Ltd., UK). The PFGE patterns were analyzed using GelCompar II software package, version 5.0 (Applied Maths BVBA, Kortrijk, Belgium). The degree of similarity between PFGE patterns was calculated using Dice correlation coefficient. The dendrogram of PFGE was constructed using the unweighted pair group method with arithmetic mean (UPGMA) with position tolerance setting at 1.5% optimization and 1.5% band position tolerance. The PFGE patterns with similarity index >85% were considered belonging to the same cluster.

The nomenclature system for PFGE banding patterns was designated by four letters. The first two letters were the abbreviation referring to *Salmonella* serotype, for example, “WE” was referred to *S.* Weltevreden, “DE” was referred to *S.* Derby, “PB” was referred to *S.* Paratyphi B, “CO” was referred to *S.* Corvallis, “AB” was referred to *S.* Albany, “BO” was referred to *S.* Bovismorbifican, “AT” was referred to *S.* Altona and “GE” was referred to *S.* Give. The third letter indicated cluster name of the serotype, shown in roman numbering system. The last letter indicated sub-cluster name of the serotype, shown in the lowercase English alphabets.

3.5 Probabilistic models

Probabilistic models were applied to describe the dynamic of *Salmonella* which is chronologically persistent in the chicken-related samples across broiler production units and to describe the dynamic of *Salmonella* contamination among the multiple sources in the broiler farm. The models presented here were formulated in a stochastic manner which combined the variation in the individual input variables in the form of a probability distribution (OIE, 2004). In order to apply the probabilistic models, the prevalence variable and concentration variable of *Salmonella* contaminated in chicken-related and environmental samples were defined in terms of “probabilistic variable” in all production units. The probability distribution was presented in the range of all possible values (x-axis) versus the probabilities (y-axis) of such corresponding values in the x-axis (Figure 2).

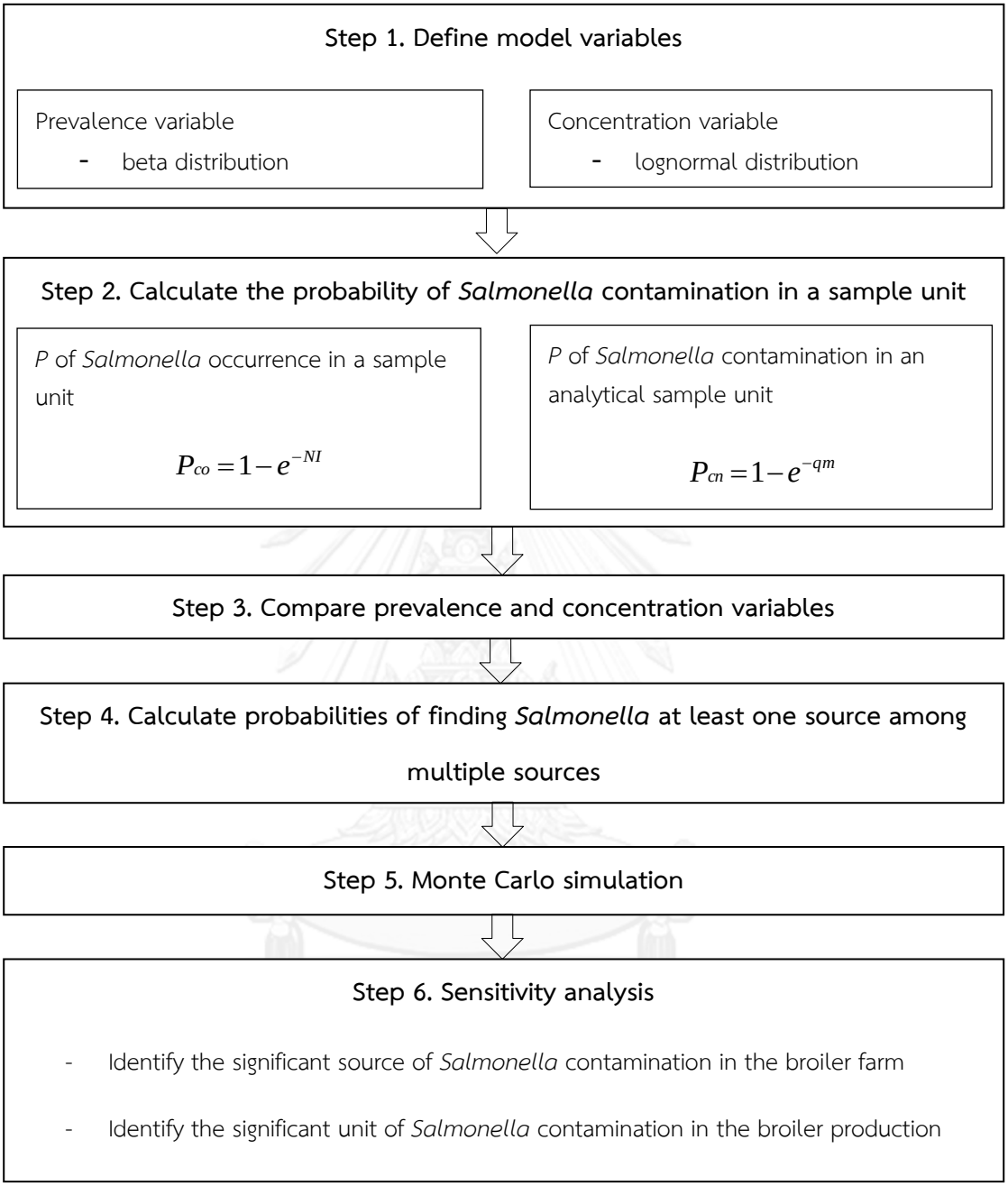


Figure 2. Step of quantitative analysis in the probabilistic model

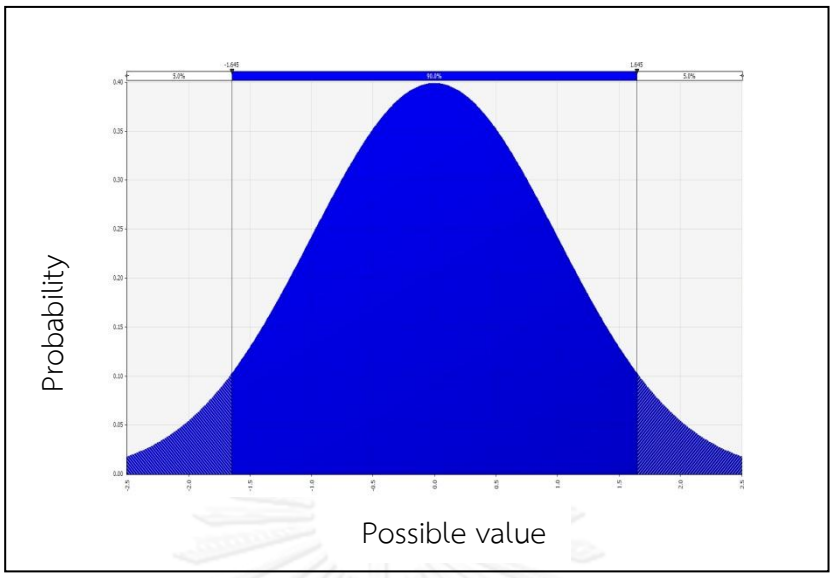


Figure 3. Probability distribution

3.5.1 Define model variables

The prevalence variable

Beta distribution was used to describe the variation of the prevalence variable (FAO/WHO, 2008b). Two parameters of beta distribution consisted of alpha (α) and beta (β) were used to define the shape of this distribution as presented in equation 1

$$P = Beta\left(\alpha, \beta\right) \quad (1)$$

According to the Bayesian inference, the alpha parameter was replaced by s+α while beta parameter was replaced by n-s+β. *s* denoted the number of successes or number of positive samples and *n* denoted the number of trials in a binomial process or number of the samples tested as presented in equation 2.

$$P = Beta\left(s + \alpha, n - s + \beta\right) \quad (2)$$

If the prior distribution is presumably an uninformed prior and likelihood function is binomial distribution in the Bayesian inference. Then this notation become a posterior distribution. Interestingly, if beta (1,1) distribution, which is equivalent to uniform (0,1) distribution, is an uninformed prior (FAO/WHO, 2008a). Then both alpha and beta parameters were replaced by 1 as shown in (3).

$$P_i = Beta\left(s+1, n-s+1\right) \quad (3)$$

P_i is the probability distribution of prevalence variable

Concentration variable

Most probable number (MPN) technique was used to quantify *Salmonella* concentration in the first sampling cycle. The number of micro-organism in terms of concentration was usually described by a lognormal distribution because the minimum concentration of *Salmonella* in samples were zero and positively skewed were found (OIE, 2004). Two parameters, which were mean (μ) and standard deviation (σ) of the lognormal distribution, were used to define the shape of the lognormal distribution as presented in equation 4.

$$P_j = log\ norm\left(\mu, \sigma\right) \quad (4)$$

P_j is the probability distribution of concentration variable

3.5.2 Probabilities of *Salmonella* contamination in a sample unit (P_c)

The presence of micro-organism in a (certain size of) sample unit has been described by Poisson distribution (Geng, 1983). The parameter of Poisson distribution could be either the number of events in an interval space, an interval time or a volume of sample (OIE, 2004). In this study, the number of events could be regarded

either as “the occurrence of *Salmonella*” or as “the number of *Salmonella* (log scale)” in a sample unit.

In the former case, two parameters used to calculate the probability that *Salmonella* presented in at least one sample unit among the total sample size (P_{co}) were the prevalence of *Salmonella* (*I*) and the corresponding sample size (*N*) as shown in equation 5 (Geng, 1983). Sample size was composed of many sample units.

$$P_{co} = 1 - e^{-NI} \quad (5)$$

In the latter case, two parameters to calculate the probability that *Salmonella* presented at least one organism (log scale) in a sample unit (P_{cn}) were the concentration (*m*) and the amount of analytical sample (*q*) as shown in equation 6 (Crepet et al., 2007). The sample unit was defined as the volume of sample tested in the laboratory.

$$P_{cn} = 1 - e^{-qm} \quad (6)$$

In order to calculate the probability of *Salmonella* contamination in a sample unit, this study defined the sample size (for P_{co}) and amount of each analytical sample (for P_{cn}) depending on the sampling plan in farm practice as shown in Table 7.

Table 7. Sample size and unit of volume to calculate P_{co} and P_{cn}

Production unit	Sample type	P_{co}	P_{cn}
		Sample size (N)	Unit of volume (q)
Breeding farm	Chicken-related		
	- Manure or cloacal swab	60	g
	- Egg	30	egg
	Environment		
	- Surface swab	5	m^2
Hatchery	Chicken-related		
	- Egg	30	egg
	Environment		
	- Surface swab	15	m^2
Broiler farm	Chicken-related		
	- Manure or cloacal swab	60	g
	Environment		
	- Surface swab	5	m^2
	- Litter	3	g
	- Feed	3	g
	- Water	3	ml
	- Pest	5	animal
	- Fomite*	5	m^2
Slaughterhouse	Chicken-related		
	- Cloacal swab or feather	60	g
	- Whole carcass	20	carcass

* cage swab, truck swab, hand swabs, water for spraying

3.5.3 Compare prevalence and concentration variables

In order to determine whether the prevalence *per se* was enough to illustrate the magnitude of *Salmonella* contamination in the broiler production. The comparison of the ranking between prevalence variables (P_{co}) and concentration variables (P_{cn}) was performed to evaluate this hypothesis. Since in this study the concentration of *Salmonella* was only enumerated in the first sampling cycle. The comparison of the ranking between P_{co} and P_{cn} was performed only for the first sampling cycle.

3.5.4 Probability of finding *Salmonella* among multiple sources

The principle applying models in this study was mainly based on the "Binomial theorem" whereby all repeated samples for each input variable consists of all identical samples (trials or *n*). Each sample would be resulted in either positive or negative (dichotomy) for *Salmonella*. A set of sampling units has been drawn from a source of *Salmonella* contamination as a population of interest. This source of *Salmonella* contamination possessed a probability of contamination (P_{co} or P_{cn}) as shown in equations (5)-(6). If repeated samplings (with equal number of sample units in all sets of sampling) have been drawn *k* times, the probability of having *Salmonella* at least one time from total *k* times was calculated as the following (Geng, 1983).

$$P_{+} = 1 - \left(1 - P_{co}\right)^{k} \qquad (7)$$

Since *Salmonella* could be disseminated from various sources either from the chicken or from the environment. The probability of finding *Salmonella* from at least one source among multiple sources (*k*) was calculated as the following equation.

$$P_{+} = 1 - \prod_{i=1}^{k} \left(1 - P_{co_i}\right) \quad (8)$$

This equation was used as a model to further identify either “which source” or “which production unit” as the significant source for *Salmonella* contamination in the broiler production

3.5.4.1 Probabilistic model of Salmonella contamination among multiple sources in the broiler farm

In the broiler farm, many possible sources of *Salmonella* can contaminate the chicken. In this study, we have identified that the important sources of *Salmonella* contamination during the broiler production consisted of the one day-old chick, contaminated equipment after cleaning and disinfection, litter after disinfection, feed, pest, water and contaminated equipment (fomite) on the slaughter day. Therefore, the probability of contamination of each source was considered to be the input variable (P_{co_i}) and calculated by equation (9). The output variable (P_{+}) as the name implied represented the result of the mathematical operation of such input variables in the model.

$$P_{+broiler\_farm} = 1 - \prod_{i=source\_1}^{source\_k} \left(1 - P_{co_i}\right) \quad (9)$$

In order to describe the dynamic of *Salmonella* persistence in the environment particularly in “the broiler farm” in chronological samplings during the rearing period, the prevalences across three sampling cycles of each individual chronological sampling were used for calculation.

For example, feed was weekly collected (weeks 1-6) in the first sampling cycle. Likewise, feed was again weekly collected (weeks 1-6) in the second and third sampling cycles. Then weekly prevalence of *Salmonella* in the feed sample was averaged across three sampling cycles to be the mean prevalence in week 1-6. Therefore feed (as a possible source of *Salmonella* contamination) would finally have 6 weekly mean prevalences corresponding to weeks 1-6. Similarly for other environmental samples, the same scheme of calculation of mean prevalence was employed.

Then, P_{co} of each *Salmonella* source from week 1-6 were used to calculate (P_{+}) for the corresponding week. P_{co} of other environmental samples (P_{co_i}) was calculated in the same way. For example, the probability (P_{+}) of finding *Salmonella* in at least one *Salmonella* source among all possible sources in the first week was the following.

$$P_{+week1} = 1 - \left(1 - P_{co\_day\_old\_chick}\right) * \left(1 - P_{co\_feed}\right) * \left(1 - P_{co\_water}\right) * \ldots \quad (10)$$

In addition, the final model for describing the probability (P_{+}) of finding *Salmonella* in at least one *Salmonella* source (*i*) among all possible sources (*k*) in the broiler farm were considered using combined data from all three sampling cycles.

3.5.4.2 Probabilistic model of Salmonella contamination in chicken-related samples across the production unit

As the transmission of *Salmonella* in the chicken from one production unit to the subsequent production unit has been apparently demonstrated in this study, the

dynamic of *Salmonella* contamination in a series of broiler production units could mathematically identify the source of *Salmonella* contamination as a whole. Therefore, the input variables were supposed to be the probability of *Salmonella* contamination in chicken-related samples (P_{co_i}) from a series of production units. These production units have been contaminated with *Salmonella* and then either horizontally or vertically transferred from one production unit to the subsequent production unit. P_{co} of a production unit was calculated from a pooled prevalence of *Salmonella* (*I*) from all chicken-related samples in the corresponding production unit (across 3 sampling cycles). Therefore, the probability of finding *Salmonella* in at least one production unit (*i*) among a series of broiler production unit (*k*) was shown in equation 11.

$$P_{+broiler\_production} = 1 - \prod_{i=unit\_1}^{unit\_k} \left(1 - P_{co_i}\right) \qquad (11)$$

In this case, k was 5 since the production units were breeder, hatchery, broiler, pre-slaughter (subunit) and slaughterhouse.

3.5.5 Monte Carlo simulation

The probability models were then simulated by means of a simulation technique using a commercial simulation software, @Risk 5.5 in Decision Tool suite 5.5 (Palisade corporation). Since, the variable was in the form of probability distribution then the basic mathematical operations of probabilistic variable could not theoretically be performed. The mechanism of simulation was to repeatedly take possible values of the input variable and substitute such values into the probabilistic model to calculate the output values. In this study, the Monte Carlo simulation was

performed up to 10,000 iterations to generate the range of possible output in the probabilistic models.

3.5.6 Sensitivity analysis

The sensitivity analysis was performed in the last step. The primary aim of sensitivity analysis was to determine input variables which consist of sources of *Salmonella* contamination in the broiler farm significantly correlated with the output variable of the probabilistic model. The result of sensitivity analysis was a set of the correlation coefficients pair-wised matched between any input variables in the model and their output variable. The correlation coefficients could be either positive (changes of input and output variables in the same direction) or negative values (changes of input and output variables in the opposite direction). The range of correlation coefficient was between -1.0 and +1.0. The closer to the both ends of correlation coefficients, the higher correlation between input and output variables. The highest correlation coefficient was listed first and then descendingly sorted regardless of positive or negative values.

3.5.6.1 Identify the significant source of Salmonella contamination in a broiler farm

By using the equation (9), day-old chicks or environment in a certain broiler farm were used as the input variables in the form of P_{co_i}. The sensitivity analysis was used to sort the correlation coefficients of day-old chicks or environment. The higher correlation coefficients of day-old chicks or environment, the higher likelihood of such factor as the significant source of *Salmonella* contamination in the broiler farm.

3.5.6.2 Identify the significant unit of Salmonella contamination in the broiler production

By using the equation (11), the *Salmonella* status of chicken-related samples in each production unit was used as the input variable in the form of P_{co_i}. The sensitivity analysis was used to sort the correlation coefficients of production units. Likewise, the higher correlation coefficients of a production unit, the higher likelihood of such product unit as the significant unit of *Salmonella* contamination in the broiler production.

The highly correlated input variable(s) to the output variable enabled risk assessors to sort out sources in the broiler farm or which production unit as significant source of *Salmonella*. And risk managers would be able to weigh and implement some possible or appropriate risk management options.

CHAPTER IV
RESULTS

4.1 Contamination level of *Salmonella*

4.1.1 Prevalence

The overall prevalences of *Salmonella* in chicken-related samples and environmental samples in the integrated broiler chicken production from 3 sampling cycles were 15.5% (225/1,449) and 15.4% (144/935), respectively. In the first sampling cycle, *Salmonella* positive samples were 53 out of 116 chicken-related samples (45.7%) and 61 out of 178 environmental samples (34.3%) (Table 8). However, in the second sampling cycle, 18 out of 692 chicken-related samples (2.6%) and 24 out of 393 environmental samples (6.1%) were found positive for *Salmonella* (Table 9). In the third sampling cycle, 154 out of 641 chicken-related samples (24.0%) and 59 out of 364 environmental samples (17.7%) were found positive for *Salmonella* (Table 10).

In the breeder production unit, the result of *Salmonella* isolation showed that only one out of three sampling cycles found *Salmonella* in manure, baskets and plates and egg trays. *S.* Albany was commonly observed in both manure and egg trays (Table 8). Whereas no *Salmonella* was found from egg samples in this study.

In the hatchery production unit, *Salmonella* were isolated from the floor of egg storage room, the hook, the egg setting stand, the hatching tray (before using), the transport belt, the hands of the workers (before and after working) and meconium in the hatching trays in two out of three sampling cycles (Tables 8, 10). The most common serotype isolated from the hatchery was *S.* Corvallis. However,

S. Havana and *S.* Agona were also found in the hatching trays before use (Tables 8, 10).

In the broiler production unit, *Salmonella* remained positive in wall, pan feeder, water nipple, water, litter (before disinfection) and lizard during the downtime period in the first sampling cycle (Table 8). For the second and third sampling cycles, *Salmonella* was also found in the litter (after disinfection) and lizards. *S.* Weltevreden was commonly found in the litter and the lizards in the second and third sampling cycles (Tables 9, 10). On the chick arrival day, *Salmonella* was isolated from the boot swab, water nipple and lizards in the second sampling cycle (Table 9) and from the meconium of the day-old-chicks on the box-liner in the third sampling cycle (Table 10). Interestingly, for the third sampling cycle, the same serotype (*S.* Corvallis) that was recovered from the meconium was also found in samples collected from the hatchery (Table 10). During the rearing period, *Salmonella* was weekly isolated from manure and cloacal swabs in the first and third samplings. For the environmental samples, *Salmonella* was recovered from pests (lizards and centipedes), boot swab, water, new feed and feed in the pan feeder. *S.* Derby and *S.* Corvallis were the most common serotypes that were isolated from the environmental samples and the chicken-related samples in the first and third samplings, respectively (Tables 8, 10). The predominant serotype isolated from pests (lizards and centipedes) in the broiler farm was *S.* Weltevreden. On the slaughter day, *Salmonella* was isolated from cages, trucks, water for spraying (to reduce heat stress during the transportation) and hands of the workers after working. Various *Salmonella* serotypes (*S.* Albany, *S.* Altona, *S.* Mbandaka and *S.* Falkensee) that have never been found in the broiler farm were detected from cages, trucks and water

before spraying in the second sampling cycle (Table 9). Furthermore, some serotypes such as *S.* Altona isolated from cages was also found in the hands of workers after catching.

In the slaughterhouse production unit, assorted *Salmonella* serotypes that were not found in the broiler flock were isolated from cloacal swab samples and whole carcass after chilling such as *S.* Kentucky, *S.* Orion, *S.* Paratyphi B and *S.* Saintpaul in the first sampling cycle (Table 8), *S.* Virginia*, S.* Agona, *S.* Give in the second sampling (Table 9). However, the same *Salmonella* serotype isolated from the cages and the hands of workers was also found in the cloacal swabs and whole carcasses at the slaughterhouse in the second sampling (Table 9). For the third sampling, the same *Salmonella* serotype (*S.* Corvallis) isolated from the cloacal swab during the rearing period was predominant serotype in the cloacal swab and whole carcass at the slaughterhouse.

4.1.2 Concentration

In this study, the minimum, mean and maximum concentration of *Salmonella* contaminated in the manure at the breeder production unit was 2.46, 3.9 and 4.38 log MPN/g. Whereas *Salmonella* concentration in the environment was 4.78 log MPN/100 cm^2 and 2.36 log MPN/100 cm^2 from egg tray and basket and plate, respectively. Among the environmental samples in the hatchery production unit, the egg storage room was highly contaminated with *Salmonella* at 3.57 log MPN /100 cm^2. In the broiler production unit, *Salmonella* concentrations in the manure were fluctuated between 1.81 and 4.03 log MPN/g depending on the age of the chicks. The highest *Salmonella* concentration in manure was at the age of two

weeks. Whereas, the highest *Salmonella* concentration in environment was found in boot swab at the age of 4 weeks. For the environmental samples, the result showed that *Salmonella* concentrations in environment were between 0.3 log MPN/g in litter (before disinfection) and 5.21 log MPN/pair in boot swab sample. In the slaughterhouse production unit, *Salmonella* concentrations in chicken-related samples were decreasing after slaughter from 2.69 log MPN/g in the feather around cloaca down to 2.62 log MPN/bird in whole carcass (Table 8). This result indicated that the slaughter process might be able to decrease *Salmonella* contamination in chicken carcasses.

4.2 Serodiversity

The result of this study showed that 27 serotypes of *Salmonella* were identified from the total of 369 positive samples (Table 11). The main serotype isolated was *S.* Corvallis (48.5%), which was ubiquitously found throughout a series of production units, followed by *S.* Derby (16.1%) and *S.* Weltevreden (9.2%), which were found in the broiler farm and slaughterhouse. A wide variety of *Salmonella* serotypes was observed and isolated from broiler farm and slaughterhouse production unit.

Table 8. Serotypes and dissemination of *Salmonella* spp. from the first sampling

Production unit	Chicken-related sample				Environment sample			
	Type	Prevalence (%)	Concentration (log MPN/ unit)	Serotype	Type	Prevalence (%)	Concentration (log MPN/ unit)	Serotype
Breeding farm	Feces	100 (9/9)	3.90/g	Albany	Egg tray	100 (5/5)	4.78/100 cm^2	Albany
	Egg	0 (15/15)	N/A*	N/A	Basket and plate	20 (1/5)	2.36/100 cm^2	Corvallis
Hatchery	Egg (before incubating)	0 (0/10)	N/A	N/A	Egg storage room	100 (1/1)	3.57/100 cm^2	Corvallis
	Egg (after incubate)	0 (0/10)	N/A	N/A	Hook	100 (1/1)	1.86/hook	Corvallis
	Meconium in tray	0 (0/12)	N/A	N/A	Egg setting stand	100 (1/1)	2.17/stand	Corvallis
					Hatching tray (before use)	8.3 (1/12)	2.58/tray	Havana
					Other samples	0 (0/33)	N/A	N/A
Broiler farm					Wall	100 (2/2)	2.99/1 m^2	Albany
• after C&D					Pan feeder	100 (5/5)	2.03/pan	Albany
					Water nipple	60 (3/5)	1.89/nipple	Albany
					Water	100 (1/1)	2.49/100 ml	Albany
					Litter (before disinfect)	40 (2/5)	0.30/g	Havana, Weltevreden
					Lizard	100 (1/1)	1.54/lizard	Albany
					Other samples	0 (0/8)	N/A	N/A
• arrival day	Meconium on box-liner	0 (0/10)	N/A	N/A	Water, Feed	0 (0/4)	N/A	N/A
• week 1	Feces	100 (5/5)	3.55/g	Derby, Caen	Boot swab	100 (5/5)	4.59/pair	Derby
					Lizard	25 (1/4)	4.46/lizard	Weltevreden
• week 2	Feces	100 (5/5)	4.03/g	Derby, Weltevreden	New feed	40 (2/5)	1.48/g	Derby, Braenderup
					Lizard	40 (2/5)	2.37/lizard	Weltevreden
• week 3	Feces	100 (5/5)	2.51/g	Derby, Albany, Bovismorbifican	Boot swab	100 (5/5)	4.96/pair	Derby
					Lizard	40 (2/5)	3.3/lizard	Derby, Hotutenae
• week 4	Feces	100 (5/5)	2.81/g	Derby, Senftenberg	Boot swab	100 (5/5)	5.21/pair	Derby, Albany, Kouka, Bovismorbifican
					Lizard	40 (2/5)	4.88/lizard	Weltevreden
• week 5	Feces	60 (3/5)	3.06/g	Derby	Boot swab	80 (4/5)	4.90/pair	Derby
					Water	100 (1/1)	0.97/100 ml	Derby
					Lizard	60 (3/5)	3.47/lizard	Weltevreden, Hotutenae
• week 6	Feces	80 (4/5)	1.81/g	Derby	Boot swab	100 (5/5)	3.93/pair	Derby, Kentucky, Bovismorbifican
					Other samples (W 1-6)	0 (0/34)	N/A	N/A
Slaughterhouse	Feather around cloaca	70 (7/10)	2.69/g	Kentucky, Derby, Altona, Bovismorbifican, Orion, Stockholm, 4,12:i:-				
	Whole carcass (rinse)	100 (10/10)	2.62/bird	Derby, Kentucky, Stanley, Agona, Weltevreden, Saintpaul, Paratyphi B				
Total	**Chicken-related sample**	**45.7 (53/116)**			**Environmental sample**	**34.3 (61/178)**		

* N/A : Not applicable

Table 9. Serotypes and dissemination of *Salmonella* spp. from the second sampling

Production unit	Chicken-related sample				Environment sample			
	Type	Prevalence (%)	Concentration (log MPN/ unit)	Serotype	Type	Prevalence (%)	Concentration (log MPN/ unit)	Serotype
Breeding farm	Cloacal swab	0 (0/120)	N/A*	N/A	Boot swab	0 (0/10)	N/A	N/A
	Egg	0 (0/50)	N/A	N/A	Other samples	0 (0/32)	N/A	N/A
Hatchery	Eggs (before incubation)	0 (0/10)	N/A	N/A	All samples	0 (0/47)	N/A	N/A
	Eggs (after incubation)	0 (0/50)	N/A	N/A				
	Meconium in hatching tray	0 (0/12)	N/A	N/A				
Broiler farm								
• after C&D					Litter (after disinfection)	20 (2/10)	N/A	Weltevreden
					Lizard	60 (3/5)	N/A	Weltevreden
					Other samples	0 (0/61)	N/A	N/A
• arrival day	Meconium on box-liner	0 (0/10)	N/A	N/A	Boot swab	20 (1/5)	N/A	Weltevreden
					Water nipple	5 (1/20)	N/A	Weltevreden
					Lizard	100 (3/3)	N/A	Weltevreden
					Other samples	0 (0/30)	N/A	N/A
• weeks 1-4	Cloacal swab	0 (0/240)	N/A	N/A	All samples (weeks 1-4)	0 (0/91)	N/A	N/A
• week 5	Cloacal swab	0 (0/60)	N/A	N/A	Boot swab	20 (1/5)	N/A	Stanley
					Lizard	80 (4/5)	N/A	IV (43;z_4,z_{23}:-)
					Other samples	0 (0/13)	N/A	N/A
• week 6	Cloacal swab	0 (0/60)	N/A	N/A	Lizard	20 (1/5)	N/A	IV (43;z_4,z_{23}:-)
					Other samples	0 (0/18)	N/A	N/A
• Slaughter day					Cage	50 (5/10)	N/A	Albany, Altona, Weltevreden
					Truck (floor swab)	100 (1/1)	N/A	Mbandaka
					Water (before spraying)	100 (1/1)	N/A	Falkensee
					Hands of worker (after work)	10 (1/10)	N/A	Altona
					Other samples	0 (0/11)	N/A	N/A
Slaughterhouse	Cloacal swab	3.3 (2/60)	N/A	Albany, Derby, Virginia				
	Whole carcass (rinse)	80 (16/20)	N/A	Albany, Agona, Give, Altona				
Total	**Chicken-related sample**	**2.6 (18/692)**			**Environmental sample**	**6.1 (24/393)**		

* N/A : Not applicable

Table 10. Serotypes and dissemination of *Salmonella* spp. from the third sampling

Production unit	Chicken-related sample				Environment sample			
	Type	Prevalence (%)	Concentration (log MPN/ unit)	Serotype	Type	Prevalence (%)	Concentration (log MPN/ unit)	Serotype
Breeding farm	Cloacal swab	0 (0/120)	N/A*	N/A	Boot swab	0 (0/10)	N/A	N/A
	Egg	0 (0/50)	N/A	N/A	Other samples	0 (0/28)	N/A	N/A
Hatchery	Egg (before incubation)	0 (0/20)	N/A	N/A	Hatching tray (before use)	8.3 (1/12)	N/A	Agona
	Meconium in hatching tray	33.3 (4/12)	N/A	Corvallis	Hands of worker (before work)	21.4 (3/14)	N/A	Corvallis
					Hands of worker (after work)	21.4 (3/14)	N/A	Corvallis
					Transferring belt (before use)	50 (1/2)	N/A	Corvallis
					Other samples	0 (0/10)	N/A	N/A
Broiler farm								
• after C&D					Litter (before disinfection)	30 (3/10)	N/A	Weltevreden
					Litter (after disinfection)	50 (5/10)	N/A	Weltevreden, Cannstatt
					Lizard	50 (2/4)	N/A	Weltevreden
					Other samples	0 (0/21)	N/A	N/A
• arrival day	Meconium on the box-liners	80 (8/10)	N/A	Corvallis	All sample	0 (0/30)	N/A	N/A
• week 1	Cloacal swab	85 (51/60)	N/A	Corvallis	Boot swab	100 (5/5)	N/A	Corvallis
					New feed	0 (0/2)	N/A	N/A
					Feed in pan feeder	80 (4/5)	N/A	Corvallis
					Water	16.7 (1/6)	N/A	Weltevreden
					Pest (centipede, lizard, cockroach	18.2 (2/11)	N/A	Weltevreden
• week 2	Cloacal swab	73.3 (44/60)	N/A	Corvallis	Boot swab	100 (5/5)	N/A	Corvallis
					Feed in pan feeder	100 (5/5)	N/A	Corvallis
					New feed, Water	0 (0/8)	N/A	N/A
					Lizard	20 (1/5)	N/A	Corvallis
• week 3	Cloacal swab	3.3 (2/60)	N/A	Corvallis	Feed in pan feeder	20 (1/5)	N/A	Corvallis
					Other samples	0 (0/18)	N/A	N/A
• week 4	Cloacal swab	10 (6/60)	N/A	Corvallis	All samples	0 (0/23)	N/A	N/A
• week 5	Cloacal swab	8.3 (5/60)	N/A	Corvallis, Weltevreden	Boot swab	20 (1/5)	N/A	Eastbourne
					Lizard	40 (2/5)	N/A	Corvallis
					Other samples	0 (0/13)	N/A	N/A
• week 6	Cloacal swab	3.3 (2/60)	N/A	Corvallis	Boot swab	100 (5/5)	N/A	Corvallis
					Feed in pan feeder	40 (2/5)	N/A	Corvallis
					New feed, Water	0 (0/8)	N/A	N/A
					Lizard	40 (2/5)	N/A	Weltevreden
• Slaughter day					Cage	6.7 (1/15)	N/A	Corvallis
					Water (before spray)	100 (1/1)	N/A	Weltevreden
					Water (after spray)	100(3/3)	N/A	Weltevreden, Corvallis
					Other samples	0 (0/36)	N/A	N/A
• Slaughterhouse	Cloacal swab	25 (15/60)	N/A	Corvallis				
	Whole carcass (rinse)	89.5 (17/19)	N/A	Corvallis				
Total	**Chicken-related sample**	**24 (154/641)**			**Environmental sample**	**17.7 (59/364)**		

* N/A : Not applicable

Table 11. Serodiversity of *Salmonella*-positive samples isolated from chicken-related and environmental samples

Serotype	No. of samples							
	Breeding farm		Hatchery		Broiler farm		Slaughterhouse	Total
	Chicken-related	Environment	Chicken-related	Environment	Chicken-related	Environment		
Corvallis		1	4	10	109	28	32	**184**
Derby					25	27	9	**61**
Weltevreden					2	32	1	**35**
Albany	9	5			1	14	2	**31**
Havana				1		1		**2**
Agona				1			9	**10**
Paratyphi							9	**9**
Altona						4	3	**7**
Give							5	**5**
IV 43:z_4,z_{23}:-						5		**5**
Bovismorbificans					1	2	1	**4**
Hotunenae						3		**3**
Kentucky						1	2	**3**
Cannstatt						3		**3**
Stanley						1	2	**3**
Orion							2	**2**
Stockholm							2	**2**
Braenderup						1		**1**
Caen					1			**1**
Seftenberg					1			**1**
Kouka						1		**1**
Mbandaka						1		**1**
Falkensee						1		**1**
Virginia							1	**1**
Eastbourne						1		**1**
Saintpaul							1	**1**
4,12:i:-							1	**1**

4.3 Antimicrobial resistance of *Salmonella*

The antimicrobial resistances of *Salmonella* serotype isolated from the first, second and third sampling cycles were summarized in Tables 12-14. Out of a total of 220 *Salmonella* isolates tested, 25.5% were susceptible to all antimicrobial agents whereas 0.9% was resistant to 1 class of antimicrobial agents, followed by 21.8% and 51.8% which were resistant to 2 classes and 3 or more than 3 classes of antimicrobial agents, respectively (Table 15). In the breeder production unit, 92.9% (13/14) of the isolates was resistant to ampicillin with the same resistant level as amoxicillin. While, in the hatchery production unit, most of isolates were equally resistant to lincomycin-spectinomycin, ampicillin, amoxicillin and gentamicin at 71.4% (10/14). In the broiler farm, the highest frequency of antimicrobial resistance was lincomycin-spectinomycin, ampicillin and amoxicillin at 59.9%, 37.3% and 37.3%, respectively (Table 15). Likewise, the frequency of antimicrobial resistant to lincomycin-spectinomycin, ampicillin and amoxicillin in the slaughterhouse were found at 92%, 78% and 78%, respectively (Table 15). Multidrug resistances (at least 3 classes) were found among isolates from all broiler production units. The highest frequency of multidrug-resistant isolates was from breeding farm (13/14), followed by slaughterhouse (39/50), hatchery (10/14) and broiler farm (52/142) (Table 15). However, no *Salmonella* isolate was resistant to ceftazidime, cefotaxime, meropenem, colistin sulphate, norfloxacin and enrofloxacin in this study.

Among all isolates from different production units, 13 antimicrobial resistance patterns were found in this study (Table 16). Three antimicrobial resistance patterns, which were LS-DC-TE pattern, LS-AMP-AMX-GEN pattern and AMP-AMX-C-NA-SXT pattern, were found in both sample types (chicken-related sample and

environmental sample). The most prevalent antimicrobial resistance pattern was LS-AMP-AMX-GEN (34.5%), followed by LS-DC-TE (21.4%) and AMP-AMX-C-NA-SXT (8.6%). Notably, LS-AMP-AMX pattern was found only in isolates from environmental sample. Therefore, it was possible that the persistence of multidrug-resistant *Salmonella* in the environment can carry over to the chicken during rearing period.

Table 12. Antimicrobial resistance patterns of *Salmonella* spp. isolates from the first sampling

Production unit	Serotype	*n*	No. of *Salmonella* resistance isolates															
			LS	AMP	AMX	CAZ	CTX	MEM	GEN	DC	TE	CT	C	NA	CIP	NOR	ENR	SXT
Breeding farm	Albany	12	0	12	12	0	0	0	0	0	0	0	12	12	0	0	0	12
	Corvallis	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Hatchery	Corvallis	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Havana	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Broiler farm	Albany	7	0	7	7	0	0	0	0	0	0	0	7	7	0	0	0	7
	Bovismorbifican	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Braenderup	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Derby	39	39	0	0	0	0	0	0	39	39	0	0	0	0	0	0	0
	Havana	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Hotutenae	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Kentucky	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Kouka	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Senftenberg	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0
	Weltevreden	8	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Slaughterhouse	Agona	1	1	1	1	0	0	0	1	0	0	0	0	0	0	0	0	0
	Altona	1	1	1	1	0	0	0	1	0	0	0	0	0	0	0	0	0
	Bovismorbifican	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Derby	8	8	0	0	0	0	0	0	8	8	0	0	0	0	0	0	0
	4,12:i:-	1	1	1	1	0	0	0	1	1	1	0	0	0	0	0	0	0
	Kentucky	2	2	2	2	0	0	0	1	0	0	0	1	0	0	0	0	0
	Orion	2	2	2	2	0	0	0	2	0	0	0	0	2	0	0	0	0
	Paratyphi	6	6	6	6	0	0	0	6	0	0	0	0	6	6	0	0	0
	Stanley	1	1	1	1	0	0	0	1	0	0	0	0	0	0	0	0	0
	Stockhlom	2	2	2	2	0	0	0	2	0	0	0	0	2	0	0	0	0
	Weltevreden	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Total		**108**	**63**	**35**	**35**	**0**	**0**	**0**	**15**	**48**	**49**	**0**	**20**	**29**	**6**	**0**	**0**	**19**
(% Total)			**58.3**	**32.4**	**32.4**	**0**	**0**	**0**	**13.9**	**44.4**	**45.4**	**0**	**18.5**	**26.9**	**5.6**	**0**	**0**	**17.6**

LS, lincomycin-spectinomycin; AMP, ampicillin; AMX, amoxicillin; CAZ, ceftazidime; CTX, cefotaxime; MEM, meropenem; GEN, gentamicin; DC, doxycycline; TE, tetracycline; CT, colistin sulphate; C, chloramphenicol; NA, nalidixic acid; CIP, ciprofloxacin; NOR, norfloxacin; ENR, enrofloxacin; SXT, Trimethoprim-sulfamethoxazole

Table 13. Antimicrobial resistance patterns of *Salmonella* spp. isolated from the second sampling

Production unit	Serotype	*n*	No. of *Salmonella* resistance isolates														
			LS	AMP	AMX	CAZ	CTX	MEM	GEN	DC	TE	CT	C	NA	CIP	NOR	ENR	SXT
Breeding farm	All negative																	
Hatchery	All negative																	
Broiler farm	IV (43:z_4,z_{23}:-)	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Albany	1	1	1	1	0	0	0	1	0	0	0	0	0	0	0	0	0
	Altona	4	4	4	4	0	0	0	3	0	0	0	0	0	0	0	0	0
	Falkensee	1	1	1	1	0	0	0	1	0	0	0	0	0	0	0	0	0
	Mbandaka	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Stanley	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Weltevreden	11	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Slaughterhouse	Agona	7	7	7	7	0	0	0	7	0	0	0	0	0	0	0	0	0
	Albany	2	1	2	2	0	0	0	2	0	0	0	0	2	0	0	0	0
	Altona	2	2	2	2	0	0	0	2	0	0	0	0	0	0	0	0	0
	Derby	1	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0
	Give	4	4	4	4	0	0	0	0	0	0	0	0	4	0	0	0	0
	Virginia	1	1	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0
Total		**39**	**21**	**22**	**22**	**0**	**0**	**0**	**16**	**0**	**0**	**0**	**1**	**7**	**0**	**0**	**0**	**0**
(% Total)			**53.8**	**56.4**	**56.4**	**0**	**0**	**0**	**41**	**0**	**0**	**0**	**2.6**	**17.9**	**0**	**0**	**0**	**0**

LS, lincomycin-spectinomycin; AMP, ampicillin; AMX, amoxicillin; CAZ, ceftazidime; CTX, cefotaxime; MEM, meropenem; GEN, gentamicin; DC, doxycycline; TE, tetracycline; CT, colistin sulphate; C, chloramphenicol; NA, nalidixic acid; CIP, ciprofloxacin; NOR, norfloxacin; ENR, enrofloxacin; SXT, Trimethoprim-sulfamethoxazole

Table 14. Antimicrobial resistance patterns of *Salmonella* spp. isolated from the third sampling

Production unit	Serotype	*n*	No. of *Salmonella* resistance isolates														
			LS	AMP	AMX	CAZ	CTX	MEM	GEN	DC	TE	CT	C	NA	CIP	NOR	ENR	SXT
Breeding farm	Agona	1	1	1	1	0	0	0	1	0	0	0	0	0	0	0	0	0
Hatchery	Corvallis	10	10	10	10	0	0	0	10	0	0	0	0	0	0	0	0	0
Broiler farm	Cannstatt	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Corvallis	40	40	40	40	0	0	0	40	0	0	0	0	0	0	0	0	0
	Eastbourne	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Weltevreden	13	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Slaughterhouse	Corvallis	7	7	7	7	0	0	0	7	0	0	0	0	0	0	0	0	0
Total		**73**	**58**	**58**	**58**	**0**	**0**	**0**	**58**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
(% Total)			**79.5**	**79.5**	**79.5**	**0.0**	**0.0**	**0.0**	**79.5**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**

LS, lincomycin-spectinomycin; AMP, ampicillin; AMX, amoxicillin; CAZ, ceftazidime; CTX, cefotaxime; MEM, meropenem; GEN, gentamicin; DC, doxycycline; TE, tetracycline; CT, colistin sulphate; C, chloramphenicol; NA, nalidixic acid; CIP, ciprofloxacin; NOR, norfloxacin; ENR, enrofloxacin; SXT, Trimethoprim-sulfamethoxazole

Table 15. The percentage of *Salmonella* resistance to antimicrobial agents in the broiler production unit (summary from all three sampling cycle)

Production unit	*n*	Number of *Salmonella* isolates (%) resistant to antimicrobial agents														
		SEN	LS	AMP	AMX	GEN	DC	TE	C	NA	CIP	SXT	Other	Multidrug resistance		
														1 class	2 class	≥ 3 class
Breeding farm	14	1 (7.1)	1 (7.1)	13 (92.9	13 (92.9)	1 (7.1)	0	0	12 (85.7)	12 (85.7)	0	12 (85.7)	0	0	0	13 (92.9)
Hatchery	14	4 (28.6)	10 (71.4)	10 (71.4)	10 (71.4)	10 (71.4)	0	0	0	0	0	0	0	0	0	10 (71.4)
Broiler farm	142	49 (34.5)	85 (59.9)	53 (37.3)	53 (37.3)	45 (31.7)	39 (27.5)	40 (28.2)	7 (4.9)	7 (4.9)	0	7 (4.9)	0	1 (0.7)	40 (28.2)	52 (36.6)
Slaughterhouse	50	2 (4)	46 (92)	39 (78)	39 (78)	33 (66)	9 (18)	9 (18)	2 (4)	17 (34)	6 (12)	0	0	1 (2)	8 (16)	39 (78)
Total	**220**	**56**	**142**	**115**	**115**	**89**	**48**	**49**	**21**	**36**	**6**	**19**	**0**	**2**	**48**	**114**
(% Total)		**(25.5)**	**(64.5)**	**(52.3)**	**(52.3)**	**(40.5)**	**(21.8)**	**(22.3)**	**(9.5)**	**(16.4)**	**(2.7)**	**(8.6)**	**(0)**	**(0.9)**	**(21.8)**	**(51.8)**

LS, lincomycin-spectinomycin; AMP, ampicillin; AMX, amoxicillin; GEN, gentamicin; DC, doxycycline; TE, tetracycline; C, chloramphenicol; NA, nalidixic acid; CIP, ciprofloxacin; SXT, Trimethoprim-sulfamethoxazole; Other = ceftazidime; cefotaxime; meropenem; colistin sulphate; norfloxacin; enrofloxacin; SEN, sensitive to all antimicrobial agents

Table 16. Antimicrobial resistance patterns of *Salmonella* isolated from all production unit

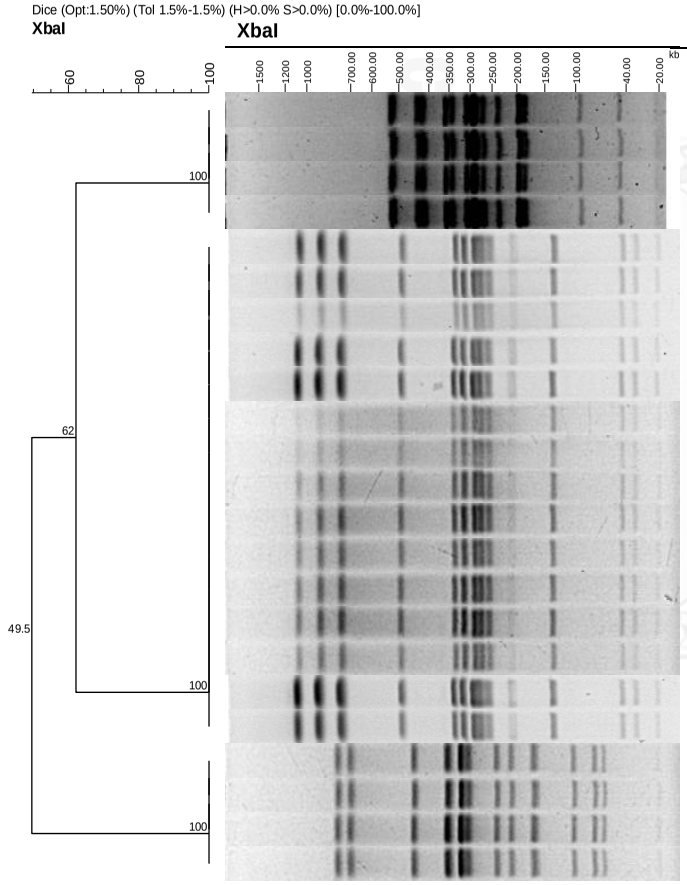
Resistance pattern	Number of isolates							
	Breeding farm		Hatchery		Broiler farm		Slaughterhouse	Total (%)
	Chicken	Environment	Chicken	Environment	Chicken	Environment		
TE					1			1 (0.5)
AMP/AMX							1	1 (0.5)
LS/AMP/AMX						1		1 (0.5)
LS/DC/TE					21	18	8	47 (21.4)
LS/C/NA							1	1 (0.5)
LS/AMP/AMX/GEN		1	3	7	16	29	20	76 (34.5)
LS/AMP/AMX/C							1	1 (0.5)
LS/AMP/AMX/NA							4	4 (1.8)
AMP/AMX/GEN/NA							1	1 (0.5)
AMP/AMX/C/NA/SXT	9	3			1	6		19 (8.6)
LS/AMP/AMX/GEN/NA							5	5 (2.3)
LS/AMP/AMX/GEN/DC/TE							1	1 (0.5)
LS/AMP/AMX/GEN/NA/CIP							6	6 (2.7)
No resistance		1		4	2	47	2	56 (25.5)
Total	**9**	**5**	**3**	**11**	**41**	**101**	**50**	**220**

LS, lincomycin-spectinomycin; AMP, ampicillin; AMX, amoxicillin; GEN, gentamicin; DC, doxycycline; TE, tetracycline; C, chloramphenicol; NA, nalidixic acid; CIP, ciprofloxacin; SXT, Trimethoprim-sulfamethoxazole

4.4 Genetic diversity and source tracking of *Salmonella* in broiler production by PFGE

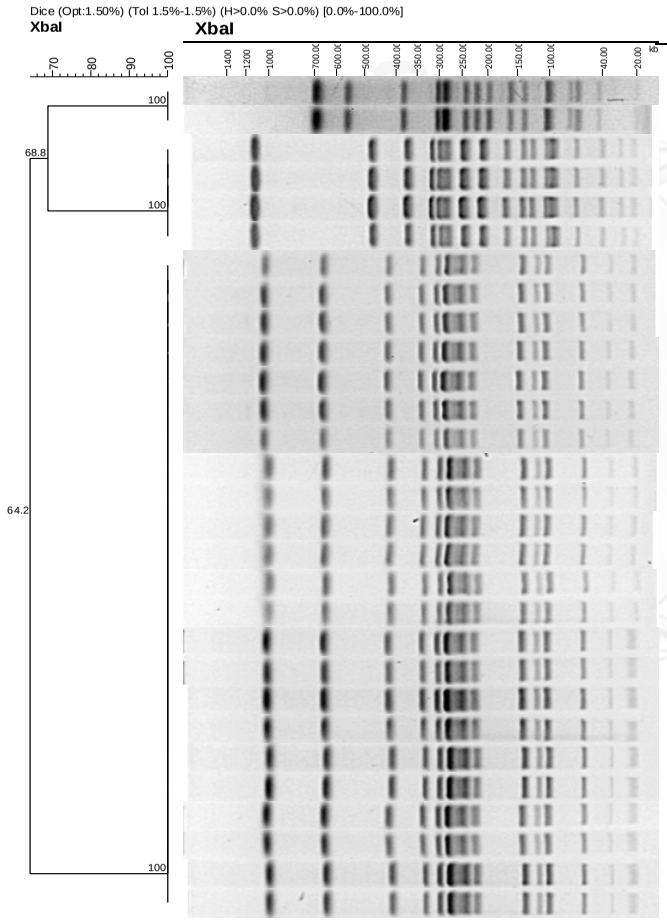
To infer source of *Salmonella* contamination, genotypic characterization and relatedness of the *Salmonella* isolates of the same serotype isolated from different sources was analyzed. A total of 202 *Salmonella* isolates sharing common serotype "between chicken-related sample and environmental sample" or "across different production units", were selected for pulse-field gel electrophoresis (PFGE).

In the first sampling, 6 serotypes which were *S.* Corvallis (4 isolates), *S.* Albany (15 isolates), *S.* Bovismorbificans (4 isolates), *S.* Derby (45 isolates), *S.* Paratyphi B (12 isolates) and *S.* Weltevreden (12 isolates), were selected for PFGE analysis. Although the *S.* Paratyphi B which contaminated only in the whole carcasses, this serotype was also selected for genetic characterization in order to investigate the diversity of this serotype in the whole carcasses. The dendrograms of *Salmonella* isolates were showed in Figures 4-5. The indistinguishable PFGE patterns by *Xba*I were then followed by *Avr*II restriction enzyme. The PFGE patterns were designated as "COIb", "ABIa", "BOI", "DEI" for *S.* Corvallis, *S.* Albany, *S.* Bovismorbificans and *S.* Derby, respectively (Figures 4-5). While, two PFGE patterns of *S.* Paratyphi B termed as "PBI" and "PBII" were identified from the isolates of whole carcasses (Figure 5). The PFGE result revealed that genetic diversity of *S.* Weltevreden was broader than that of other serotypes since six patterns of *S.* Weltevreden were identified from 12 isolates tested (Figure 6). Notably, all PFGE patterns of *S.* Weltevreden were obtained from lizard isolates. This result indicated the diversity of *S.* Weltevreden in the lizard.



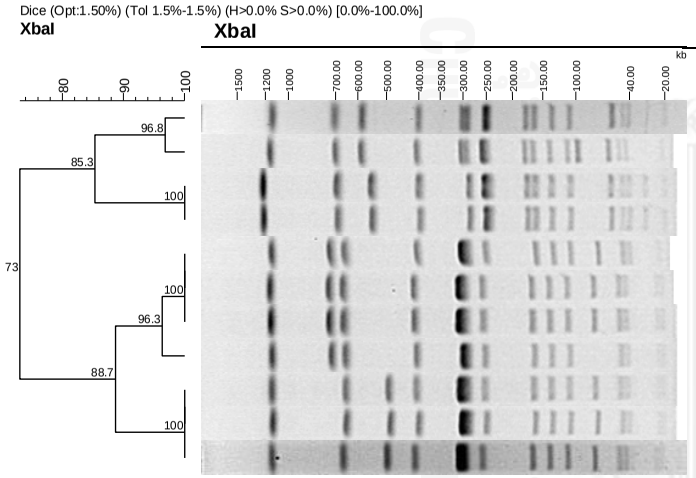
Serotype	Unit	Sample type	Stage	Pattern	
				PFGE	ABO
S. Corvallis	Hatchery	Egg storage room	After C&D	COIb	Susceptible
S. Corvallis	Hatchery	Hook	Before use	COIb	Susceptible
S. Corvallis	Hatchery	Stand	Before use	COIb	Susceptible
S. Corvallis	Breeding farm	Basket and plate	Before use	COIb	Susceptible
S. Albany	Broiler farm	Wall	After C&D	ABIa	AMP/C/NA/AML/SXT
S. Albany	Broiler farm	Pan feeder	After C&D	ABIa	AMP/C/NA/AML/SXT
S. Albany	Broiler farm	Water nipple	After C&D	ABIa	AMP/C/NA/AML/SXT
S. Albany	Broiler farm	Pest (lizard)	Down time	ABIa	AMP/C/NA/AML/SXT
S. Albany	Broiler farm	Water	Down time	ABIa	AMP/C/NA/AML/SXT
S. Albany	Breeding farm	Feces	55 weeks old	ABIa	AMP/C/NA/AML/SXT
S. Albany	Breeding farm	Feces	55 weeks old	ABIa	AMP/C/NA/AML/SXT
S. Albany	Breeding farm	Feces	55 weeks old	ABIa	AMP/C/NA/AML/SXT
S. Albany	Breeding farm	Feces	55 weeks old	ABIa	AMP/C/NA/AML/SXT
S. Albany	Breeding farm	Feces	55 weeks old	ABIa	AMP/C/NA/AML/SXT
S. Albany	Breeding farm	Egg tray	Before use	ABIa	AMP/C/NA/AML/SXT
S. Albany	Breeding farm	Egg tray	Before use	ABIa	AMP/C/NA/AML/SXT
S. Albany	Breeding farm	Egg tray	Before use	ABIa	AMP/C/NA/AML/SXT
S. Albany	Broiler farm	Feces	3 weeks	ABIa	AMP/C/NA/AML/SXT
S. Albany	Broiler farm	Boot swab	4 weeks	ABIa	AMP/C/NA/AML/SXT
S. Bovismorbificans	Broiler farm	Feces	3 weeks	BOI	Susceptible
S. Bovismorbificans	Broiler farm	Boot swab	4 weeks	BOI	Susceptible
S. Bovismorbificans	Broiler farm	Boot swab	6 weeks	BOI	Susceptible
S. Bovismorbificans	Slaughterhouse	Feather around cloaca	Slaughter day	BOI	Susceptible

Figure 4. Dendrogram of *S.* Corvallis, *S.* Albany and *S.* Bovismorbificans from the 1st sampling with *Xba*I restriction enzyme



Serotype	Unit	Sample type	Stage	Pattern	
				PFGE	ABO
S. Paratyphi B	Slaughterhouse	Whole carcass (rinse)	Slaughter day	PBI	LS/AMP/NA/AML/CN/CIP
S. Paratyphi B	Slaughterhouse	Whole carcass (rinse)	Slaughter day	PBI	LS/AMP/NA/AML/CN/CIP
S. Paratyphi B	Slaughterhouse	Whole carcass (rinse)	Slaughter day	PBII	LS/AMP/NA/AML/CN/CIP
S. Paratyphi B	Slaughterhouse	Whole carcass (rinse)	Slaughter day	PBII	LS/AMP/NA/AML/CN/CIP
S. Paratyphi B	Slaughterhouse	Whole carcass (rinse)	Slaughter day	PBII	LS/AMP/NA/AML/CN/CIP
S. Paratyphi B	Slaughterhouse	Whole carcass (rinse)	Slaughter day	PBII	LS/AMP/NA/AML/CN/CIP
S. Derby	Broiler farm	Feces	1 week	DEI	LS/DO/TE
S. Derby	Broiler farm	Boot swab	1 week	DEI	LS/DO/TE
S. Derby	Broiler farm	Feces	2 weeks	DEI	LS/DO/TE
S. Derby	Broiler farm	New feed	2 weeks	DEI	LS/DO/TE
S. Derby	Broiler farm	Boot swab	3 weeks	DEI	LS/DO/TE
S. Derby	Broiler farm	Feces	3 weeks	DEI	LS/DO/TE
S. Derby	Broiler farm	Pest (lizard)	3 weeks	DEI	LS/DO/TE
S. Derby	Broiler farm	Boot swab	4 weeks	DEI	LS/DO/TE
S. Derby	Broiler farm	Boot swab	4 weeks	DEI	LS/DO/TE
S. Derby	Broiler farm	Feces	4 weeks	DEI	LS/DO/TE
S. Derby	Broiler farm	Feces	4 weeks	DEI	LS/DO/TE
S. Derby	Broiler farm	Boot swab	5 weeks	DEI	LS/DO/TE
S. Derby	Broiler farm	Boot swab	5 weeks	DEI	LS/DO/TE
S. Derby	Broiler farm	Feces	5 weeks	DEI	LS/DO/TE
S. Derby	Broiler farm	Water	5 weeks	DEI	LS/DO/TE
S. Derby	Broiler farm	Boot swab	6 weeks	DEI	LS/DO/TE
S. Derby	Broiler farm	Boot swab	6 weeks	DEI	LS/DO/TE
S. Derby	Broiler farm	Feces	6 weeks	DEI	LS/DO/TE
S. Derby	Broiler farm	Feces	6 weeks	DEI	LS/DO/TE
S. Derby	Slaughterhouse	Whole carcass (rinse)	Slaughter day	DEI	LS/DO/TE
S. Derby	Slaughterhouse	Whole carcass (rinse)	Slaughter day	DEI	LS/DO/TE
S. Derby	Broiler farm	Feather around cloaca	Slaughter day	DEI	LS/DO/TE
S. Derby	Slaughterhouse	Feather around cloaca	Slaughter day	DEI	LS/DO/TE

Figure 5. Dendrogram of *S.* Paratyphi B and *S.* Derby from the 1st sampling with *Xba*I restriction enzyme



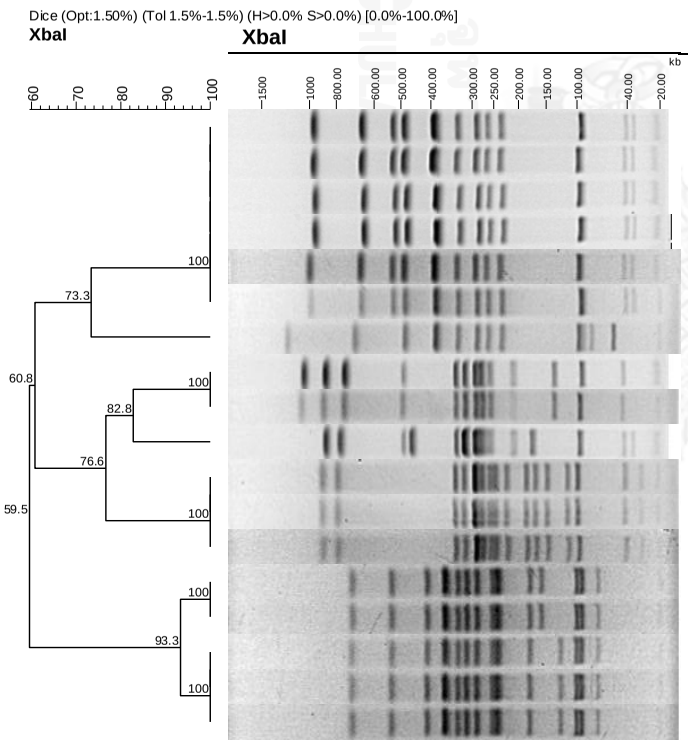
Serotype	Unit	Sample type	Stage	Pattern	
				PFGE	ABO
S. Weltevreden	Broiler farm	Pest (lizard)	1 weeks	.WEIIa2	Susceptible
S. Weltevreden	Broiler farm	Feces	2 weeks	.WEIIa3	Susceptible
S. Weltevreden	Broiler farm	Pest (lizard)	5 weeks	.WEIIb1	Susceptible
S. Weltevreden	Broiler farm	Pest (lizard)	5 weeks	.WEIIb1	Susceptible
S. Weltevreden	Broiler farm	Litter	Before disinfection	.WEIa1	Susceptible
S. Weltevreden	Broiler farm	Pest (lizard)	2 weeks	.WEIa1	Susceptible
S. Weltevreden	Broiler farm	Pest (lizard)	2 weeks	.WEIa1	Susceptible
S. Weltevreden	Broiler farm	Pest (lizard)	4 weeks	.WEIa2	Susceptible
S. Weltevreden	Broiler farm	Pest (lizard)	4 weeks	.WEIa3	Susceptible
S. Weltevreden	Slaughterhouse	Whole carcass (rinse)	Slaughter day	.WEIa3	Susceptible
S. Weltevreden	Slaughterhouse	Whole carcass (rinse)	Slaughter day	.WEIa3	Susceptible

Figure 6. Dendrogram of *S.* Paratyphi B and *S.* Derby from the 1st sampling with *Xba*I restriction enzyme

In the second sampling cycle, 5 serotypes which were *S.* Altona (7 isolates), *S.* Albany (3 isolates), *S.* Give (3 isolates), *S.* Agona (5 isolates), and *S.* Weltevreden (11 isolates) were selected for PFGE analysis. The dendrograms of *Salmonella* isolates were showed in Figures 7-8. *S.* Altona showed two PFGE *Xba*I macrorestriction patterns called "ATI" and "ATII" pattern. The same "ATI" pattern from the isolates of cage, hand swab (after work) and whole carcass indicated that the contaminated cage was a possible source for *Salmonella* contamination to whole carcasses. Furthermore, it was possible that the contaminated hand of worker could spread the *Salmonella* to other chicks during catching process. When, "ATI" pattern was further subtyped using the *Avr*II enzyme, two patterns were obtained (Figure 9). It turned out that PFGE pattern from cage was different from the same PFGE patterns from both hand swab and whole carcass. This result indicated that the cage might not play a significant role of the spread of *Salmonella* between the hand of workers and chicken carcasses. Moreover, the noticeably different PFGE pattern of *S.* Albany from the cage as "ABII" and from the chicken-related sample as "ABIb" at slaughterhouse re-confirmed that the cage might not be the major source of *Salmonella* contamination to the chicken before slaughter. However, the result of this study had not enough evidence to identify the source of *Salmonella* contamination for both the hand of workers and whole carcasses. For isolates of *S.* Give, the indistinguishable PFGE patterns termed as "GEI" was observed from the isolates of whole carcass (Figure 7). While, two PFGE patterns of *S.* Agona termed as "AGIa" and "AGIb" were identified from the isolates of whole carcass (Figure 7).

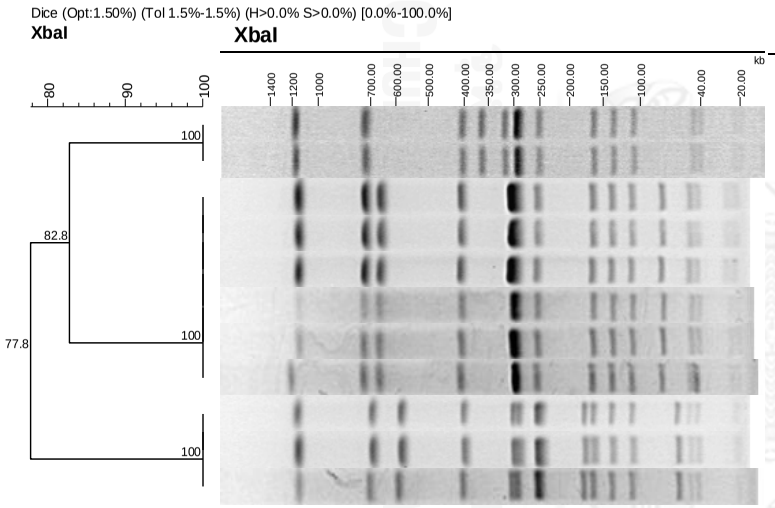
For *S.* Weltevreden, the shared PFGE pattern, "WEIa1", among isolates of lizard, litter after disinfection, boot swab was observed (Figure 8). This result showed

that the lizard was the important reservoir of *Salmonella* re-contamination of the clean litter after disinfection. In addition, the common PFGE pattern, “WEIb2”, between water nipple and cage (before use) was also found. This result indicated that the water supply in the broiler farm that was used for broiler drinking and equipment spraying (before entering the broiler farm), was the source of *Salmonella* contamination in the cage.



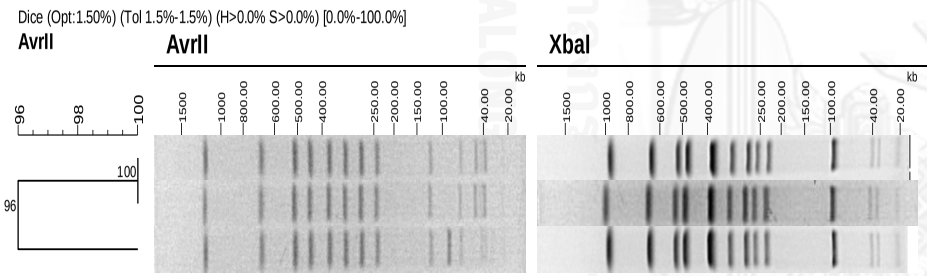
Serotype	Unit	Sample type	Stage	Pattern	
				PFGE	ABO
S. Altona	Broiler farm	Cage	Before use	ATI	LS/AMP/AML
S. Altona	Broiler farm	Cage	Before use	ATI	LS/AMP/AML/CN
S. Altona	Broiler farm	Cage	Before use	ATI	LS/AMP/AML/CN
S. Altona	Broiler farm	Hand swab	After work	ATI	LS/AMP/AML/CN
S. Altona	Slaughterhouse	Whole carcass (rinse)	Slaughter day	ATI	LS/AMP/AML/CN
S. Altona	Slaughterhouse	Whole carcass (rinse)	Slaughter day	ATI	LS/AMP/AML/CN
S. Altona	Slaughterhouse	Whole carcass (rinse)	Slaughter day	ATII	LS/AMP/AML/CN
S. Albany	Slaughterhouse	Cloacal swab	Slaughter day	ABIb	LS/AMP/NA/AML/CN
S. Albany	Slaughterhouse	Whole carcass (rinse)	Slaughter day	ABIb	LS/AMP/NA/AML/CN
S. Albany	Broiler farm	Cage	Before use	ABII	LS/AMP/AML/CN
S. Give	Slaughterhouse	Whole carcass (rinse)	Slaughter day	GEI	LS/AMP/NA/AML
S. Give	Slaughterhouse	Whole carcass (rinse)	Slaughter day	GEI	LS/AMP/NA/AML
S. Give	Slaughterhouse	Whole carcass (rinse)	Slaughter day	GEI	LS/AMP/NA/AML
S. Agona	Slaughterhouse	Whole carcass (rinse)	Slaughter day	AGIa	LS/AMP/AML/CN
S. Agona	Slaughterhouse	Whole carcass (rinse)	Slaughter day	AGIa	LS/AMP/AML/CN
S. Agona	Slaughterhouse	Whole carcass (rinse)	Slaughter day	AGIb	LS/AMP/AML/CN
S. Agona	Slaughterhouse	Whole carcass (rinse)	Slaughter day	AGIb	LS/AMP/AML/CN
S. Agona	Slaughterhouse	Whole carcass (rinse)	Slaughter day	AGIb	LS/AMP/AML/CN

Figure 7. Dendrogram of *S.* Altona, *S.* Albany, *S.* Give and *S.* Agona from the 2nd sampling with *Xba*I restriction enzyme



Serotype	Unit	Sample type	Stage	Pattern	
				PFGE	ABO
S. Weltevreden	Broiler farm	Nipple	On arrival day	WEIb2	Susceptible
S. Weltevreden	Broiler farm	Cage	Before use	WEIb2	Susceptible
S. Weltevreden	Broiler farm	Pest (lizard)	Down time	WEIa1	Susceptible
S. Weltevreden	Broiler farm	Litter	After disinfection	WEIa1	Susceptible
S. Weltevreden	Broiler farm	Litter	After disinfection	WEIa1	Susceptible
S. Weltevreden	Broiler farm	Boot swab	On arrival day	WEIa1	Susceptible
S. Weltevreden	Broiler farm	Pest (lizard)	On arrival day	WEIa1	Susceptible
S. Weltevreden	Broiler farm	Pest (lizard)	On arrival day	WEIa1	Susceptible
S. Weltevreden	Broiler farm	Pest (lizard)	Down time	WEIIa2	Susceptible
S. Weltevreden	Broiler farm	Pest (lizard)	Down time	WEIIa2	Susceptible
S. Weltevreden	Broiler farm	Pest (lizard)	On arrival day	WEIIa2	Susceptible

Figure 8. Dendrogram of *S.* Weltevreden from the 2nd sampling with *Xba*I restriction enzyme



Serotype	Unit	Sample type	Stage	ABO pattern
S. Altona	Broiler farm	Hand swab	After work	LS/AMP/AML/CN
S. Altona	Slaughterhouse	Whole carcass (rinse)	Slaughter day	LS/AMP/AML/CN
S. Altona	Broiler farm	Cage	Before use	LS/AMP/AML

Figure 9. Dendrogram of *S.* Altona from the 2nd sampling with *Xba*I and *Avr*II restriction enzyme

In the third sampling cycle, two main serotypes which were *S.* Corvallis (66 isolates), and *S.* Weltevreden (14 isolates), were identified for PFGE. The PFGE dendrogram was slightly adjusted with position tolerance setting with 1.1% optimization values and 1.1% band position tolerances in order to get the proper PFGE pattern for *S.* Weltevreden isolates. The dendrograms of these *Salmonella* isolates were shown in Figures 10-11.

For *S*. Corvallis, the majority of the isolates exhibited the same PFGE pattern (COIa2). This pattern has been found from the hatchery, broiler farm to the slaughterhouse. In hatchery, the COIa2 pattern was found in meconium, hand swab of worker (before and after work) and transporting belt. In the broiler farm, the COIa2 pattern was also found in meconium (box liner), cloacal swab of chicken (weeks 1, 2, 4, 5, 6), boot swab (weeks 1, 6), feed form pan feeder (weeks 1, 2, 3, 6), pest (weeks 2, 5) and water after chicken spraying. In the slaughterhouse, the isolates from cloacal swab and whole carcass were also showed the same PFGE pattern (COIa2). This result indicated that the main source of *Salmonella* contamination throughout the broiler production in this sampling cycle was originally disseminated from the hatchery to the slaughterhouse via broiler farm.

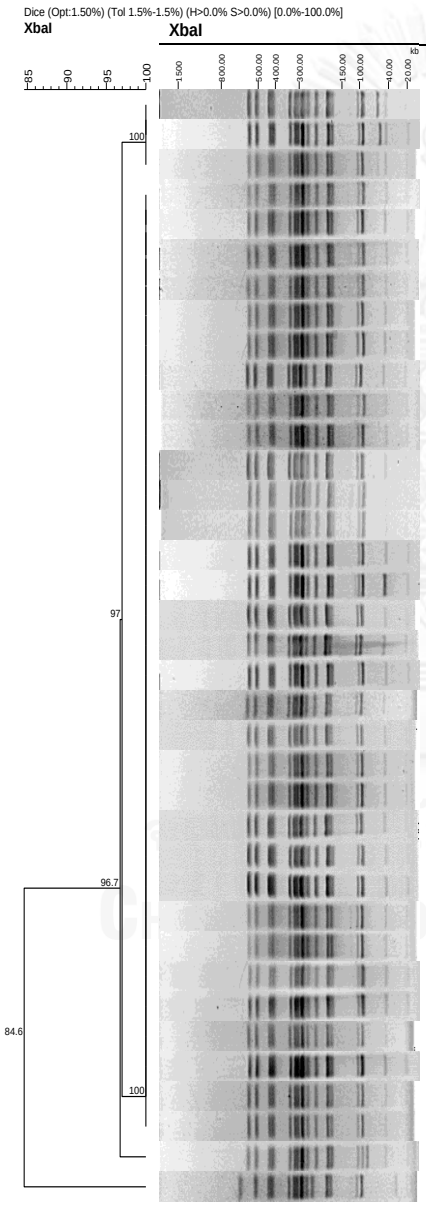
The contaminated belt and hand of worker (before working) indicated the lack of effective hygiene management in the hatchery thus causing *Salmonella* dissemination to the newly hatched chick. Then, the contaminated baby chicks were sent to the broiler farm, the horizontal transmission of *Salmonella* between chicken and its environment can cause the wide spread of *Salmonella* within the broiler farm and to the slaughterhouse. In addition to the hatchery which was identified as the important source of *S.* Corvallis in this production cycle, other minor sources of

S. Corvallis may co-existed and were indicated by 3 additional different PFGE patterns from the isolates of cloacal swab in weeks 1-3 (Figure 10).

For *S.* Weltevreden, similar to the first sampling cycle, high diversity of this serotype was observed since 7 PFGE patterns were identified. Although the pest, water, and litter were found positive for *S.* Weltevreden, the broiler was positive on only one occasion (week 5) with isolate of different PFGE pattern. It is possible that this serotype may not be able to compete with *S*. Corvallis that was already occupied in the contaminated chicks. Interestingly, the litter before and after disinfection were found to be contaminated with *Salmonella* of the same PFGE pattern. This result indicated that the disinfectant spray was not effective enough to disinfect *Salmonella* in the litter. For the water samples, two PFGE patterns were found thus indicating that the water supply possessed various subtypes of *S.* Weltevreden. However, no obvious evidence justified whether the source of *S.* Weltevreden in broiler chicken had been originated from contaminated litter or contaminated water.

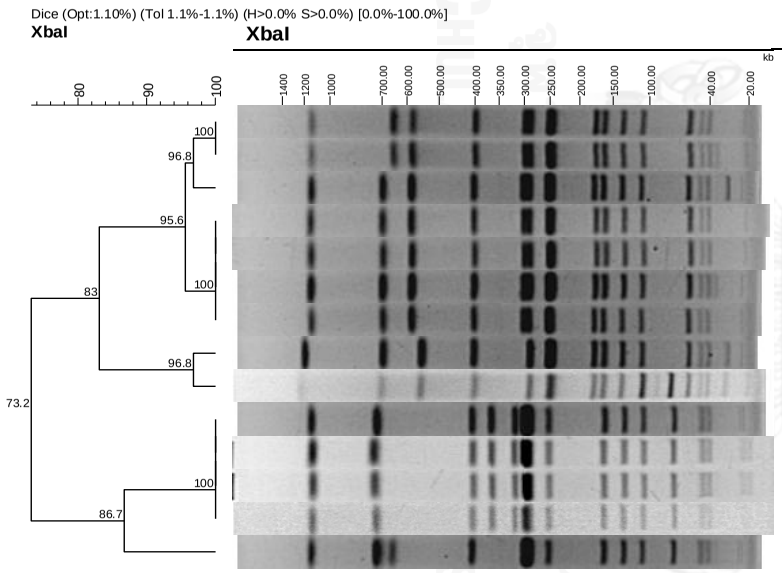
Interestingly, when comparing the PFGE patterns from the pest (lizard and centipede) found in all three sampling cycles, three indistinguishable PFGE patterns were found among the pest isolates (Figure 12). The shared PFGE patterns, “WEIIa2” and “WEIa1”, were found in the isolates of lizards from all three sampling cycles. Furthermore, the shared PFGE pattern, “WEIIb1”, between the lizard in the first sampling and the centipede in the third sampling was observed. This result indicated that the lizard and centipede could be the importance reservoir for *Salmonella* dissemination and transmission across the broiler flocks. In addition, a variety of PFGE

patterns was found in the lizard thus indicating the diversity of *S.* Weltevreden in this reservoir.



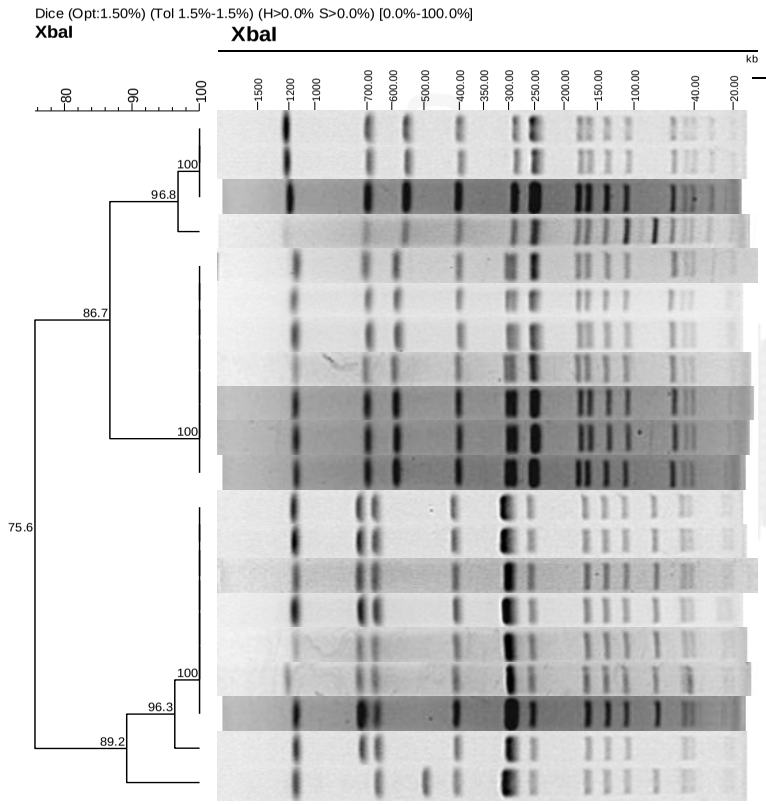
Unit	Sample type	Stage	Pattern	
			PFGE	ABO
Broiler farm	Cloacal swab	1 week	COIa1	LS/AMP/AML/CN
Broiler farm	Boot swab	2 weeks	COIa1	LS/AMP/AML/CN
Broiler farm	Cage	After use	COIa1	LS/AMP/AML/CN
Hatchery	Meconium	Hatching day	COIa2	LS/AMP/AML/CN
Hatchery	Meconium	Hatching day	COIa2	LS/AMP/AML/CN
Hatchery	Hand swab	Before work	COIa2	LS/AMP/AML/CN
Hatchery	Hand swab	Before work	COIa2	LS/AMP/AML/CN
Hatchery	Hand swab	After work	COIa2	LS/AMP/AML/CN
Hatchery	Hand swab	After work	COIa2	LS/AMP/AML/CN
Hatchery	Belt	Before use	COIa2	LS/AMP/AML/CN
Broiler farm	Meconium (box liner)	On arrival day	COIa2	LS/AMP/AML/CN
Broiler farm	Meconium (box liner)	On arrival day	COIa2	LS/AMP/AML/CN
Broiler farm	Cloacal swab	1 week	COIa2	LS/AMP/AML/CN
Broiler farm	Cloacal swab	1 week	COIa2	LS/AMP/AML/CN
Broiler farm	Boot swab	1 week	COIa2	LS/AMP/AML/CN
Broiler farm	Feed from pan	1 week	COIa2	LS/AMP/AML/CN
Broiler farm	Cloacal swab	2 weeks	COIa2	LS/AMP/AML/CN
Broiler farm	Cloacal swab	2 weeks	COIa2	LS/AMP/AML/CN
Broiler farm	Feed from pan	2 weeks	COIa2	LS/AMP/AML/CN
Broiler farm	Pest (lizards)	2 weeks	COIa2	LS/AMP/AML/CN
Broiler farm	Feed from pan	3 weeks	COIa2	LS/AMP/AML/CN
Broiler farm	Cloacal swab	4 weeks	COIa2	LS/AMP/AML/CN
Broiler farm	Cloacal swab	5 weeks	COIa2	LS/AMP/AML/CN
Broiler farm	Pest (lizards)	5 weeks	COIa2	LS/AMP/AML/CN
Broiler farm	Cloacal swab	6 weeks	COIa2	LS/AMP/AML/CN
Broiler farm	Boot swab	6 weeks	COIa2	LS/AMP/AML/CN
Broiler farm	Feed from pan	6 weeks	COIa2	LS/AMP/AML/CN
Broiler farm	Water after spray	Slaughter day	COIa2	LS/AMP/AML/CN
Broiler farm	Water after spray	Slaughter day	COIa2	LS/AMP/AML/CN
Slaughterhouse	Cloacal swab	Slaughter day	COIa2	LS/AMP/AML/CN
Slaughterhouse	Cloacal swab	Slaughter day	COIa2	LS/AMP/AML/CN
Slaughterhouse	Cloacal swab	Slaughter day	COIa2	LS/AMP/AML/CN
Slaughterhouse	Whole carcass (rinse)	Slaughter day	COIa2	LS/AMP/AML/CN
Slaughterhouse	Whole carcass (rinse)	Slaughter day	COIa2	LS/AMP/AML/CN
Slaughterhouse	Whole carcass (rinse)	Slaughter day	COIa2	LS/AMP/AML/CN
Broiler farm	Cloacal swab	2 weeks	COIa3	LS/AMP/AML/CN
Broiler farm	Cloacal swab	3 weeks	COII	LS/AMP/AML/CN

Figure 10. Dendrogram of *S.* Corvallis from the 3rd sampling with *Xba*I restriction enzyme



Serotype	Unit	Sample type	Stage	Pattern	
				PFGE	ABO
S. Weltevreden	Broiler farm	Water before spray	Slaughtering day	WEIIa1	Susceptible
S. Weltevreden	Broiler farm	Water after spray	Slaughtering day	WEIIa1	Susceptible
S. Weltevreden	Broiler farm	Cloacal swab	5 weeks	WEIIa4	Susceptible
S. Weltevreden	Broiler farm	Pest (lizard)	Down time	WEIIa2	Susceptible
S. Weltevreden	Broiler farm	Pest (lizard)	Down time	WEIIa2	Susceptible
S. Weltevreden	Broiler farm	Water	1 week	WEIIa2	Susceptible
S. Weltevreden	Broiler farm	Pest (lizard)	6 weeks	WEIIa2	Susceptible
S. Weltevreden	Broiler farm	Pest (centipede)	1 week	WEIIb1	Susceptible
S. Weltevreden	Broiler farm	Pest (lizard)	6 weeks	WEIIb2	Susceptible
S. Weltevreden	Broiler farm	Litter	Before disinfection	WEIb1	Susceptible
S. Weltevreden	Broiler farm	Litter	Before disinfection	WEIb1	Susceptible
S. Weltevreden	Broiler farm	Litter	After disinfection	WEIb1	Susceptible
S. Weltevreden	Broiler farm	Litter	After disinfection	WEIb1	Susceptible
S. Weltevreden	Broiler farm	Pest (centipede)	1 week	WEIa1	Susceptible

Figure 11. Dendrogram of *S.* Weltevreden from the 3rd sampling with *Xba*I restriction enzyme



Cycle	Serotype	Unit	Sample type	Stage	Pattern	
					PFGE	ABO
1	S. Weltevreden	Broiler farm	Pest (lizard)	5 weeks	WEIIb1	Susceptible
1	S. Weltevreden	Broiler farm	Pest (lizard)	5 weeks	WEIIb1	Susceptible
3	S. Weltevreden	Broiler farm	Pest (centipede)	1 week	WEIIb1	Susceptible
3	S. Weltevreden	Broiler farm	Pest (lizard)	6 weeks	WEIIb2	Susceptible
1	S. Weltevreden	Broiler farm	Pest (lizard)	1 weeks	WEIIa2	Susceptible
2	S. Weltevreden	Broiler farm	Pest (lizard)	Down time	WEIIa2	Susceptible
2	S. Weltevreden	Broiler farm	Pest (lizard)	Down time	WEIIa2	Susceptible
2	S. Weltevreden	Broiler farm	Pest (lizard)	On arrival day	WEIIa2	Susceptible
3	S. Weltevreden	Broiler farm	Pest (lizard)	Down time	WEIIa2	Susceptible
3	S. Weltevreden	Broiler farm	Pest (lizard)	Down time	WEIIa2	Susceptible
3	S. Weltevreden	Broiler farm	Pest (lizard)	6 weeks	WEIIa2	Susceptible
1	S. Weltevreden	Broiler farm	Pest (lizard)	2 weeks	WEIa1	Susceptible
1	S. Weltevreden	Broiler farm	Pest (lizard)	2 weeks	WEIa1	Susceptible
1	S. Weltevreden	Broiler farm	Pest (lizard)	2 weeks	WEIa1	Susceptible
2	S. Weltevreden	Broiler farm	Pest (lizard)	Down time	WEIa1	Susceptible
2	S. Weltevreden	Broiler farm	Pest (lizard)	On arrival day	WEIa1	Susceptible
2	S. Weltevreden	Broiler farm	Pest (lizard)	On arrival day	WEIa1	Susceptible
3	S. Weltevreden	Broiler farm	Pest (centipede)	1 week	WEIa1	Susceptible
1	S. Weltevreden	Broiler farm	Pest (lizard)	4 weeks	WEIa2	Susceptible
1	S. Weltevreden	Broiler farm	Pest (lizard)	4 weeks	WEIa3	Susceptible

Figure 12. Dendrogram of *S.* Weltevreden from all sampling cycle with *Xba*I restriction enzyme

4.5 Probability of contamination of *Salmonella* (P_{co} vs. P_{cn})

The probabilities of contamination in terms of occurrence (P_{co}) and number of microorganism (P_{cn}) along the broiler production chain in the first sampling cycle were shown in Tables 17. In the breeding farm, the probability of *Salmonella* contamination from the occurrence (P_{co}) in manure was 1.000. It can be interpreted that in every 60 pooled of manure samples were collected, the probability that *Salmonella* has been contaminated at least one pooled manure samples among the entire 60 pooled manure samples was 1.000. The highest probability of *Salmonella* contamination from the occurrence (P_{co}) was found in the manure samples in both the breeding farm and broiler farm. For the slaughterhouse, the highest P_{co}, 1.0, was found in the feather around cloaca and whole carcass samples. Whereas, the highest P_{cn} was found in the surface swab samples in the breeding farm (0.992) followed by manure samples (0.980) in the same production unit. In general, P_{co} was higher than P_{cn} among various samples except the environmental samples in the breeding farm. P_{co} in the second and third sampling cycle were shown in Tables 18-19.

Table 17. Probability of *Salmonella* contamination (P_{co} vs. P_{cn}) in the 1st sampling

Production unit	Sample type	*Salmonella* by occurrence			*Salmonella* by number		
		Prevalence (%) *(l)*	Sample size *(N)*	P_{co}	Concentration (log MPN/unit) *(m)*	Unit of volume *(q)*	P_{cn}
Breeding farm	Chicken related						
	Manure	90.91	60	1.000	3.90	g	0.980
	Egg	5.88	30	0.829	0.46	egg	0.370
	Environment						
	Surface swab*	58.33	5	0.946	4.81	m^2	0.992
Hatchery	Chicken related						
	Egg	2.94	30	0.586	0.46	egg	0.370
	Environment						
	Surface swab**	10	15	0.777	0.73	m^2	0.518
Broiler farm	Chicken related						
	manure	66.67	60	1.000	2.17	g	0.886
	Environment						
	Surface swab***	64.71	5	0.961	1.61	m^2	0.800
	Litter (before)	42.86	3	0.724	0.47	g	0.374
	Litter (after)	14.29	3	0.349	0.46	g	0.370
	Feed	8.57	3	0.227	0.52	g	0.408
	Water	25.00	3	0.528	0.21	ml	0.192
	Pest	42.31	5	0.879	1.77	pest	0.829
Slaughterhouse	Chicken related						
	Before slaughter	66.67	60	1.000	2.02	g	0.867
	After slaughter	91.67	20	1.000	2.62	carcass	0.927

*Surface swab: egg tray, basket and plate

**Surface swab: room swab, stand, hook, hand swab, trolley, water spray, egg illuminating plate, egg transferring plate, hatching tray, chick box, belt, truck

***Surface swab: wall, floor, pan feeder, water nipple and cup

Table 18. Probability of *Salmonella* contamination (P_{co}) in the 2nd sampling cycle

Production unit	Sample type	*Salmonella* by occurrence		
		Prevalence (%) *(I)*	Sample size *(N)*	P_{co}
Breeding farm	Chicken related			
	Manure	0.82	60	0.388
	Egg	1.92	30	0.438
	Environment			
	Surface swab*	2.50	5	0.118
Hatchery	Chicken related			
	Egg	1.35	30	0.333
	Environment			
	Surface swab**	2.94	15	0.357
Broiler farm	Chicken related			
	manure	0.27	60	0.149
	Environment			
	Surface swab***	1.96	5	0.093
	Litter (before)	8.33	3	0.221
	Litter (after)	25.00	3	0.528
	Feed	2.17	3	0.063
	Water	2.50	3	0.072
	Pest	26.47	5	0.734
	Fomite (slaughter day)****	33.33	5	0.811
Slaughterhouse	Chicken related			
	Before slaughter	4.84	60	0.945
	After slaughter	77.27	20	1.000

*Surface swab: egg tray, basket and plate, boot swab, belt, hand swab

**Surface swab: room swab, stand, hook, hand swab, water spray, hatching tray, belt

***Surface swab: wall, floor, pan feeder, water nipple and cup

****Fomite : cage, truck, hand swab, water before spraying

Table 19. Probability of *Salmonella* contamination (P_{co}) in the 3rd sampling cycle

Production unit	Sample type	*Salmonella* by occurrence		
		Prevalence (%) *(I)*	Sample size *(N)*	P_{co}
Breeding farm	Chicken related			
	Manure	0.82	60	0.388
	Egg	2.38	30	0.510
	Environment			
	Surface swab*	2.63	5	0.123
Hatchery	Chicken related			
	Egg	14.71	30	0.988
	Environment			
	Surface swab**	16.22	15	0.912
Broiler farm	Chicken related			
	manure	31.99	60	1.000
	Environment			
	Surface swab***	4.76	5	0.212
	Litter (before)	33.33	3	0.632
	Litter (after)	50.00	3	0.777
	Feed	28.26	3	0.572
	Water	5.00	3	0.139
	Pest	18.60	5	0.606
	Fomite (slaughter day)****	6.90	5	0.292
Slaughterhouse	Chicken related			
	Before slaughter	25.81	60	1.000
	After slaughter	85.71	20	1.000

*Surface swab: egg tray, basket and plate, boot swab, belt, hand swab

**Surface swab: room swab, stand, hook, hand swab, water spray, hatching tray, belt

***Surface swab: wall, floor, pan feeder, water nipple and cup

****Fomite : cage, truck, hand swab, water before spraying

4.5.1 Comparison between prevalence and concentration variables

Ranking between prevalence-based probability of contamination (P_{co}) and concentration-based probability of contamination (P_{cn}) was shown in Tables 20-21. In terms of occurrence, P_{co} were highest in the surface swab after cleaning and disinfection (0.961) followed by pest (0.879), water (0.528) and feed (0.227) (Table 20). Whereas, in terms of concentration, P_{cn} were highest in pest (0.829) followed by the surface swab after cleaning and disinfection (0.800), feed (0.408) and water (0.192) (Table 21). This result indicated that the orders of P_{co} and P_{cn} were different depending on prevalence or concentration of *Salmonella* in various sources. Therefore, the prevalence *per se* may be not enough to profoundly illustrate the magnitude of *Salmonella* contamination in the broiler production.

Table 20. The probability of *Salmonella* contamination (P_{co}) in the broiler production unit

Variable	Source	P_{co}
Prevalence	Surface swab (after C&D*)	0.961
	Pest	0.879
	Water	0.528
	Feed	0.227

* cleaning and disinfection

Table 21. The probability of *Salmonella* contamination (P_{cn}) in the broiler production unit

Variable	Source	P_{cn}
Concentration	Pest	0.829
	Surface swab (after C&D*)	0.800
	Feed	0.408
	Water	0.192

* cleaning and disinfection

4.5.2 Probabilistic model of *Salmonella* contamination among multiple sources in the broiler farm

Probabilities of finding *Salmonella* in at least one source among the various sources (P_+) along a chronological sampling in the broiler farm were shown in Table 22. For the uncertainty of P_+ was shown in Table 23. In the first week of sampling, the highest probability of *Salmonella* contamination (P_{co}) was found in day-old chick sample corresponding to its correlation coefficient. However, during the rearing period (weeks 2-6), the highest probability of *Salmonella* contamination was found in pest samples in weeks 2-6. Among various sources of *Salmonella* during the rearing period, the pest possessed the highest correlation coefficient in every week of sampling. This result indicated that the pest was a constant and significant source of *Salmonella* contamination and dissemination in the environment of the broiler farm. The overall range of P_+ was between 0.704 and 1.000. The highest P_+ (1.0) was found in the first week of sampling across three sampling cycles (Table 22). It can be interpreted that among multiple sources of *Salmonella* contamination during the first week, i.e., day-old chick, pest, litter (after disinfection), feed, the persistence of

Salmonella after cleaning and disinfection and water, P_+ among all these multiple sources was extremely high. In addition, the status of *Salmonella* in day-old chick also played a significant role of *Salmonella* contamination in the first week.

Compared with all possible sources of *Salmonella* contamination in the broiler farm, the highest P_{co} was undoubtedly found in day-old chick followed by pest, litter (after disinfection) and contaminated equipment and environment on slaughter day (fomite). In addition, the highest correlation coefficient was found in day-old chick, pest, litter and fomite. This result indicated that the day-old chick was a highly significant source of *Salmonella* contamination in the broiler farm.

Table 22. Probability of finding *Salmonella* among the multiple sources in the broiler farm (all three sampling cycles)

Sampling time	Source	Pco	Correlation coefficient	P+
Week 1	Day-old chick	1.000	0.99	1.000
	Pest	0.689	0.07	
	Litter (after disinfection)	0.589	0.07	
	Feed	0.404	0.04	
	C&D*	0.339	0.02	
	Water	0.259	0.04	
Week 2	Pest	0.713	0.82	0.921
	Feed	0.664	0.46	
	Water	0.181	0.26	
Week 3	Pest	0.586	0.85	0.742
	Feed	0.239	0.33	
	Water	0.181	0.34	
Week 4	Pest	0.586	0.88	0.704
	Water	0.181	0.33	
	Feed	0.127	0.24	
Week 5	Pest	0.947	0.89	0.969
	Water	0.329	0.37	
	Feed	0.127	0.16	
Week 6	Pest	0.811	0.82	0.964
	Fomite**	0.586	0.33	
	Feed	0.430	0.33	
	Water	0.193	0.20	
Overall	Day-old chick	1.000	0.99	1.000
	Pest	0.731	0.04	
	Litter (after disinfection)	0.589	0.04	
	Fomite**	0.586	0.04	
	C&D*	0.339	0.03	
	Feed	0.306	0.02	
	Water	0.102	0.01	

* surface swab of wall, floor, pan feeder and water nipple and cup
**surface swab of cage, truck, water (before spraying) and hand swab (before working)

Table 23. Statistical values of probability of finding *Salmonella* among the multiple sources in the broiler farm

Sampling time	Statistical value				
	Minimum	5^{th} percentile	Mean	95^{th} percentile	Maximum
Week 1	0.999	0.999	1.000	1.000	1.000
Week 2	0.492	0.794	0.905	0.974	0.995
Week 3	0.172	0.452	0.708	0.900	0.983
Week 4	0.101	0.395	0.668	0.883	0.973
Week 5	0.738	0.909	0.962	0.989	0.998
Week 6	0.683	0.878	0.952	0.991	0.998
All week	0.998	0.999	1.000	1.000	1.000

4.5.3 Probabilistic model of *Salmonella* contamination in chicken-related samples across the production unit

P_+ in chicken-related samples among the different production unit was shown in Table 24. The highest P_{co} in the chicken-related samples was found in whole chicken carcasses at the slaughterhouse. Whereas, the highest correlation coefficient was found in the feather around cloaca or cloacal swab samples before slaughter.

Table 24. Probability of finding *Salmonella* in chicken-related samples among the different production unit

Production unit (Type of sample)	Pco	Correlation coefficient	P+
Breeder (egg)	0.244	0.05	1.000
Hatchery (egg or meconium)	0.663	0.10	
Broiler (feces or cloacal swab)	0.999	0.16	
Pre-slaughter subunit (feather or cloacal swab)	0.999	0.35	
Slaughterhouse (whole carcass)	1.000	0.22	

For the egg samples in the breeder production unit, the lowest P_{co} corresponded to the lowest correlation coefficient in this study. This result indicated that the vertical transmission was not supposed to be the main route for *Salmonella* contamination and transmission to the broiler in subsequent production units in this integrated broiler production.

CHAPTER V
DISCUSSION

The result of this study presented comprehensive information regarding prevalence and concentration of *Salmonella* in an integrated broiler production throughout a series of production units starting from a breeding farm, a hatchery, a broiler farm to a slaughterhouse. The *Salmonella* isolated from both chicken-related and environmental samples were characterized for both genetic and antimicrobial susceptibility profiles in order to reveal special and temporal relationship between isolates. The level and extent of *Salmonella* contamination throughout this series of production units were utilized as input variables for the probabilistic model in order to describe the dynamic of *Salmonella* contamination in the chicken and their environment along the broiler production.

5.1. Prevalence and concentration of *Salmonella*

In this study, the overall *Salmonella* prevalences in the breeding farm, the hatchery and the slaughterhouse were 3.4%, 5.7% and 37.4%, respectively. These values were lower than previous studies that reported as high as 100% prevalence in the breeder flock (Sasipreeyajan et al., 1996), 16.6% in the hatchery (Mulika and Yuwapanichsampan, 2008) and 42% in the slaughterhouse in Thailand (Padungtod and Kaneene, 2006). In contrast to other production units, the broiler farm in this study showed higher prevalence of *Salmonella* than previously reported (Padungtod and Kaneene, 2006). These discrepancies might be depended on many factors such as different management practices, sampling time, sample type, sampling area and

sample size. Therefore these factors played an important role to determine the prevalence among production units.

In terms of magnitude of *Salmonella* contamination in the broiler farm, the mean *Salmonella* concentrations in the chicken manure were between 1.81 and 4.03 log MPN/g during the rearing period. The highest mean concentration of *Salmonella* in chicken manure was observed at the age of two weeks. This finding was likely attributable to the intermittent shedding of *Salmonella* after chicken had acquired *Salmonella* from the environment. A previous study showed that the intermittent long-term shedding of *Salmonella* can be found in the chicken after infected with low dose of *S.* Enteritidis (Van Immerseel et al., 2004). Moreover, it is possible that the vaccination program, which was commonly done at the age of two weeks of the chicken, can somehow elicit the stress condition and augment *Salmonella* shedding in the manure. Among the environmental samples, the highest levels of *Salmonella* contamination during rearing period were found in the boot swabs between 3.93 and 5.21 log MPN/pair. This finding suggested that the boot swab better represented the magnitude of *Salmonella* in the broiler flock status than manure. It was possible that the boot swab might collect fecal material from many chickens in the house compared to the manure which was taken from few chickens in the house. After slaughter process, the result of this study showed that the *Salmonella* prevalence in whole chicken carcasses increased whereas the *Salmonella* concentration decreased. The enhancement of *Salmonella* prevalence in the slaughterhouse may be as a result of the cross contamination from the slaughter equipment or the transport crates, which were reported to be the main cause for *Salmonella* contamination in chicken carcasses (Rasschaert et al., 2008). However, the reduction of *Salmonella*

concentration indicated that steps in the slaughter process can somehow get rid of the *Salmonella* load in the chicken carcasses. A previous study reported that the *Salmonella* concentration in broiler carcasses were gradually decreased in the processing steps such as evisceration (1.56 log MPN/carcass), washing (<1.53 log MPN/carcass) and chilling (<1.08 log MPN/carcass) (Svobodová et al., 2012).

5.2 Serodiversity and genetic diversity of *Salmonella*

Various *Salmonella* serotypes were found in this study. Among the 27 serotypes found in various production units, the most prevalence serotypes were *S.* Corvallis. This finding was different from other studies that reported *S.* Blockley and *S.* Weltevreden were the most prevalence serotypes contaminated in broiler farm in Thailand (Sasipreeyajan et al., 1996; Padungtod and Kaneene, 2006). This difference may be associated with the differences of the sampling time and the sampling location. In addition, a vast variety of *Salmonella* serotypes in the slaughterhouse was observed in this study. This serodiversity implied that the cross-contamination in the slaughterhouse was the main source for *Salmonella* contamination in chicken carcasses (Rasschaert et al., 2008).

Nowadays, many molecular typing methods for *Salmonella* subtyping such as multilocus sequence typing (MLST), multiple locus VNTR analysis (MLVA) are available with different discriminatory powers (Foley et al., 2009; Stepan et al., 2011; Wattiau et al., 2011). However, PFGE technique using both *Xba*I and *Avr*II restriction enzymes combined has been shown to be a promising and suitable tool for studying the genetic characterization of *Salmonella* in present study. In the breeder production unit, the identical PFGE patterns of *S.* Albany were observed from both

manure and egg trays (before using) thus indicating that cleaning and disinfection (C&D) of the equipment in this production unit was not adequate. Furthermore, it was very possible that pests commonly found in the breeding farm such as rodents, lizards, cockroaches, house flies and insects were the reservoir for *Salmonella* recontamination to the breeding farm equipment. Unfortunately, all pests were not found during the sampling in this study, only lizard and rodent manures were found in the housing area. For the egg samples which were also collected in the breeding farm, no *Salmonella* was found. Therefore, it can be inferred that the vertical transmission was not the main route of *Salmonella* transmission in this integrated broiler production.

In the hatchery, *S.* Corvallis was the predominant serotype isolated from the chicken-related samples and environmental samples. The identical PFGE patterns were found between the meconium on the hatching tray, hand swab (before work and after work) and transferring belt (in the third sampling cycle) indicating that the contaminated hatchery environment and equipment were the main sources of *Salmonella* dissemination and contamination in the newly hatched chicks. A previous study revealed that the contaminated environment and equipment such as egg trolleys and trays in the hatchery could lead to the widespread dissemination of *Salmonella* within an integrated poultry organization (Davies et al., 1997). In addition, the identical PFGE patterns of *S.* Corvallis isolated from the hatchery, from the broiler farm, and from the whole carcasses in the slaughterhouse production unit indicating that the contaminated chicks from the hatchery play an important role in *Salmonella* spreading to the subsequent production units.

In the broiler farm of the first sampling cycle, an indistinguishable PFGE pattern of *S.* Albany between the environmental isolates (wall, pan feeder, water, water nipple, lizard, and boot swab) especially from inlet water at the farm preparation step and the chicken-related isolate (manure in week 3) indicated that the contaminated water supply may be the source of *Salmonella* contamination in the broiler house and its equipment during the cleaning and disinfection step. In the same sampling cycle, an identical PFGE pattern between the isolates of *S.* Derby found in the environmental samples, especially in water supply and new feed and the isolates found in the chicken-related samples indicated that the water supply and new feed were the likely sources of *Salmonella* infection in the broiler. Although the *Salmonella* was found in the water supply and new feed after the chickens were positive, the water and feed supply could not be ruled out from the list of potential sources of *Salmonella* contamination. The discrepancy of the detection time could be explained by intermittent nature of the contamination and adequacy of the sampling procedure.

For the water samples, various serotypes of *Salmonella* (*S.* Albany and *S.* Derby in the first sampling cycle, *S.* Weltevreden and *S.* Falkensee in the second sampling cycle and *S.* Weltevreden in the third sampling cycle) were found in several circumstances. This finding indicated that the water supply was repeatedly contaminated with a mixture of *Salmonella* serotypes. Therefore, it was highly recommended to check and monitor the efficiency of water chlorination in the broiler production units. Similarly, for the feed sample, adequate heat treatment and hygienic practice during the feed production should be emphasized.

In the third sampling cycle, an identical PFGE pattern of *S.* Corvallis was found in three production units, from the hatchery, the broiler farm and chicken carcasses at the slaughterhouse. The persistent of this specific *S.* Corvallis pattern indicated that the horizontal transmission was the main route of *Salmonella* contamination starting from the day-old chicks in the hatchery to the chicken carcasses after slaughter. This finding was similar to a previous study revealing that the serotype and PFGE patterns of *Salmonella* isolates from positive flocks in the broiler farm were the same as the isolates from gastrointestinal tracts and neck skins in the slaughterhouse (Rasschaert et al., 2008).

The present study also revealed high genetic diversity of *S.* Weltevreden isolates from the broiler farm production unit. For the pest (lizard), more than five PFGE patterns were found across the sampling cycles indicating the genetic diversity of this serotype in this host. Moreover, the indistinguishable PFGE pattern of *S.* Weltevreden among the lizards from different sampling cycles indicated that lizard can act as a reservoir for the *Salmonella* serotype which can persist in the environment and also can transmit the *Salmonella* within and between broiler production cycles. In the first sampling cycle, identical PFGE pattern of the isolates from the litter (before disinfection) and the isolates from the lizard at week 2 indicated that the litter could be the source of the *Salmonella* contamination and transferred the bacterium to the lizard or vice versa. Moreover, PFGE pattern from the lizard (week 4) was in common with that from the whole carcass indicated that the lizard could also acted as the source of *Salmonella* contamination in the chicken carcasses. In the second sampling cycle, the same PFGE pattern of *S.* Weltevreden was found in pest (lizard), litter (after disinfection) and boot swab (on

chick arrival day) indicating that the lizard can be the source for *Salmonella* recontamination after disinfection. Moreover, the lizard can transmit *Salmonella* to the water source which was revealed by the indistinguishable PFGE pattern in the third sampling cycle. Interestingly, a slight difference of PFGE pattern between lizard and cloacal swab at week 5 was observed. The different but closely related genetic pattern of the isolates from the two sample types cannot rule out the pest as a source of the serotype in the chicken. The slight change in the genetic pattern may be resulted from single genetic event such as point mutation, insertion, deletion, or acquiring of new plasmid that could easily be happened when the bacterium was passing through the chicken host.

On the slaughter day, many studies concluded based on serotyping results that the transport cage was a possible source for *Salmonella* contamination to the chicken carcass (Slader et al., 2002; Rasschaert et al., 2008). However, the present study revealed that although the serotypes found in the transport cages and the chicken carcasses were the same, the PFGE patterns within the serotype were different. This result indicated that the role of transport cage in *Salmonella* dissemination to the broiler carcass may be less profound than previously thought.

In the slaughterhouse, several serotypes such as *S.* Bovismorbificans, *S.* Derby (in the first sampling), and *S.* Corvallis (in the third sampling) with the same PFGE patterns were found from the isolates from previous broiler production units and the isolates from the chicken carcasses in the slaughterhouse. This result was the evidence confirming that *Salmonella* in the broiler farm can persist and then transmit to the chicken carcasses in the slaughterhouse. In addition, there were serotypes which had never been found in any prior production units but were

detected in the whole carcasses. This result indicated that the cross-contamination during the slaughter process was another explanation for *Salmonella* contamination in the broiler carcasses.

5.3 Antimicrobial resistance of *Salmonella*

In this study, different classes of antimicrobial drugs commonly used in the poultry farm and used as therapeutic agents in human were selected for antimicrobial susceptibility testing. Although CLSI and EUCAST guidelines recommended either ampicillin or amoxicillin for antimicrobial resistance monitoring, both antibiotics were tested in this study in order to cover all drugs which were commonly used in the poultry farm. The results of this study showed that the antimicrobial resistance of *Salmonella* was found in all units of the broiler production. The most common pattern among the *Salmonella* isolates in this study was Lincomycin-spectinomycin resistance. This result was not unexpected since lincomycin-spectinomycin has been used frequently in broiler farms (Persoons et al., 2012). Additionally, an extra-label use of lincomycin-spectinomycin was recommended for treating *E. coli* infection (Agunos et al., 2012) in the broiler. Therefore, these practices could promote the antimicrobial resistance of bacteria especially *Salmonella* which was commonly found in the poultry farm.

Furthermore, high proportions of *Salmonella* isolates with resistance to ampicillin and amoxicillin were also found in this study. Despite administration of the drug by human, the ampicillin resistant trait could be a result of natural selection process (Rosser and Young, 1999; Bani et al., 2007). This finding is in agreement with some previous study indicating that 56.9% and 55.2% of *Salmonella* isolates in the

conventional broiler farm were resistant to ampicillin and amoxicillin-clavulanic acid, respectively (Alali et al., 2010). In Thailand, the prevalence of amoxicillin resistance in this study was obviously contradicted that of amoxicillin in a previous study where amoxicillin resistance was not even detected in the poultry farm (Padungtod and Kaneene, 2006). This substantial dissimilarity may be associated with some factors such as the geography of sampling location (northeastern in this study versus northern in previous study), types of antibiotic recommended in individual poultry farm and analytical methods for antimicrobial susceptibility testing (disk diffusion method in this study versus microbroth dilution method in previous study).

Approximately 40.5% of *Salmonella* isolates in this study were resistant to gentamicin. This prevalence was higher than that reported from previous studies in Thailand (Chuanchuen et al., 2008; Mulika and Yuwapanichsampan, 2008). This result might reflect the long-term and routine utilization of gentamicin particularly in this broiler production. This antimicrobial agent has been directly administered into the hatching eggs or day-old chick against *Mycoplasma* and bacterial contaminations (McEwen and Fedorka-Cray, 2002). Moderate level of tetracycline (22.3%) and doxycycline (21.8%) resistance were reported in the present study. This finding agreed with the resistance rates, which were 24.3% and 22.6%, respectively of a previous study (Mulika and Yuwapanichsampan, 2008).

Although chloramphenicol has been prohibited in the production of food animals since 1984 (Payne et al., 1999) because of the adverse effect to human health, the chloramphenicol residue in the broiler meat could be found in many countries such as Korea (14.1%), Brazil (6%), Australia (0.1-1.5%) and even Thailand (0.9-54.5%) (Boonmar et al., 1998; Cheong et al., 2007; Page, 2009; Medeiros et al.,

2011). In this study, 9.5% of the isolates resistant to chloramphenicol which was slightly lower than chloramphenicol resistance in a previous result conducted in a broiler farm (Chuanchuen et al., 2008). In fact, all chemical agents used for either sanitation or animal health, must be approved and tightly controlled by veterinarian authority in this integrated broiler production. However the microorganism could also multiply or persist in the presence of different classes of antimicrobial agents due to possession of various resistance mechanisms so-called “co-resistance”. Likewise, a microorganism could somehow multiply or persist in the presence of other members of a particular class of antimicrobial agents or across different classes due to a shared mechanism of resistance so-called “cross-resistance”. These two mechanisms of antimicrobial resistance could possibly explain the resistance of chloramphenicol taking place in this integrated broiler production. An earlier evidence showed that the cross-resistance between biocides and chloramphenicol could be found in *Salmonella* isolates (Braoudaki and Hilton, 2004). Therefore, choices of biocides used in broiler farm should be carefully selected to avoid accumulation of the chloramphenicol resistant bacteria.

For nalidixic acid, the resistance from this study was relatively lower than that of some previous studies reported in Thailand (Boonmar et al., 1998; Padungtod and Kaneene, 2006; Mulika and Yuwapanichsampan, 2008). On the other hand, low levels of ciprofloxacin resistance was found (2.7%), which was in agreement with a previous study reported in poultry farms (1.6%) (Chuanchuen et al., 2008).

Multidrug-resistant (MDR) *Salmonella* in chicken meat has been reported in many countries such as Korea (87.2%), Spain (65.4), and Japan (94.8) (Carraminana et al., 2004; Shahada et al., 2006; Kim et al., 2012). In Thailand, the high proportion of

MDR in the poultry isolates were also reported (Boonmar et al., 1998; Padungtod and Kaneene, 2006; Chuanchuen et al., 2008). In this study, the highest proportion of the multidrug-resistant isolates was found in breeding farm (92.9%), followed by slaughterhouse (78%), hatchery (71.4%) and broiler farm (36.6%). The high proportion of the MDR isolates in the breeding farm may be the effect of the longer period of antimicrobial use in this production unit. Long-term exposure to many different drugs of the breeders can provide selective advantage for the MDR isolates to survive and become the major population over the sensitive isolates.

Next, the noticeable proportion of multidrug resistance in the slaughterhouse manifested the likely cross-contamination of *Salmonella* during the slaughter process. Then the spread of the multidrug-resistant isolates to other carcasses was inevitable. A variety of antibiotic resistance patterns that are commonly found in the slaughterhouse emphasize the cross-contamination of the resistance isolates during the slaughter process.

5.4 Probabilistic model of *Salmonella*

The probabilistic approach was used to describe the uncertainty and variability of the prevalence and concentration of *Salmonella* contamination in the broiler production in the present study. This approach inherently provided a broader information than a deterministic (a point estimate) approach (FAO/WHO, 2008b). Therefore, the result of this study provided a comprehensive range of useful information in order to determine the exposure assessment of *Salmonella* contamination in the integrated broiler production in the future.

Although the qualitative *Salmonella* assay was routinely used for *Salmonella* detection (Carrique-Mas and Davies, 2008), the information is not enough to reveal the magnitude of *Salmonella* contamination. The quantitative value can help prioritize management resources to the most significant source of the *Salmonella* contamination. Therefore, this study aimed at comparing the information on qualitative approach in terms of the probability of *Salmonella* occurrence in a sample unit (P_{co}) and the quantitative approach in terms of the probability of *Salmonella* present in an analytical sample unit (P_{cn}) in order to determine whether prevalence *per se* was enough to explain the level of *Salmonella* contamination in the broiler production. However, the result of this study could not locate the association between P_{co} and P_{cn} in the broiler production.

Since the limitation of concentration in this study which may not be able to enumerate the true concentration of *Salmonella* in the sample. Especially, when the low percentage of *Salmonella* positive samples was found, the further enumeration on a small number of positive samples may not represent of the real range of *Salmonella* concentrations. Therefore, in order to compare the association between prevalence variables and concentration variables, larger sample sizes might warrant the true ranges of *Salmonella* concentrations. Nevertheless, according to the result of this study, the risk assessors and risk managers should simultaneously consider both the qualitative and quantitative data in order to weigh, select, and implement some appropriate *Salmonella* control measures in the broiler production unit.

To date, the probabilistic model in the primary production chain was limited. Some studies focused only on transmission of *Campylobacter* within a chicken flock without considering the source of *Campylobacter.* This may influence the result of

the model (Hartnett et al., 2001). Even though a previous study developed a probabilistic model describing transmission of *Salmonella* in the primary broiler production chain, this model cannot be directly used in the countries where high prevalence of *Salmonella* has been recognized (Ranta and Maijala, 2002). Therefore, the aim of this study was to describe the dynamic of *Salmonella* existence among the various sources in the broiler farm with high prevalence of *Salmonella*. The probability of finding *Salmonella* among various sources as an output from the model simulation was chronologically compared throughout the broiler production. The result of our model found that the day-old chicks play the most significant role for *Salmonella* contamination in the first week of sampling. This finding was similar to a previous study thus indicating that the *Salmonella* contamination of day-old chicks was significantly associated with *Salmonella* contamination of the broiler flock at the end of the rearing period (Rose et al., 1999). This conclusion also supported by the genetic profiles of *Salmonella* which showed indistinguishable pattern between the day-old chicks and the chicken-related samples in the broiler farm and subsequently found in the whole carcasses at the slaughterhouse. Moreover, during the rearing period (weeks 2-6), the result of sensitivity analysis suggested that the pest (lizard) was a significant source of *Salmonella* contamination and dissemination in the broiler farm. This finding was in line with the PFGE pattern showing that the pest (lizard) was a main reservoir for *Salmonella* contamination in the broiler farm.

In the broiler farm, the probability of finding *Salmonella* in at least one source among the multiple sources was 1.0. This result suggested that the *Salmonella* control measure should be implemented in this integrated broiler production particularly from the contaminated day-old chicks. Even though, among

the multiple sources of *Salmonella,* the pest showed only low level of correlation with that of the output variable. However, the pest control measure such as trap or physical control measure needed to implement in this broiler farm because the pest can circulate and re-contaminate *Salmonella* throughout the rearing period.

A probabilistic model describing probability of finding *Salmonella* positive sample among the production units was also constructed. The model output revealed that the probability of finding *Salmonella* in chicken-related samples among the different production units was extremely high. The result of sensitivity analysis revealed that the pre-slaughter subunit and slaughterhouse production unit were the most important unit contributing to high *Salmonella* contamination in the chicken carcasses. This finding indicated that this integrated broiler production should pay more attention to the cross-contamination during transportation and slaughter process which may cause *Salmonella* spreading to the whole carcasses.

However, in terms of model application, our models separately consider the *Salmonella* contamination among the various sources in the broiler farm and among chicken-related sources across the production unit. Therefore, in order to describe the dynamic of *Salmonella* interaction between chicken and environment, further studies were recommended to fulfill this information gaps.

Conclusion and suggestion

The present study demonstrated probabilistic model and the genetic characteristics of *Salmonella* isolated throughout an integrated broiler production. The result of this study presented the essential information for the risk assessors and risk managers to get insight into the pathway and dynamic of *Salmonella* contamination in an entire broiler production process. In addition, the information on antibiotic resistance patterns of the *Salmonella* isolates can be used to assist establishment of effective *Salmonella* control measures specific for the integrated broiler production in Thailand.

This study revealed that *Salmonella* control measures should be implemented and enforced in the entire broiler production process. Hygienic measures e.g. cleaning and disinfection in the animal production environment and all equipment should be revisited and improved particularly in the hatchery production unit which was the initial step in the broiler production and was identified to be the main source of *Salmonella* that can be transmitted to the subsequent production units. In addition, pest control measure should be revised for better controlling of the *Salmonella* reservoir. Similarly, the source of water supply and the water treatment procedure used in the broiler farm should be revised. Quality of the water used should be regularly checked and monitored to confirm the efficiency of water disinfection (chlorination). In addition, quality of the feed including pellet feed (from extruder with heat treatment) should be closely monitored and the feed supplies should be from a trustworthy feed mill where the guarantee of *Salmonella*-free feed was promptly obtainable. Finally, HACCP in the slaughterhouse should be revised or

strictly enforced to reduce the cross contamination of *Salmonella* during the slaughter process.

Furthermore, high prevalence of multidrug-resistant (MDR) *Salmonella* found in this study emphasized the importance of prudent use of antibiotics in the broiler production. To reduce the risk to human health due to MDR *Salmonella*, alternative to antimicrobial agents such as the use of probiotic and competitive exclusion may be used to reduce the frequency of the MDR in the broiler production.

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APPENDICES

APPENDIX A

Sample collection

Table A-1. Breeding farm and hatchery sample collection procedure

Production unit	Sample category	Sample type
Breeding farm	Chicken-related	Manure: nine pooled fecal samples (ca. 300 g per 1 pooled sample) (1st sampling) Cloacal swab: 1 chicken/swab (2nd and 3rd sampling) Egg: pooled samples (5 eggs per sample) (1st sampling), individual egg (2nd and 3rd sampling)
	Environmental	Boot swab: five pairs of boot swab Egg trays: pooled swab from 10 tray per sample (swab 100 cm^2/tray) Basket and plate: pooled swab from 5 baskets and 5 plates per sample Hand swab: before and after work (2nd and 3rd sampling) Egg transferring belt: swab 1000 cm^2/belt, collected 2 belts per flock
Hatchery	Chicken-related	Egg: pooled samples (5 eggs/sample) (before incubation, after 18 days of incubation, Meconium: swab from hatching tray (1 tray/sample)
	Environmental	Floor swab (egg storage room, incubating room, hatching room): 1000 cm^2/per room egg trolley: swab 1000 cm^2/trolley Transferring plate: swab 1000 cm^2/plate

Table A-2. Hatchery and broiler farm sample collection procedure

Production unit	Sample category	Sample type
Hatchery	Environmental	Illuminating plates: swab 1000 cm^2/plate Transferring belt: swab 1000 cm^2/belt Hook: swab surface area of hook Egg setting stand: swab surface area of egg setting stand Hatching tray: swab all area of hatching tray (1 tray/sample) Hand swab: swab 1 person per sample (before and after working) Water: collect 200 ml/sample Chick box: swab 4 boxes/sample (ca. 1000 cm^2) Truck: swab 1000 cm^2/truck
Broiler farm	Chicken-related	Meconium in box liner: 10 boxes/sample Manure: five pooled fecal samples (ca. 300 g/pooled sample) (1^{st} sampling) cloacal swab: 1 chicken/swab (2^{nd} and 3^{rd} sampling)
	Environmental	Floor: swab 1 m^2/floor Wall: swab 1 m^2/wall Pan feeder: swab 5 pan feeder/sample (1^{st} and 3^{rd} sampling), swab 1 pan feeder/sample (2^{nd} sampling) Litter: collect 300 gram/sample

Table A-3. Broiler farm and slaughterhouse sample collection procedure

Production unit	Sample category	Sample type
Broiler farm	Environmental	Boot swab: five pairs of boot swab Water nipple swab: swab 5 nipples and 5 cups/sample (1st and 3rd sampling), swab 1 nipple and 1 cup/sample (2nd sampling) New feed in hopper: collect feed from new package or hopper (300 gram/ sample) Feed in pan feeder: collect feed from 5 pan feeders per sample (300 gram/ sample) Water: collect 200 ml of water from 10 nipples/sample or collect 100 ml of water before entry the house Pest: collect 1 pest/ sample Water for chicken spraying: collect 200 ml of water (before and after spray) Transport cage: swab 1 cage/sample Hand swab: swab 1 person/sample (before and after catch the chicken) Truck: floor swab 1000 cm^2/truck
Slaughterhouse	Chicken-related	Feathers around cloaca: collect 3 g of feather/chicken/sample Cloacal swab: 1 chicken/swab (2nd and 3rd sampling) Whole carcass: rinse with 400 ml BPW/carcass

APPENDIX A

Sample collection

Figure A-1. Manure sample

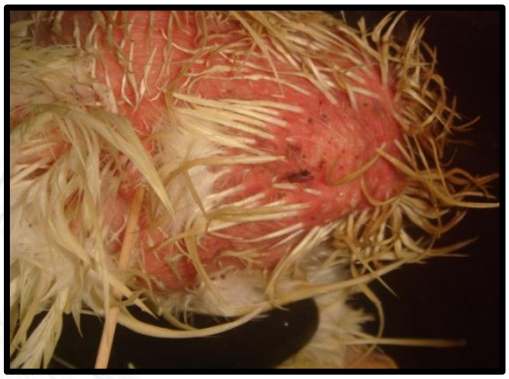


Figure A-2. Cloacal swab sample

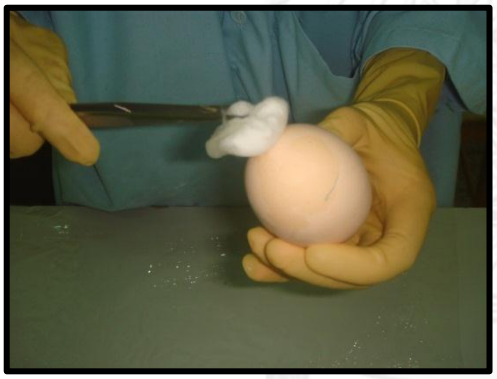


Figure A-3. Egg sample

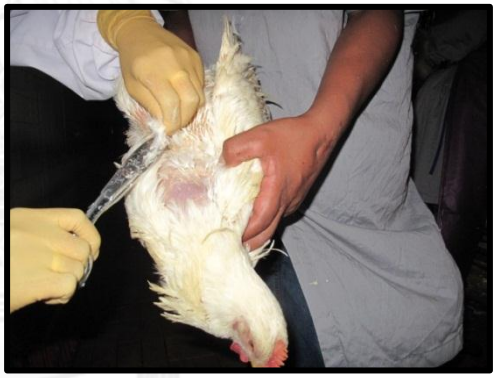


Figure A-4. Feather sample

Figure A-5. Boot swab sample

Figure A-6. Hand swab sample

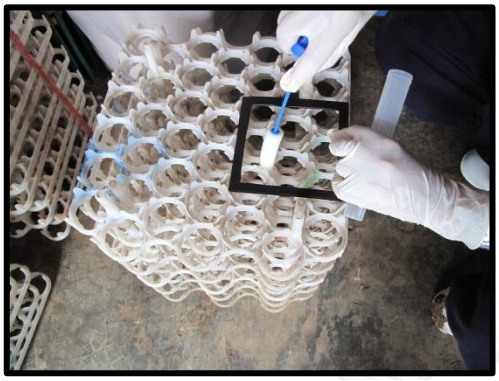


Figure A-7. Egg tray sample

Figure A-8. Basket and plate sample

Figure A-9. Floor swab sample

Figure A-10. Egg trolley sample

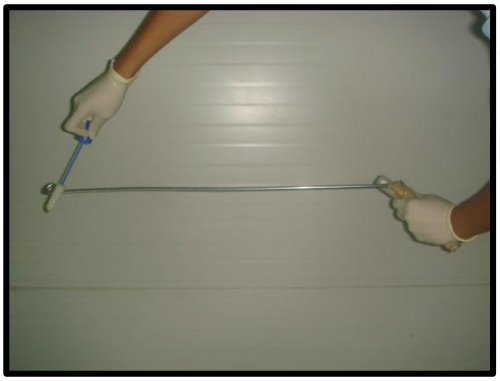


Figure A-11. Hook sample

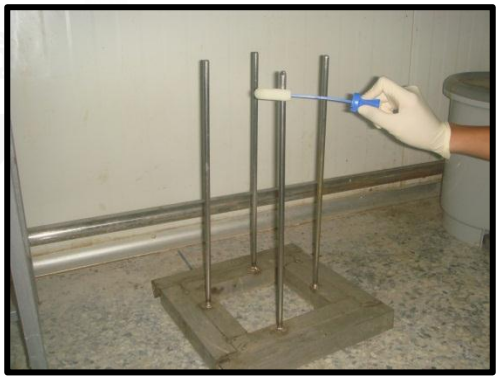


Figure A-12. Egg setting stand sample

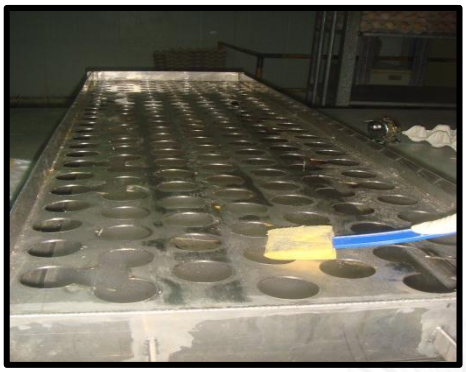


Figure A-13. Egg illuminating plate sample

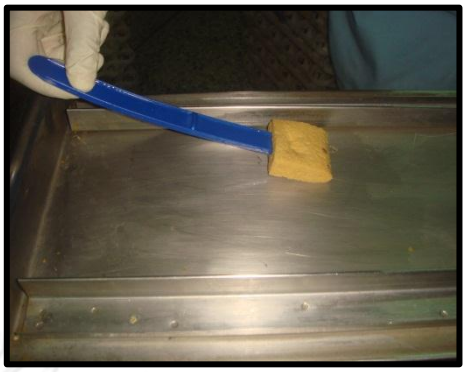


Figure A-14. Egg transferring plate sample

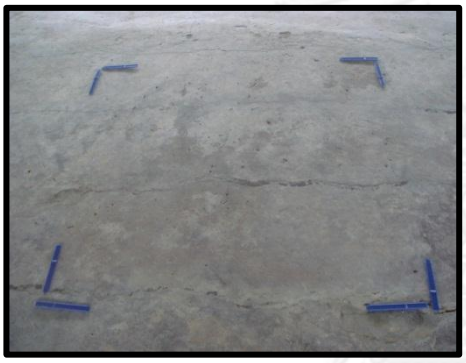


Figure A-15. Floor swab sample

Figure A-16. Wall swab sample

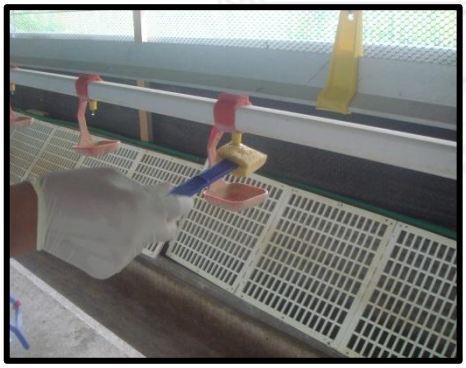


Figure A-17. Water nipple swab sample

Figure A-18. Pan feeder swab sample

Figure A-19. Water sample

Figure A-20. Cage swab sample

Figure A-21. Truck swab sample

APPENDIX B

Salmonella detection and enumeration procedure

Salmonella detection was performed according to ISO 6579:2002/Amd 1:2007 (Annex D) standard method which consist of four steps. (Figure B-1)

1. Pre-enrichment: All samples were mixed with Buffered peptone water (BPW, Merck KGaA; Darmstadt, Germany). For the samples of manure, litter and feed sample, 25 grams of sample were mixed with 225 ml of BPW. For the swab samples, the swabs were put into 5-30 ml of BPW (depend on size of swab). For the egg and pest samples, the whole samples were mixed with 225 ml of BPW and 100 ml of BPW, respectively. For the water sample, 100 ml of water was mixed with 225 ml of double-strength BPW. Then, all mixtures were incubated at 37°C ± 1°C for 18 ± 2 hours.

2. Selective enrichment: Modified semi-solid Rappaport Vassiliadis medium (MSRV, Merck, KGaA; Darmstadt, Germany) and 10 ml of Rappaport Vassiliadis with soya broth (RVS, Merck, KGaA; Darmstadt, Germany) were inoculated with 0.1 ml of the culture in the first step. In addition, 1 ml of the culture were also inoculated into Muller-Kauffmann tetrathionat-novobiocin broth (MKTTn, Merck, KGaA; Darmstadt, Germany). The MSRV and RVS broth were incubated at 42°C ± 1°C for 24 hours and MKTTn at 37°C ± 1°C for 24 hours.

3. Isolation step: The cultures obtained in the second step were streaked on xylose lysine deoxycholate agar (XLD, Merck, KGaA; Darmstadt, Germany) and chromagar *Salmonella* medium plate (Microbiology, Paris, France). Then, the plates were incubated at 37°C ± 1°C for 24 hours.

4. Confirmation step: Approximately 3-5 suspected colonies were selected and streaked on the nutrient agar (NA) plates. In addition, Triple sugar iron agar (TSI), Lysine iron agar (LIA) and Sulfide-Indole-Motility medium (SIM) were inoculated for biochemical testing. The colonies that show typical reaction of *Salmonella* were identified by slide agglutination test following the Kauffmann-White scheme.

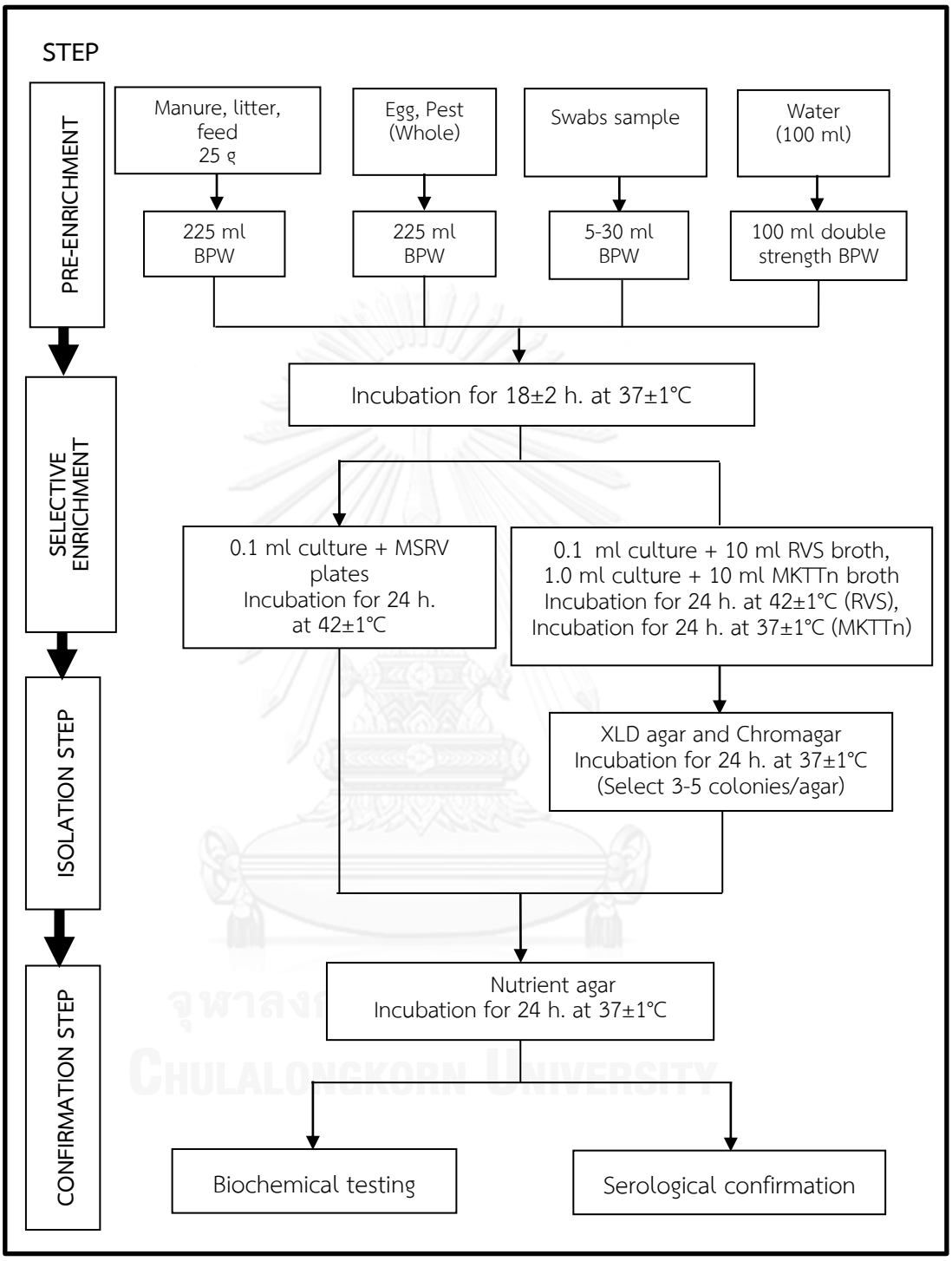


Figure B-1. The process for *Salmonella* spp. detection

The *Salmonella* enumeration in this study was performed by three-tube series MPN technique. The positive pattern of *Salmonella* was consulted with MPN table. The process was shown in Figure B-2.

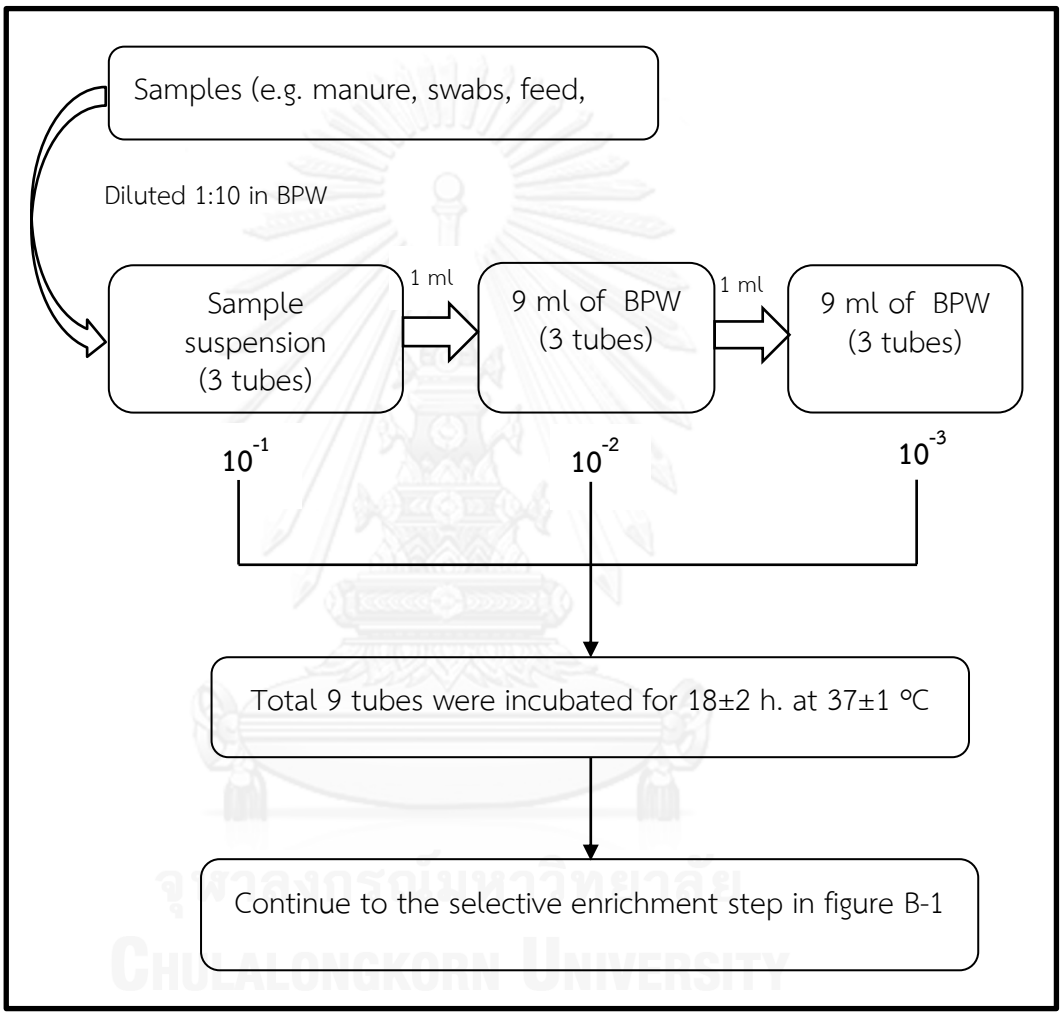


Figure B-2. The process for Salmonella enumeration by 3-tubes series MPN technique

APPENDIX C

Antimicrobial susceptibility testing

Table C-1. Antimicrobial agents, substances and equipment for antimicrobial susceptibility testing

Antimicrobial drug	Substance or equipment
Lincomycin-spectinomycin (109 µg)	*Escherichia coli* ATCC®25922
Ampicillin (10 µg)	Sterile saline
Amoxicillin (10 µg)	Sterile swabs
Ceftazidime (30 µg)	95% ethanol
Cefotaxime (5 µg)	0.5 McFarland standard
Meropenem (10 µg)	Wickerham card
Gentamicin (10 µg)	Mueller Hinton agar (MHA) plates
Doxycycline (10 µg)	Forceps
Tetracycline (30 µg)	antibiotic disk dispenser
Colistin sulphate (10 µg)	Ruler
Chloramphenicol (30 µg)	Inoculating loop
Nalidixic acid (30 µg)	Vortex
Ciprofloxacin (5 µg)	
Norfloxacin (10 µg)	
Enrofloxacin (5 µg)	
Trimethoprim-sulfamethoxazole (25µg)	

Disk diffusion susceptibility test protocol (following the Kirby-Bauer protocol)

1. Culture *Salmonella* isolates on Muller Hinton agar (MHA) and incubate at 37 ºC for 18-24 hours.
2. Pick up four to five single colonies of *Salmonella* from the surface of MHA by using a sterile inoculating loop.
3. Suspend the organism in 2 ml of sterile saline.
4. Vortex the saline and adjust the turbidity of the suspension to 0.5 McFarland standard. (use the suspension within 15 minutes after preparation).
5. Dip a sterile swab into the inoculum tube.
6. Rotate the swab against the side of the tube to remove the excess fluid.
7. Inoculate the swab into the surface of a MHA by streaking the swab three times over the entire agar surface, rotate the plate approximately 60 degree each time to ensure a thoroughly spreading.
8. Rim the plate with the swab to pick up any excess liquid
9. Allow the inoculate plate to sit at room temperature about 3-5 minutes (no longer than 15 minutes) for the surface of the agar plate to dry before place the antimicrobial disks.
10. Place the antimicrobial disk on the surface of the agar by using multidisc dispenser to dispense multiple disks at one time.
11. Place the dispenser over the agar plate and firmly press the plunger to dispense the disks onto the surface of the plate.
12. Gentle press the disk with the forceps to ensure complete contact with the agar surface.
13. Invert the plates and place them at 37º C for 16 to 18 hours.

14. Test all antimicrobial agents with *Escherichia coli* ATCC®25922 for standard control.
15. Read the diameters after 16 hours by using a ruler.

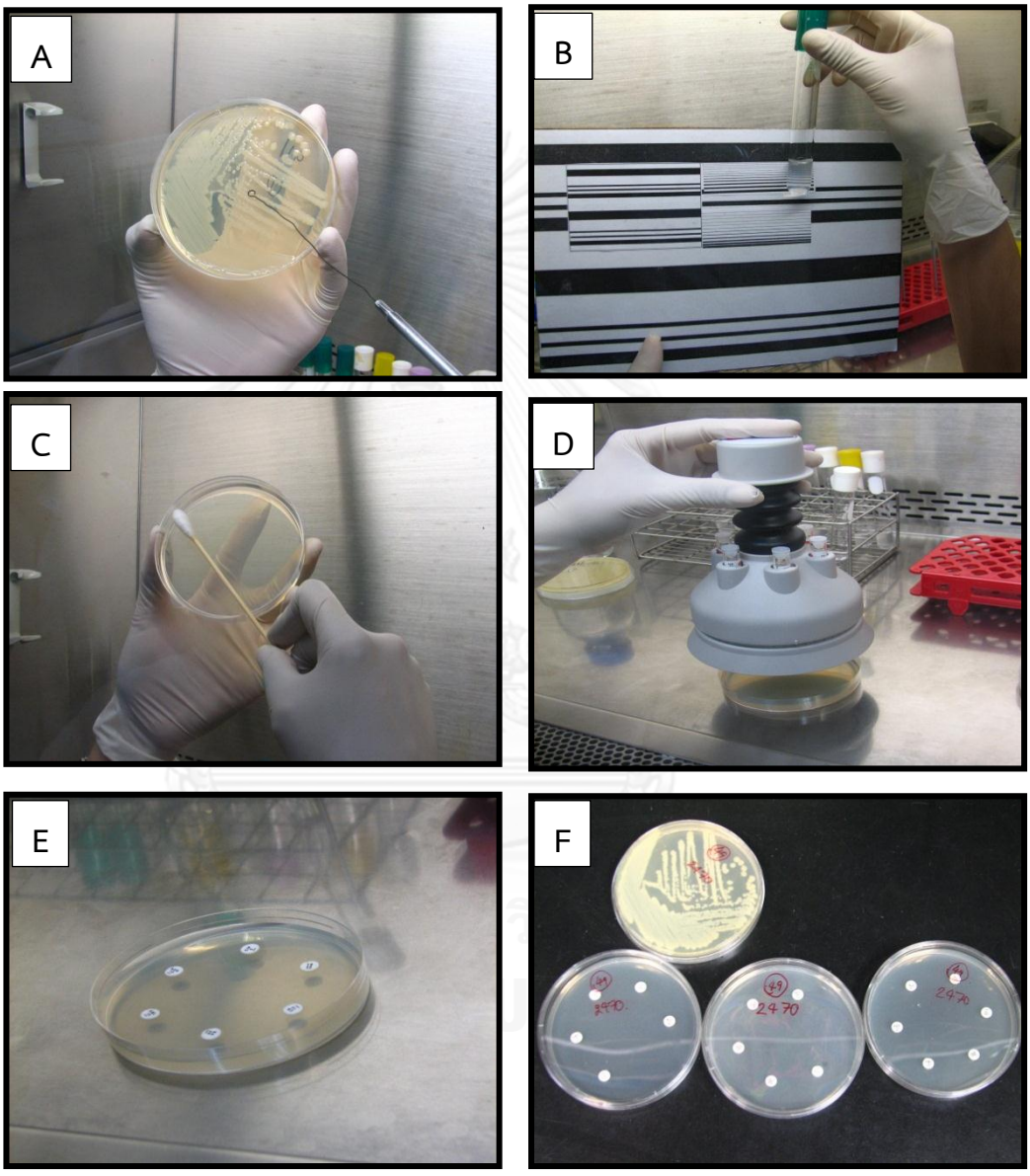


Illustration of antimicrobial susceptibility test. A: pick up 4-5 isolated of *Salmonella* colonies from Mueller Hinton agar, B: adjust the turbidity of the suspension to 0.5 McFarland standard, C: inoculate the surface of MHA with *Salmonella* swab (streak the swab three times over the entire agar surface), D: place the antimicrobial disks on the surface of the agar by using antibiotic disk dispenser, E: the MHA plate after placing the antimicrobial disks, F: the total disks for each sample testing

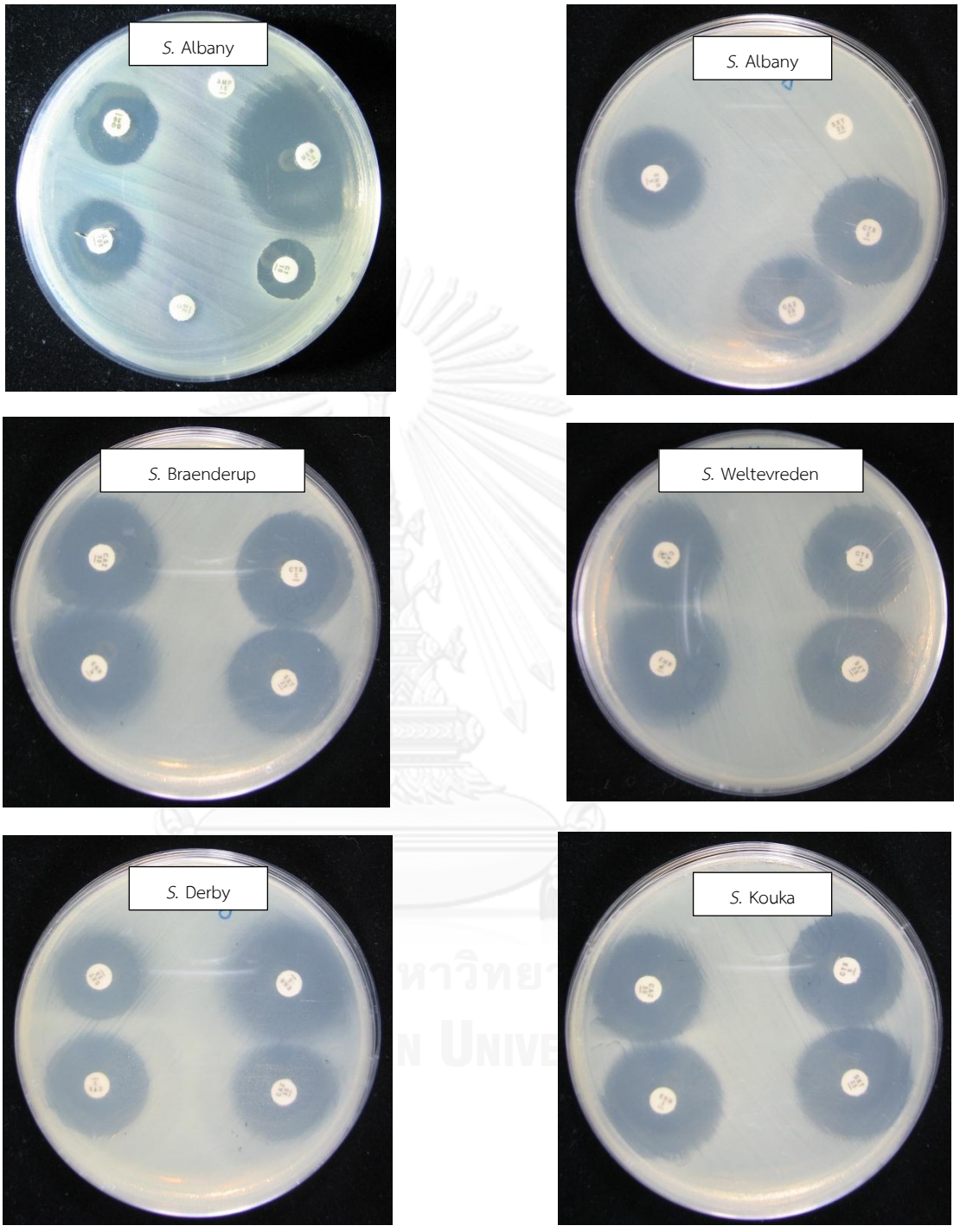


Figure C-1. Antimicrobial resistance pattern of *Salmonella* isolates

APPENDIX D

Pulsed-Field Gel Electrophoresis (PFGE)

Chemical substances for PFGE protocol

1. 1 M Tris-HCl, pH 8.0

   121.1 g Tris base

   Dissolve in 650-700 ml ultrapure water

   Add approximately 80 ml of 6N HCl

   Let solution come to room temperature.

   Make final adjustments to pH 8.0

   Dilute to 1,000 ml with ultrapure water. Sterilize by autoclaving

2. 0.5 M EDTA, pH 8.0

   186.1 g $Na_2EDTA\text{-}2\ H_2O$

   Add 800 ml ultrapure water

   Mix and adjust pH to 8.0 with approximately 50 ml of 10 N NaOH

   Dilute to 1,000 ml with ultrapure water. Sterilize by autoclaving

3. 20 mg/ml Proteinase K stock solution

   100 mg proteinase K powder

   5 ml sterile ultrapure water

   Mix and dispense in 500-600 µl volumes in 1.5 ml microcentrifuge tubes

   Storage at -20°C

4. 10% N-Lauroylsarcosine, Sodium salt (Sarcosyl)

   10 g sarcosyl

   90 ml sterile ultrapure water

   Carefully add sarcosyl to water in sterile container

   Dissolve by mixing gently and warming to 50°-60° C

5. Tris:EDTA Buffer (TE), pH 8.0

   10 mM Tris-HCl: 1 mM EDTA, pH 8.0

   10 ml 1 M Tris-HCl, pH 8.0

   2 ml 0.5 M EDTA, pH 8.0

   Dilute to 1,000 ml with sterile ultrapure water

6. Cell suspension buffer (CSB)

   100 mM Tris-HCl: 100 mM EDTA, pH 8.0

   10 ml 1 M Tris-HCl, pH 8.0

   20 ml 0.5 M EDTA, pH 8.0

   Dilute to 100 ml with sterile ultrapure water

7. Cell lysis buffer

   25 ml 1 M Tris-HCl, pH 8.0

   50 ml 0.5 M EDTA, pH 8.0

   50 ml 10% Sarcosyl

   Dilute to 500 ml with sterile ultrapure water

Add 25 µl Proteinase K stock solution (20 mg/ml) per 5 ml of cell lysis buffer just before use.

8. 5X Tris-Borate EDTA Buffer (TBE), pH 8.3

   0.9 M Tris base 54 g

   0.9 M Boric Acid 27.5 g

   0.02 M EDTA, pH 8.0 (20 ml 0.5 M)

   Dilute to 1,000 ml with sterile ultrapure water. Sterilize by autoclaving

9. 0.5X TBE Buffer

   200 ml 5X TBE Buffer

   Dilute to 2,000 ml with ultrapure water

10. Ethidium bromide

    Dilute 10 mg/ml stock solution 1:10,000 with ultrapure water

11. Molecular grade water (Hyclone, USA)
12. *Xba*I restriction enzyme and buffer (New England BioLabs, USA
13. *Avr*II restriction enzyme and buffer (New England BioLabs, USA)
14. SeaKem® gold agarose gel (Lonza, Switzerland)
15. Pulsed field certified agarose (Biorad, Canada)
16. Mueller Hinton agar (Difco, USA)

Pulsed-Field Gel Electrophoresis (PFGE) protocol

1. Culture *Salmonella* isolates on Muller Hinton agar (MHA) and incubate at 37 ºC for 14-18 hours.
2. Pick up *Salmonella* colonies from the surface of MHA by sterile cotton swab. Suspend colonies in 2 ml of Cell Suspension Buffer (CSB) by gentle spinning until evenly dispersion. Adjust the optical density of cell suspension to 0.8 to 1.0 at 610 nm wavelength.
3. Transfer 200 µl of adjusted cell suspensions into microcentrifuge tubes. Add 10 µl of 20 mg/ml stock proteinase K (USBiological, USA) to adjusted cell suspensions and mix gently with pipette. (Keep the microcentrifuge tubes on the ice box)
4. Add 200 µl of 1% melted SeaKem® Gold agarose into cell suspensions and gently pipette the mixture approximately 5-10 times. (Avoid the bobble forming during pipette). Then, dispense the mixture into plug mold immediately and allow the plugs to solidify at room temperature for 10 to 15 minutes.
5. Prepare master mix by adding 20 mg/ml stock proteinase K into cell lysis buffer (2.5 µl of proteinase K and 5 µl of cell lysis buffer needed for 1 tube). In the case that 15 tubes need to prepare, the total of master mix must to contain 37.5 µl of proteinase K and 7.5 ml of cell lysis buffer. Then, pipette 500 µl of the mixture into the new microcentrifuge tube.
6. Transfer plugs from mold and put into the microcentrifuge tubes which contain the mixture of proteinase K and cell lysis buffer.

7. Incubate the plug at 54 ºC for 2 hours with constantly and vigorous agitation (300 rpm)
8. Pre-heat sterile ultrapure water and TE buffer at 54 ºC in water bath. The volume of sterile ultrapure water and TE buffer was calculated for washing the plug two times and four times, respectively. (20-25 ml/tube for washing the plug/1 time)
9. Pour off lysis buffer from plug slides and hold the plug in tube with a CHEF® screened caps (Biorad, Canada). Add 20-25 ml of sterile ultrapure water to each tube and shake the tubes in water bath at 54 ºC for 10-15 minutes. Then, pour off water from the tube and repeat this step for one more time.
10. Add 20-25 ml of TE buffer to each tube and shake the tubes in water bath at 54 ºC for 10-15 minutes. Then, pour off TE buffer and repeat washing step with TE buffer for four more times.
11. After washing, the plugs can be used or stored in 5-10 ml of TE buffer at 4 ºC for 6 months to 1 year.
12. Prepare a master mix of pre-restriction buffer by diluting 10X restriction buffer 1:10 with molecular grade water (Hyclone, USA). The total volume of pre-restriction buffer was 100 µl per tube. Then, add diluted pre-restriction buffer to each microcentrifuge tube.
13. Remove plugs from TE buffer and cut plugs into 2 mm-wide pieces with a razor blade on the Petri dish. Then, transfer each cutting plug to microcentrifuge tube in 12.
14. Incubate the plug slice at 37 ºC for 5-10 minutes. Then, discard the pre-restriction buffer by using pipette.

15. Prepare a master mix of restriction enzyme as follow: (1 reaction)

Reagent	*Xba*I* (50 Unit/sample)	*Avr*II** (30 Unit/sample)
Molecular grade water (µl)	177.5	174
Restriction Buffer (µl)	20	20
Enzyme (µl)	2.5	6
Total volume (µl)	200	200

**Xba*I (New England Biolabs, USA) size 20 unit/µl

***Avr*II (New England Biolabs, USA) size 5 unit/µl

16. Add 200 µl of restriction enzyme mixture into each microcentrifuge tube. The plug slices must be under restriction enzyme mixture. Then, incubate plug slice at 37 ºC for 3 hours.
17. Prepare 1% pulsed-field certified agarose (Biorad, Canada) approximately 1 hour before the restriction reaction is finished and incubate the agarose at 55 ºC in water bath.
18. After incubation, remove enzyme mixture and add 200 µl of 0.5X TBE in each tube. Then, incubate at room temperature for 5 minutes.
19. Remove plug slices from 0.5X TBE and use tissue (KimWipes™, USA) to absorb excess buffer from the plug. Then, load plug slices in the bottom of the comb teeth. For *S.* Braendreup H 9812 Standard plug slices, load on teeth at the 1st, 8th and 15th line. Allow all plug slices to air dry on the comb for 10 minutes.
20. Position comb in gel form and pour 1% pulsed-field certified agarose into the gel casting apparatus. Allow gel to solidify for 30-45 minutes.

21. Put gel frame in electrophoresis chamber and pour 2 liters of 0.5X TBE buffer into the chamber. Then, turn on the Chef Mapper (Biorad, Canada), cooling module at 14 ºC and pump (set at 70 to get a flow rate of 1 liter/minute) approximately 30 minutes before gel is to be run.
22. After gel solidified, place gel inside gel frame in the chamber. Set the running condition (auto algorithm mode, 30 kb for low molecular weight, 700 kb for high molecular weight, 18 hours for running time, 2.16 seconds for initial switch time and 63.8 seconds for final switch time).
23. When eletrophoresis run is over. Turn off machine. Then, strain the gel with ethidium bromide for 20 minutes. Destain the gel with 500-800 ml of distilled water for three times (each time approximately 20 minutes).
24. Capture image under UV light with Gel Doc (Synoptics, Ltd., UK).

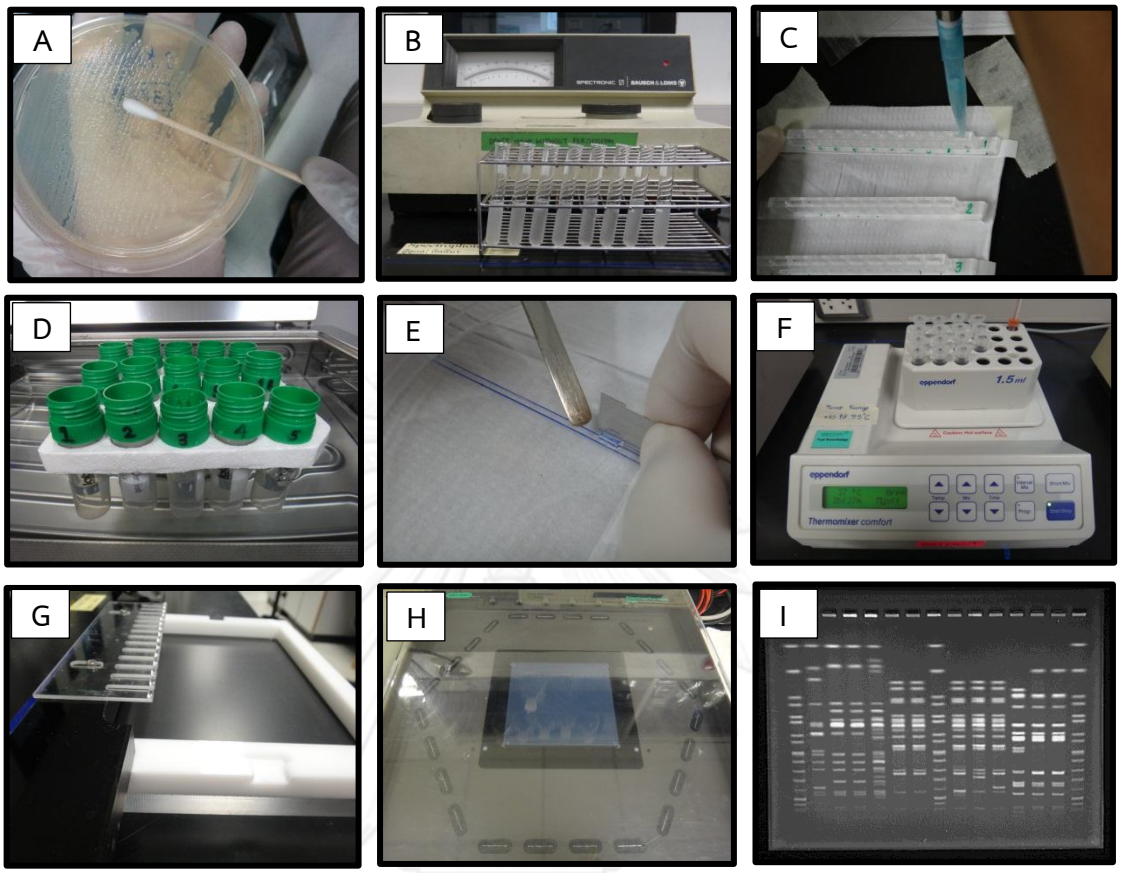


Illustration of PFGE process. A: pick up *Salmonella* colonies from Mueller Hinton agar, B: adjust the optical density of cell suspension to 0.8 to 1.0 at 610 nm, C: immobilize *Salmonella* cell with 1% SeaKem agarose gel (Lonza, Switzerland) into plug molds, D: wash the plugs with sterile water and TE buffer, E: cut the plug into 2 mm-wide for enzyme digestion, F: digest *Salmonella* DNA with restriction enzyme, G: load plug slices on comb teeth, H: run electrophoresis for 18 hours, I: capture image under UV light with Gel Doc (Synoptics, Ltd., UK).

APPENDIX E

Probabilistic model

Table E-1. Summary of simulation model values of probability of finding *Salmonella* among the multiple sources in the broiler farm (P_+)

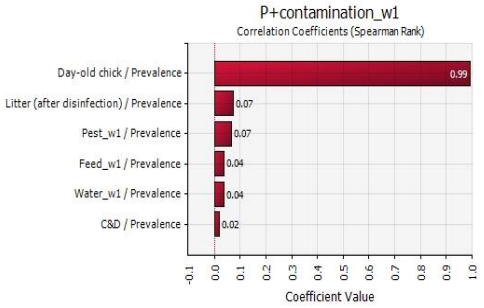
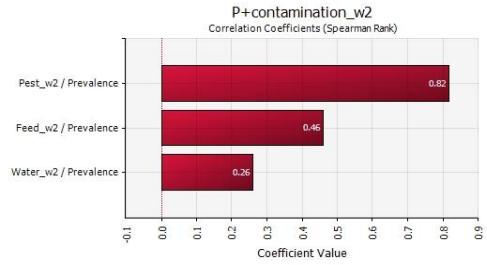
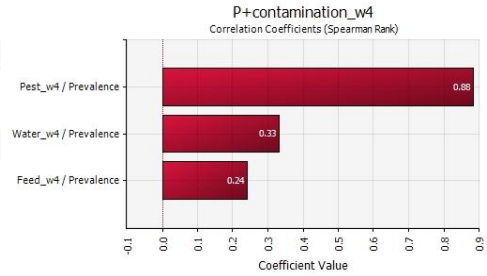
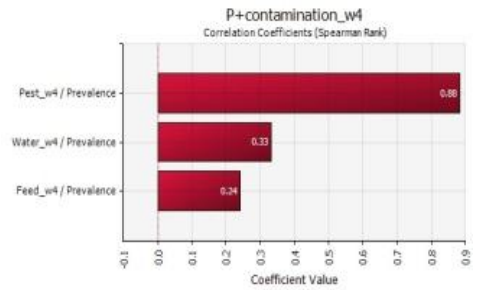
Sampling time	Statistical value (P_+)					Sensitivity analysis
	Min	5%	Mean	95%	Max	
Week 1	0.999	0.999	1.000	1.000	1.000	
Week 2	0.492	0.794	0.905	0.974	0.995	
Week 3	0.172	0.452	0.708	0.900	0.983	
Week 4	0.101	0.395	0.668	0.883	0.973	

Table E-1. Summary of simulation model values of Probability of finding *Salmonella* among the multiple sources in the broiler farm (P_+) (continued)

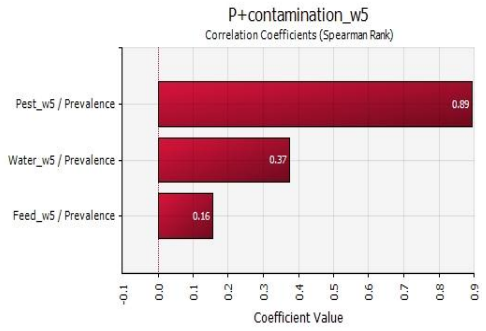
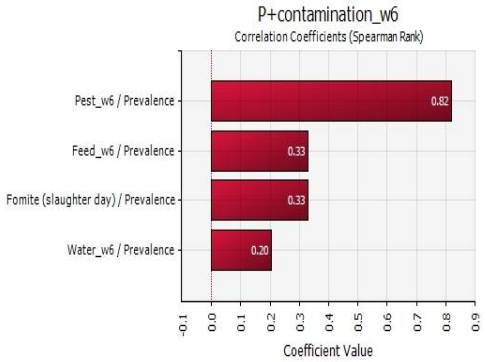
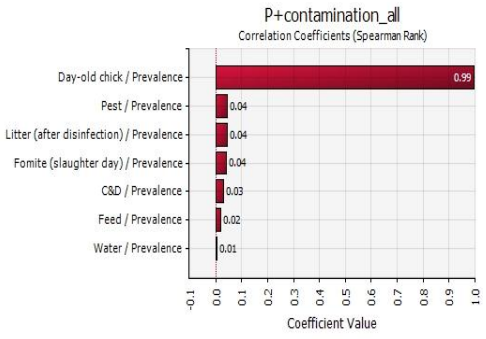
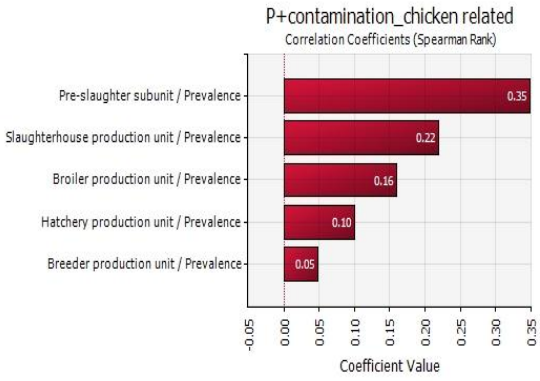
Sampling time	Statistical value (P_+)					Sensitivity analysis
	Min	5%	Mean	95%	Max	
Week 5	0.738	0.909	0.962	0.989	0.998	
Week 6	0.683	0.878	0.952	0.991	0.998	
All week	0.998	0.999	1.000	1.000	1.000	

Table E-2. Summary of simulation model values of Probability of finding *Salmonella* in chicken-related sample across the different production unit (P_+)

Statistical value (P_+)					Sensitivity analysis
Min	5%	Mean	95%	Max	
0.999	1.000	1.000	1.000	1.000	

VITA

Miss Nion Boonprasert was born on October 12, 1981 in Kamphaeng Phet province, Thailand. She earned degree in the Doctor of Veterinary Medicine (D.V.M.) with 1st class honour from the Faculty of Veterinary Medicine, Chiang Mai University, Chiang Mai, Thailand in 2006. Later, she got degree of Master of Veterinary Public Health from Faculty of Veterinary Medicine, Chiang Mai University, Thailand and Freie Universität, Berlin, Germany. After that, she has pursued the Doctor of Philosophy Program in the Department of Veterinary Public Health, Faculty of Veterinary Science, Chulalongkorn University since academic year 2010.