# การวิเคราะห์หาแหล่งที่มาของเชื้อ *Salmonella enterica* ในกระบวนการผลิตไก่เนื้อด้วยเทคนิค Pulsed-Field Gel Electrophoresis (PFGE)

นางสาวร้อยขวัญ สุนทราวาณิชย์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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ปีการศึกษา 2555

# SOURCE TRACKING OF *SALMONELLA ENTERICA* IN BROILER PRODUCTION BY PULSED-FIELD GEL ELECTROPHORESIS (PFGE)

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A Thesis Submitted in Partial Fulfillment of the Requirements
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| Thesis Title | SOURCE TRACKING OF *SALMONELLA ENTERICA* IN BROILER PRODUCTION BY PULSED-FIELD GEL ELECTROPHORESIS (PFGE) |
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ร้อยขวัญ สุนทราวาณิชย์ : การวิเคราะห์หาแหล่งที่มาของเชื้อ *Salmonella enterica* ในกระบวนการผลิตไก่เนื้อด้วยเทคนิค Pulsed-Field Gel Electrophoresis (PFGE). (SOURCE TRACKING OF *SALMONELLA ENTERICA* IN BROILER PRODUCTION BY PULSED-FIELD GEL ELECTROPHORESIS (PFGE)) อ.ที่ปรึกษาวิทยานิพนธ์หลัก : รศ. น.สพ.ดร.ศุภชัย เนื้อนวลสุวรรณ, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม : ดร.นิภา โชคสัจจะวาที, 89 หน้า.

การศึกษานี้มีวัตถุประสงค์เพื่อศึกษาหาแหล่งที่มาของเชื้อซัลโมเนลลาที่สำคัญในกระบวนการผลิตไก่เนื้อตามลำดับเวลาตลอดช่วงการเลี้ยงด้วยเทคนิค Pulse-Field Gel Electrophoresis (PFGE) โดยเก็บตัวอย่างจากฟาร์มไก่เนื้อแห่งหนึ่งที่เลี้ยงในระบบอุตสาหกรรมเป็นจำนวน 3 รุ่นการผลิต ในช่วงปี 2010-2012 ซึ่งแต่ละรุ่นได้เก็บตัวอย่างจากโรงเรือนเดิม ตัวอย่างที่เก็บมาทั้งหมดเป็นตัวอย่างจากตัวไก่ 1,350 ตัวอย่าง และจากสิ่งแวดล้อมในฟาร์มไก่เนื้อ 697 ตัวอย่าง จากนั้นแยกเชื้อและตรวจพิสูจน์ซีโรไทป์ของเชื้อซัลโมเนลลาที่ตรวจพบ และคัดเลือกเชื้อซัลโมเนลลาที่มีซีโรไทป์เหมือนกันระหว่างไก่เนื้อและสิ่งแวดล้อม เพื่อวิเคราะห์หาแหล่งที่มาโดยอาศัยเทคนิค PFGE ในการเปรียบเทียบรูปแบบพันธุกรรม (PFGE subtype) ผลการศึกษาพบว่าสิ่งแวดล้อมที่อาจจะเป็นแหล่งของการปนเปื้อนในฝูงไก่ที่ 1 ได้แก่ น้ำ และอาหาร เนื่องจากพบว่าน้ำและอาหารใหม่ มีการปนเปื้อนเชื้อซัลโมเนลลาที่มี PFGE subtype ที่ตรงกันกับเชื้อในตัวไก่ ได้แก่ *S.* Albany PFGE subtype ABa1 และ *S.* Derby PFGE subtype Da1 นอกจากนี้ยังพบว่าน้ำที่ปนเปื้อนอาจเป็นสาเหตุที่ทำให้โรงเรือนและอุปกรณ์อื่นๆ มีการปนเปื้อนด้วย เนื่องจากเชื้อที่ปนเปื้อนมี PFGE subtype เดียวกับเชื้อจากน้ำที่ใช้ในฟาร์ม (*S.* Albany PFGE subtype ABa1) สำหรับการเลี้ยงไก่ฝูงที่ 2 ไม่พบการปนเปื้อนในตัวไก่ระหว่างการเลี้ยง อย่างไรก็ตามได้มีการพบเชื้อ PFGE subtype เดียวกันกับที่พบในการเลี้ยงรอบแรกในจิ้งจกและสิ่งแวดล้อม (*S.* Weltevreden PFGE subtype Wa1 และ Wa2) ดังนั้นคาดว่าสัตว์พาหะ เช่น จิ้งจก อาจเป็นแหล่งของการแพร่เชื้อซัลโมเนลลาระหว่างรุ่นการเลี้ยงได้ การเลี้ยงในรอบที่ 3 พบการปนเปื้อน *S.* Corvallis PFGE subtype Ca1 ในลูกไก่ตั้งแต่เริ่มการเลี้ยง และตรวจพบเชื้อนี้ในตัวไก่ตลอดการเลี้ยง รวมถึงยังพบในสัตว์พาหะและสิ่งแวดล้อมในโรงเรือน จึงสรุปได้ว่าลูกไก่เป็นแหล่งการปนเปื้อนหลักของเชื้อในการเลี้ยงรอบนี้ และอาจเป็นแหล่งในการแพร่กระจายเชื้อไปสู่โรงเรือนซึ่งอาจปนเปื้อนข้ามไปยังการเลี้ยงรอบต่อไปได้ ดังนั้นจากการเก็บตัวอย่างทั้งสามรอบสามารถสรุปได้ว่า ลูกไก่ อาหารและน้ำที่ปนเปื้อนเป็นแหล่งของเชื้อซัลโมเนลลาที่สำคัญในการเลี้ยงไก่เนื้อ นอกจากนี้การจัดการฟาร์มอื่นๆ เช่น ความสะอาดของโรงเรือน วัสดุรองพื้น อุปกรณ์ในการขนส่ง และสัตว์พาหะยังอาจเป็นแหล่งสะสมและทำให้มีการปนเปื้อนเชื้อในไก่เนื้อได้อีกด้วย

ภาควิชา สัตวแพทยสาธารณสุข ลายมือชื่อนิสิต .....

สาขาวิชา สัตวแพทยสาธารณสุข ลายมือชื่ออ.ที่ปรึกษาวิทยานิพนธ์หลัก .....

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KEYWORDS : Broiler production / Pulsed-Field Gel Electrophoresis (PFGE) / *Salmonella enterica* / Source tracking

ROIKHWAN SOONTRAVANICH : SOURCE TRACKING OF *SALMONELLA ENTERICA* IN BROILER PRODUCTION BY PULSED-FIELD GEL ELECTROPHORESIS (PFGE)

ADVISOR : ASSOC. PROF. SUPHACHAI NUANUALSUWAN, D.V.M., Ph.D., CO-ADVISOR : NIPA CHOKESAJJAWATEE, Ph.D., 89 pp.

The objectives of this study were to determine the chronological dissemination and the main sources of *Salmonella* introduction to broiler production throughout rearing period by Pulsed-Field Gel Electrophoresis (PFGE). The samples were collected from a commercial broiler farm up to 3 cycle productions from a commercial broiler farm in Northeastern part of Thailand during 2010-2012. The samples were collected from the same broiler house and the total number of samples from broiler and environment were 1,350 and 697, respectively. The isolates with common *Salmonella* serotypes between broiler and environment were chosen for source tracking by subtyping with PFGE. The result from the first flock showed that contaminated water and new feed were possibly the primary sources of *Salmonella* to the broiler flock because of the identical PFGE pattern of *S.* Albany (subtype ABa1) and *S.* Derby (subtype Da1) among feed, water and broiler. The contaminated water was also possibly the source of *Salmonella* contamination to broiler house and equipment because *S.* Albany PFGE subtype ABa1 was found from both water and broiler house equipment after disinfection. *Salmonella* isolates were not detected from broiler in the second flock. However, the same PFGE subtypes of *S.* weltevreden (subtype Wa1, Wa2) from house lizards and environment between the first and the second flock were found. The result indicated that house lizards may act as a reservoir between flocks. In the third flock, *S.* Corvallis (PFGE subtype Ca1) was found from the day old chick. This PFGE subtype was also found throughout the rearing period from broiler and also from pest and environment in the broiler house. So, the contaminated day old chick was the main source of *Salmonella* contamination in this flock. In conclusion, this study suggested that contaminated water and feed including infected day-old chick were among the main sources of *Salmonella* contamination in broiler farm. In addition, the contamination of house and equipment, litter, transportation equipment and pest should also be concerned as a possible source of *Salmonella* contamination in broiler flock.

Department : Veterinary Public Health Student's Signature .....

Field of Study : Veterinary Public Health Advisor's Signature .....

Academic Year : 2012 Co-advisor's Signature .....

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# LIST OF ABBREVIATION

| | |
|---|---|
| bp | base pair |
| °C | degree Celsius |
| CDC | The Center for Disease Control and Prevention, USA |
| DNA | deoxyribonucleic acid |
| EDTA | Ethylene diaminetetraacetic acid |
| EFSA | European Food Safety Authority |
| et al. | et alibi and others |
| h | hour (s) |
| i.e. | id est, that is |
| kb | kilo base pair |
| PCR | polymerase chain reaction |
| PFGE | Pulsed-Field Gel Electrophoresis |
| ml | milliliter (s) |
| mm | millimeter (s) |
| µl | micro liter (s) |
| µm | micro meter (s) |
| NSSC | WHO National *Salmonella* and *Shigella* Center |
| *S.* | *Salmonella* |
| TE | Tris-EDTA |
| TBE | Tris-Borate EDTA |
| U | unit |
| UPGMA | Unweighted Pair Group Method with Arithmetic Mean |
| WHO | World Health Organization |

# CHAPTER I

# INTRODUCTION

## 1.1 Importance and Rationale

*Salmonella* is a bacterial member of the family Enterobacteriaceae. It is a gram-negative facultative anaerobic rod-shaped bacterium with peritrichous flagella. The importance of *Salmonella* is that it is one of the major causes of foodborne disease throughout the world (WHO, 2007). The clinical symptoms of human salmonellosis are acute onset of fever, abdominal pain, diarrhea, nausea and vomiting. Salmonellosis can become severe and life-threatening for infants, elderly and those with impaired immune system. Especially, young children (ages 0-4 years) had the highest rate of Salmonellosis (EFSA, 2011). In the United States of America, it was estimated that there were 1.4 million non-typhoidal *Salmonella* infections resulting in 580 deaths each year (WHO, 2007). Among outbreak-related foodborne illness, *Salmonella* was the most frequent cause of hospitalized cases in the USA between 2008 and 2011 (CDC, 2011a) The European Union also reported 108,614 cases of human Salmonellosis in 2009 (EFSA, 2011). Apart from being a public health problem, economic loss from Salmonellosis in human is costly, the total estimated

cost associated with human Salmonellosis cases is US$ 3 billion and US$ 15.5 million per year in the United States and Denmark respectively (WHO, 2007).

Humans generally get infected with *Salmonella* by ingestion of contaminated food of animal origin such as meat, pork, poultry products and milk. Among food of animal origin, poultry product (i.e. eggs and poultry meat) are the most common sources of Salmonellosis in human (EFSA, 2011; CDC, 2011a). Many countries have attempted to control *Salmonella* in poultry production in order to protect consumers from illness. For instance, the European Union has the regulations to control the prevalence of zoonotic agent including *Salmonella*, especially at the farm level to ensure that proper and effective measures are taken to detect and to control *Salmonella* at all relevant stages of poultry production. The target is to reduce *Salmonella* Enteritidis and *Salmonella* Typhimurium positive flock to 1% or less in broiler.

In Thailand, poultry production is among the top export industries. In 2010, Thailand exported up to 432,230 tons of poultry products valued at 52,230 million baht (OAE, 2011). The major importing countries of poultry products from Thailand are the European countries and Japan. In order to achieve the customers' satisfaction and to stay ahead of other poultry exporting countries, Thailand as a leading poultry producer, is expected to efficiently control *Salmonella* in poultry production at the farm level in order to maintain customer's confidence in Thai poultry products.

Controlling *Salmonella* in broiler production is complicated, since *Salmonella* can be introduced to broiler flocks from many different sources such as contaminated day-old chicks, contaminated feed, farm pests such as rodents, invertebrates and wild birds (Rose et al., 1999; Heyndrickx et al., 2002; Gast, 2003; Namata et al., 2009; Marin et al., 2011). Moreover, *Salmonella* can persist for long period without proper cleaning and proper disinfection of broiler houses and equipments occur (Rose et al., 1999; Marin et al., 2011). Additionally, transportation of broiler to slaughterhouse is associated with *Salmonella* detection in broiler meat as well (Heyndrickx et al., 2002).

Though many studies revealed possible sources of *Salmonella* in broiler production, *Salmonella* control in broiler flocks is not well achieved. For example, European Union found 5.0 % of the tested broiler flocks were *Salmonella*-positive flock in 2009 (EFSA, 2011). In Thailand, the study that aims to track the sources of *Salmonella* introduction to broiler production throughout the rearing period has never been done before. Therefore, this study intended to investigate the main sources of *Salmonella* in broiler production at each step of rearing by using serotyping and Pulsed-Field Gel Electrophoresis (PFGE) technique. PFGE was used to determine genetic clonality of *Salmonella* isolates from broiler production. This technique is not only a method of choice for epidemiologic subtyping pathogenic bacteria including *Salmonella* (Fakhr et al., 2005; CDC, 2011) but it also has a high discriminatory

power with reproducible, standardized protocol and the shared interlaboratory results. This technique requires rare-cutting restriction enzymes to cleave bacterial DNA, then separates DNA fragments by a special electrophoresis that is constantly changing the direction of the electrical field (Peters, 2009). PFGE was successfully used for tracking *Salmonella* sources in poultry production e.g. broiler, layer and turkey in several countries (Kim et al., 2007; Lapuz et al., 2007; Nayak and Stewart-King, 2008). This study was beneficial to control *Salmonella* in broiler production of Thailand more effectively.

### 1.2 Objectives

The objectives of the study were to determine the chronological dissemination and the main sources of *Salmonella* introduction to broiler production throughout rearing period by Pulsed-Field Gel Electrophoresis (PFGE).

# CHAPTER II

# LITERATURE REVIEW

## 1. *Salmonella spp.*

*Salmonella* is a member of the bacterial family Enterobacteriaceae. It is a gram-negative facultative anaerobic rod-shaped bacterium with approximately 0.7 to 1.5 μm in diameter, 2.0 to 5.0 μm long, generally motile with peritrichous flagella, except the poultry specific-serotypes, *Salmonella* Gallinarum and *Salmonella* Pullorum. *Salmonella* can ferment glucose and often produce gas, reduce nitrate to nitrite and is catalase positive, but oxidase negative. Moreover, *Salmonella* and other microorganism of the family Enterobacteriaceae are more resistant to novobiocin, selenite, tergitol and bile salts than other bacteria. In addition, *Salmonella* are more resistant to brilliant green and malachite green than other bacteria in family Enterobacteriaceae. The optimum temperature for *Salmonella* to multiply is 37$^{o}$C, but the microorganism can grow in temperature ranging from 5 to 45 $^{o}$C and pH range between 4.0 and 9.0. The optimum pH for growth is 7.0 (Grimont et al., 2000; Gast, 2003).

The genus *Salmonella* is composed of two species, which are *S. bongori* and *S. enterica*. *S. enterica* can be classified further into 6 subspecies based on phenotypic

characters. Six subspecies of *S. enterica* are *enterica* (I), *salamae* (II), *arizonae* (IIIa), *diarizonae* (IIIb), *houtenae* (IV) and *indica* (VI).

Since 2007, the genus *Salmonella* can be differentiated to 2,579 serotypes, based on Kaufmann-White scheme where the somatic (O), the flagellar (H) and the capsular (Vi) antigens to identify the serotypes (Table 1).

*S. bongori* has been classified into 22 serotypes and *S. enterica* could be differentiated to 2,557 serotypes. Among *S.* enterica, *S. enterica subspecies enterica* has the most serotypes (1,531 serotypes) (Grimont and Weill, 2007).

-The O-antigens are determined by lipopolysaccharides (LPS) on the cell wall of bacteria, which are named with Arabic number. For instance, O: 1, O: 2, O: 12.

-The H-antigens are associated with flagella proteins which are heat-labile proteins. *Salmonella* typically contains 2 phases *of* H-antigens. The first phase is called specific phase and named by small type letter from “a to z” and “$z_1$ to $z_{66}$”. The second phase is called non-specific phase and named with Arabic number.

-The Vi antigen presents only in *S.* Typhi, *S.* Paratyphi C and *S.* Dublin (Grimont et al., 2000; D'Aoust et al., 2001). *Salmonella* serotypes with Vi antigen have more pathogenicity than those without Vi antigen.

Table 1. *Salmonella* subspecies and species

| Species | Subspecies | No. of serotypes |
|---|---|---|
| *Salmonella enterica* | *Enterica* | 1,531 |
| | *Salamae* | 505 |
| | *Arizonae* | 99 |
| | *Diarizonae* | 336 |
| | *Houtanae* | 73 |
| | *Indica* | 13 |
| | Subtotal | 2,557 |
| *Salmonella bongori* | | 22 |
| | Total | 2,579 |

(Modified from Grimont and Weill, 2007)

*Salmonella* serotypes are divided into three groups based on host range (Uzzau et al., 2000).

1. Host-restricted group: This group typically causes disease in one particular host species, for example, *S.* Typhi in human, *S.* Gallinarum in avian and *S.* Typhisuis in swine.

2. Host-adapted group: *Salmonella* serotypes in host-adapted group are mainly associated with a specific host species but can sometimes cause disease in other host species such as, *S.* Choleraesuis which generally causes disease in swine, but also causes disease in human infrequently.

3. Un-restricted group: *Salmonella* serotypes in this group can cause disease in a wide range of host species. For example, *S.* Typhimurium and *S.* Enteritidis

The main route of *Salmonella* infection in humans is through ingestion of contaminated food. *Salmonella* normally multiply in the mucosa of ileum, cecum, colon and mesenteric lymph node of infected animals. Subsequently, most *Salmonella* will be cleared by the host immune system, however subclinical infection may persist and these animals can shed *Salmonella* in feces. Subclinical infection may develop clinical disease if the infected hosts are under stress.

## 2. Salmonellosis in poultry

*Salmonella* species that affect poultry health is *S. enterica*. The main serotypes associated with poultry health are *S.* Pullorum and *S.* Gallinarum. Both serotypes are avain host specific. *S.* Pullorum causes Pullorum disease (PD), while *S.* Gallinarum causes Fowl typhoid (FT).

*S.* Pullorum (PD) mainly causes disease in young chicks around the first few weeks of age. The symptoms of the disease are high prevalence of dead-in-shell chicks, and high mortality rate of chicks after hatching. Affected chicks show signs of depression, weakness with white sticky feces. On the other hand, FT normally affects growing and adult chickens, but it may cause mortality and clinical sign in young chicks. The clinical signs in adult chicken are increase in mortality rate, depressed, and watery to mucoid yellow diarrhea. Both diseases can spread among chickens by vertical (transovarian) and horizontal transmission (Vegad, 2008; Gast, 2003)

Besides *S.* Pullorum and *S.* Gallinarum, there are more than 2,500 *Salmonella* serotypes, known as non-typhoidal *Salmonella* which are important causes of food-borne disease in human. As stated above, poultry product is a major source of *Salmonella* contamination (Gast, 2003; EFSA, 2011; CDC, 2011a)

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Table 2. *Salmonella* serotypes in broiler carcasses in the European Union

| *Salmonella* serotypes | Percentage |
|---|---|
| *S.* Infantis | 29.2 |
| *S.* Enteritidis | 13.6 |
| *S.* Kentucky | 6.2 |
| *S.* Typhimurium | 4.4 |
| *S.* Bredeney | 4.3 |
| *S.* Virchow | 4.1 |
| *S.* Hadar | 3.8 |
| *S.* Paratyphi var Java | 3.8 |
| *S.* Agona | 3.0 |
| *S.* Indiana | 2.9 |
| Other serotypes and non-typeable | 27.3 |

(Modified from EFSA, 2011)

In the European Union, *Salmonella* prevalence in broiler carcasses was 15.6% from January to December 2008. The predominant serotypes on broiler carcasses and meat were *S*. Infantis followed by S. Enteritidis, S. Kentucky and S. Typhimurium as shown in Table 2. The average prevalence of *Salmonella* Enteritidis and/or *Salmonella* Typhimurium on broiler carcasses in the European Union was 3.6 % (EFSA, 2011). Likewise, the study in South Australia indicated that *S*. Infantis was the most frequently found serotype in chicken meat (Fearnley et al., 2011). However, *S*. Enteritidis followed by *S*. Hadar were the most common serotypes from chicken meat in Spain and poultry products in Belgium (Uyttendaele et al., 1998; Dominguez et al., 2002). The United States of America also found that *S*. Enteritidis in broiler carcass rinses increased from 17% to 25% during 2000 to 2005 (Altekruse et al., 2006). In Thailand, Boonmar, et al. (1998) and Bangtrakulnonth, et al. (2004) found that *S*. Enteritidis was the most common serotype in frozen chicken meat in Thailand from 1993 to 1996 and from 1993 to 2002 and in chicken manures in Thailand from 1993 to 1995 (Boonmar et al., 1998).

The prevalence of *Salmonella*-positive broiler flock in northern Thailand was 91.6% for day-old chicks and 98.6% for chicken less than 3 weeks before slaughtering and the major serotype was *S*. Enteritidis (Chaengprachak, 2009). The European Union reported that 5.0% of tested broiler flocks were Salmonella-positive; 0.6% and 0.1% of the tested

flocks were positive for *S*. Enteritidis and *S*. Typhimurium, respectively (Table 3; EFSA, 2011).

Table 3. *Salmonella* serotypes in broiler flocks in European Union

| ***Salmonella* serotypes** | **Percentage** |
|---|---|
| *Salmonella positive (all serotypes)* | 5.0 |
| *S*. Enteritidis and/or *S*. Typhimurium | 0.7 |
| *S*. Enteritidis | 0.6 |
| *S*. Typhimurium | 0.1 |
| Other serotypes | 4.2 |

(Modified from EFSA, 2011)

### 3. Salmonellosis in humans

Salmonellosis is one of the major foodborne diseases in humans. According to a European Union report in 2009, there were 108,614 cases of human Salmonellosis (EFSA, 2011) The United States of America reported that among foodborne illness, *Salmonella* was the most common source of outbreak-related hospitalizations during 2008 and 2011(CDC, 2011a) In addition, the US estimated 1.4 million *Salmonella* infections, and 580 deaths annually (WHO, 2007). In Thailand, there were 3,083 isolates from humans confirmed as *Salmonella* case in 2008 (NSSC, 2008).

Non-Typhoidal *Salmonella* is the major cause of Salmonellosis in humans. Humans generally get infected with *Salmonella* by ingestion of contaminated food. Poultry products (eggs and poultry meat) are among the most common sources of Salmonellosis in humans (EFSA, 2011). The clinical signs of this disease are fever, abdominal pain, diarrhea, nausea and vomiting. The symptoms usually develop 12-72 hours after ingestion and last approximately one week. However, this disease can become more severe and life-threatening in young children, elderly and immunocompromised people (CDC, 2010). EFSA (2011) reported that young children ages 0-4 years had the highest rate of Salmonellosis (112.4 per 100,000 population) compare to other age groups (EFSA, 2011). In Thailand,

from 2002 to 2007, the majority of all *Salmonella* infection cases (32.6%) were also observed among children age 0 to 5 years according to figure 1 (Hendriksen et al., 2009).

According to global monitoring of *Salmonella* serotypes distribution in humans during 2002-2007, *S.* Enteritidis and *S.* Typhimurium were the most common serotypes found in all regions throughout the world (Foley and Lynne, 2008). The European Union also reported that *S.* Enteritidis and *S.* Typhimurium were the most frequently found in 2009 (EFSA, 2011), as well as in United States (Foley et al., 2008) and these 2 serotypes were most commonly associated with contaminated food of animal origin including poultry (EFSA, 2011). Additionally, in the US, *S.* Enteritidis was the most common of *Salmonella* serotype causing single-etiology outbreak during 2008 to June 2011. In Thailand, the annual report of confirmed *Salmonella and Shigella* in 2008 of Thailand indicated that the most common *Salmonella* isolates from human in Thailand was *S.* Enteritidis, followed by *S.* Cholerasuis, *S.* Stanley, *S.* Welterveden and *S.* Typhimurium (NSSC, 2008).

Table 4. Most common *Salmonella* serotypes in humans in Thailand

| *Salmonella* serotypes | Percentage |
|---|---|
| *S.* Enteritidis | 18.62 |
| *S.* Cholerasuis | 9.60 |
| *S.* Stanley | 8.92 |
| *S.* Weltevreden | 7.10 |
| *S.* Typhimurium | 6.78 |
| *S.* Rissen | 6.10 |
| *S.* I. 4,5,12:i:- | 5.90 |
| *S.* Anatum | 4.35 |
| *S.* Corvallis | 3.54 |
| *S.* Kedougou | 2.47 |

(Modified from NSSC, 2008)

4. Sources of *Salmonella* contamination in broiler flock

*Salmonella* can be introduced into broiler flocks via many routes such as infected day-old chicks (Rose et al., 1999; Namata et al., 2009; Marin et al., 2011) and contaminated feed (Gast, 2003; Marin et al., 2011). Moreover, improper cleaning and disinfection procedure for broiler houses and equipment (Marin et al., 2011) and contamination of the houses before restocking (Rose et al., 1999) are important factors. Furthermore, rodents, wild birds and various invertebrates (i.e. darkling beetle, cockroach, and centipede) can carry *Salmonella* to poultry flocks as well (Gast, 2003; Lapuz et al., 2007).

Kim, et al. (2007) investigated key interventions to control *Salmonella* in broiler production in Korea by determining genetic clonality of *S.* Enteritidis by using PFGE technique. The result showed that breeder farms and hatcheries were important sources of the *Salmonella* infection. Therefore, *Salmonella*-free breeding flocks were recommended as source of broiler. Besides, inadequate biosecurity practices in hatchery may lead to increase probability of horizontal transmission such as mixing eggs from various parent flocks, the high temperatures of egg storage room, distance and duration of chick transportation to the broiler farms. The mechanical separation of egg shells and chicks and disinfection of transport vehicles may reduce probability of *Salmonella* infection in chicks (Volkova et al., 2011). Other sources of *Salmonella* contamination such as dust in the

ventilation filters, the nest boxes and the wall of the houses had also been implicated in the *Salmonella* contaminations (Kim et al., 2007; Namata et al., 2009).

**5. Molecular techniques for tracking sources of *Salmonella***

Phenotype-based techniques for subtyping of pathogenic bacteria are lacking in the discriminatory power and reproducibility (Wiedmann, 2002; Sirichote et al., 2010). Moreover, some techniques require high amount of specific reagent (Xia et al., 2011). Molecular-based techniques are therefore developed to overcome these drawbacks.

At present, there are several molecular-based techniques available for tracking sources of bacterial foodborne pathogens.

**5.1 Amplification-based methods**: Amplified Fragment Length Polymorphism (AFLP), Random amplified polymorphic DNA (RAPD), Repetitive element Polymerase chain reaction (Rep-PCR) and Variable number of tandem repeat (VNTR) and Multiple locus VNTR analysis (MLVA).

These techniques are primarily based on polymerase chain reaction (PCR) amplification of bacterial DNA. They are susceptible to factors such as chemical reagents and annealing temperature which can influence their reproducibility (Hunter et al., 2005; Foley et al., 2009; Shi et al., 2010). Moreover, some techniques are complicated. AFLP

consists of several steps of procedures. VNTR and MLVA require whole genome sequence prior to design the protocol (Ross and Heuzenroeder, 2005).

5.2 **Sequencing-based methods:** Multilocus sequence typing (MLST)

The variability of DNA sequences among bacterial strains is used to determine genetic relatedness of bacteria. Mostly, housekeeping genes are used for sequencing because they are found in all isolates and not easily changed. MLST is effective when proper genes with adequate number of genes are used to sequence. Fakhr et. al., (2005) found that PFGE had more discriminatory power than MLST for subtyping *Salmonella* Typhimurium. Other studies found that MLST had better ability to distinguish *S.* Typhimurium. The variability of MLST ability to distinguish *S.* Typhimurium was due to selection and number of genes used for sequencing (Wiedmann, 2002; Foley et al., 2009).

**5.3 Restriction-based methods:** Restriction fragment length polymorphism (RFLP) and Pulsed-field gel electrophoresis (PFGE)

These techniques utilize restriction enzymes to digest bacterial DNA, then separate DNA fragments by electrophoresis. Since RFLP employs frequent cutting restriction enzymes, too many DNA fragments are generated. Consequently, it is sometimes too difficult to interpret the result (Foley et al., 2009).

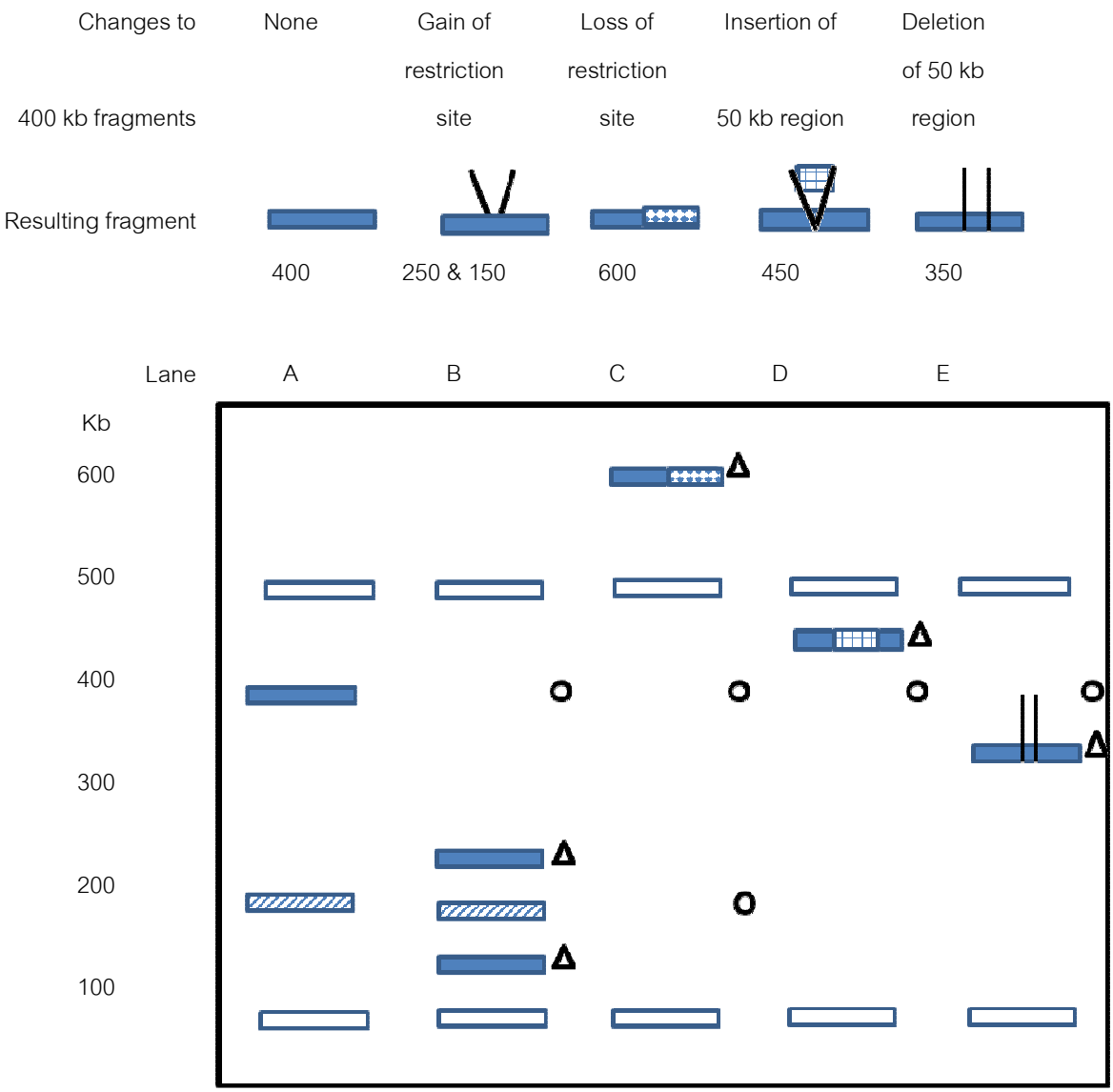
On the other hand, PFGE utilizes rare cutting restriction enzymes to digest genomic DNA, which generates only 10-20 restriction fragments. The pattern of DNA fragments can differentiate genetic clonality among bacterial strains and thus the result is easier to interpret than that of RFLP. Because the bacterial DNA fragments digested by rare cutting restriction enzymes are too large (exceeding 20,000 bp), they cannot migrate through the conventional agarose gel. PFGE resolves this problem by constantly changing the direction of the electrical field to allow large DNA fragments to migrate through agarose gel (Peters, 2009). Varies PFGE patterns derived from the different genetic events (Figure 1).

According, to the Center for Disease Control and Prevention (CDC), PFGE is the gold standard in epidemiological studies of bacterial pathogens including *Salmonella* (Fakhr et al., 2005; CDC, 2011; Xia et al., 2011).

The most common system of PFGE is contour-clamped homogenous electric field system (CHEF) where CDC adopted this system as the standard genotyping technique for *Salmonella* and other six foodborne pathogens (Hunter et al., 2005). CHEF has twenty-four point electrodes around hexagonal contour. As a result, the electric field is periodically switched 120° between two directions, the DNA fragments up to 7,000 kb can be separated (Basim and Basim, 2001).

In addition, CDC recommends that *S.* Braenderup (H9812) restricted with *Xbal* should be used as the "universal" standard strain. Because bands generated from DNA fragments of *S.* Braenderup (H9812) are distributed over the entire range commonly found in foodborne pathogen tracked by the PulseNet, the international database of PFGE. This strain is also genetically stable when subcultured (Hunter et al., 2005).

In this study, the PFGE technique was used because of its discriminatory power, reproducibility, and globally accepted status.




(Modified from Tenover et al., 1995)

Figure 1. Diagram of different PFGE patterns of an isolate as a result of different genetic events. Lane A, reference isolate; Lane B, gain of restriction site; lane C, loss of a restriction site; lane D, insertion of DNA in an existing fragment; lane E, deletion of DNA from an existing fragment. The circles indicate fragments present in the reference pattern and missing from the test isolate after a genetic event; triangles indicate fragment present after a genetic event but absent from the outbreak pattern.

# CHAPTER III

## MATERIALS AND METHODS

### 1. Sample collection

Samples were obtained from a commercial broiler farm in northeastern part of Thailand. The broiler house is close with evaporative cooling system. The size of the house is 10 meters in width and 100 meters in length, which can accommodate approximately 10,000 birds. Sample collections were done at 3 different time periods from the same broiler house for 3 cycle productions during August-October 2010, March-May 2011 and January-March 2012. This broiler house had been used for rearing broiler for 13 flocks during 2010-2012.

From each flock, samples were collected from broiler and farm environment at 4 different steps consecutively of broiler production including bird house preparation, chick arrival, ongoing rearing period (weekly) and slaughter day. Types and number of samples collected from each flock are shown in Table 5 and 6.

Table 5. Samples collected in a chronological order at broiler farms for each flock

| Chronology | Category | Types of sample | *Salmonella* status |
|---|---|---|---|
| **Bird house preparation** | Environment | Floor, Wall, Pan feeder, Watering system, Water, Litter, Pest | Contamination status after cleaning and disinfection |
| **Chick arrival day** | Environment | Floor, Wall, Pan feeder, Watering system, Water, Litter, Pest | Contamination status before placing new chicks |
| | Broiler | Meconium on box liner after transportation | Contamination status of new chicks |
| **Ongoing rearing period (weekly)** | Environment | Floor and litter, Feed, Water | Contamination status during rearing period |
| | Broiler | Cloacal swab or feces | Contamination status of broiler during rearing period |
| **Slaughter day** | Environment | Transportation related environment | Contamination status of environment before birds transportation |
| | Broiler | Feather around cloaca or feces at catch and after arrival at slaughterhouse | Contamination status or broiler at before and after transportation |

Table 6. Sampling plan*

| Chronology | Category | Types of sample | Number of sample | | |
|---|---|---|---|---|---|
| | | | Flock 1 | Flock 2 | Flock 3 |
| **Bird house preparation** | Environment | Floor | 3 | 3 | 3 |
| | | Wall | 2 | 6 | 6 |
| | | Feeding pan | 5 | 20 | 5 |
| | | Nipple | 5 | 20 | 5 |
| | | Water entry the house | - | 1 | 1 |
| | | Water from nipple | 1 | 1 | 1 |
| | | Litter before disinfectant | 5 | 10 | 10 |
| | | Litter after disinfectant | 5 | 10 | 10 |
| | | Pest | 5 | 5 | 5 |
| **Chick arrival day** | Environment | Floor/Litter/Boot swab | - | 5 | 5 |
| | | Wall | - | 6 | 6 |
| | | Feed from hopper/New feed | - | 3 | 2 |
| | | Feed in feeding pan | - | 20 | 5 |
| | | Nipple | - | 20 | 5 |
| | | Water entry the house | - | 1 | 1 |
| | | Water from nipple | - | 1 | 1 |
| | | Pest | - | 5 | 5 |
| | Broiler | Meconium on box-liner | 20 | 10 | 10 |

Table 6. Sampling plan* (continued)

| Chronology | Category | Types of sample | Number of sample | | |
|---|---|---|---|---|---|
| | | | Flock 1 | Flock 2 | Flock 3 |
| **Ongoing rearing period** (D1, 3, 5 and every 7 days) for 6 weeks** | Environment | Floor/Litter/Boot swab | 5 | 5 | 5 |
| | | Feed from hopper/New feed | 3 | 3 | 2 |
| | | Feed from feeding pan | 3 | 5 | 5 |
| | | Water entry the house | - | 1 | 1 |
| | | Water from nipple | 1 | 5 | 5 |
| | | Pest | 5 | 5 | 5 |
| | Broiler | Cloacal swab or feces | 5 | 60 | 60 |
| **Slaughter day** | Environment | Transporting cage | - | 10 | 15 |
| | | Workers' hands before working | - | 10 | 10 |
| | | Workers' hands after working | - | 10 | 10 |
| | | Transporting truck | - | 1 | 1 |
| | | Water for spraying | - | 1 | 1 |
| | | Water after spraying | - | 1 | 3 |
| | Broiler at farm | Feather around cloaca or Cloacal swab | 10 | - | - |
| | Broiler at slaughterhouse | Feather around cloaca or Cloacal swab | 10 | 60 | 60 |
| | Environment | | 115 | 307 | 275 |
| | Broiler | | 70 | 670 | 610 |

* See appendix B for sample collection procedure

** During rearing period shows the number of sample per sampling time

2. Subtyping of *Salmonella* serotypes by PFGE technique

After collecting samples from broiler farm, *Salmonella* was isolated following ISO 6579 and serotype identified following Kauffman-White scheme at National Institute of Health (NIH), Ministry of Public Health. *Salmonella* serotypes which were found in common between broilers and farm environment were selected in this study for investigating their genetic clonality by PFGE.

PFGE is then performed according to the One-Day (24-28 h) Standardized Laboratory for Molecular Subtyping of *Salmonella* serotypes by PFGE, which is defined by the PulseNet (Hendriksen et al., 2010) on a CHEF Mapper (Bio-Rad Laboratories, USA). PFGE patterns in this study were obtained with *Xba*I. In addition, *Salmonella* serotypes which all isolates showed identical PFGE pattern when digested with *Xba*I were re-confirmed that they are indistinguishable by obtained with the second enzyme, *Bln*I. The recognition site of *Xba*I and *Bln*I are shown in Table 7. Pulsed time was ramped from 2.2 s to 63.8 s during 18 hours run at 6.0 V/cm. PFGE patterns were analyzed for genetic similarity by GelCompar II software package (Applied Maths Inc., USA). Moreover, the 3 standard samples (*S.* Braenderub H9812) for each PFGE run were also done according to the PulseNet protocol. PFGE process is summarized as following and also shown in Figure 2.

1) Culture *Salmonella* isolates onto Mueller Hinton agar (MHA) and incubate at 37 $^{o}$C, 14-18 hours.
2) Mix bacterial cells with cell suspension buffer (CSB) (Appendix A). Adjust concentration of cell suspensions to optical density of 0.8-1.0 at 610 nm wavelength.
3) Immobilize bacterial cells in agarose plugs by mixing cell suspension with agarose gel.
4) Lyse bacterial cells in the agarose plugs with detergent (sarcosine) and proteinase K (Appendix A).
5) Wash lysed bacterial cells with water and Tris EDTA (TE) buffer (Appendix A).
6) Digest bacterial DNA with restriction enzyme *XbaI* and *BlnI*.
7) Load the agarose plugs that contain bacterial DNA into agarose gel and run the electrophoresis for 18 hours.

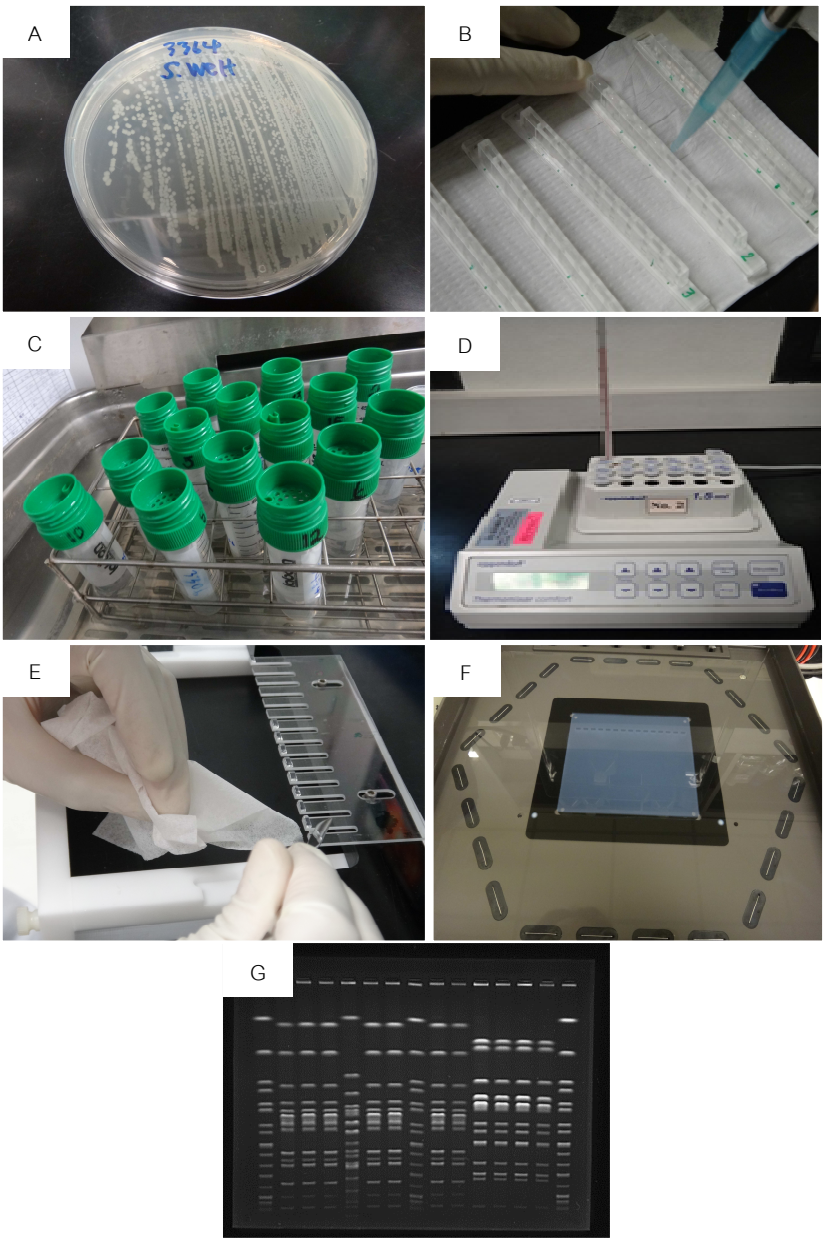



Figure 2. Illustrations of PFGE process; A: Culture *Salmonella* isolates onto Mueller Hinton agar (MHA), B: Immobilize bacterial cells in 1% SeaKem (Lonza, Switzerland) agarose gel, C: After lysis bacterial cells, wash lysed cells with water and TE buffer in 55°C water bath, D: Digest bacterial DNA with restriction enzymes in 37°C water bath, E: Load plugs that contain DNA on to comb teeth, F: Place gel in electrophoresis chamber, and G: Visualize DNA banding patterns of PFGE under UV light.

Table 7. Recognition sites of *Xba*I and *Bln*I

| Restriction enzyme | Recognition sites |
|---|---|
| *Xba*I | 5'….T'C T A G A.…3' |
| | 3'….A G A T C'T.…5' |
| *Bln*I | 5'….C'C T A G G.…3' |
| | 3'….G G A T C'C.…5' |

3. **Interpretation of PFGE banding patterns**

Genetic relatedness analysis of *Salmonella* isolates were categorized into 4 categories following Tenover et al. (1995) as summarized below and shown in Table 8.

1. *Indistinguishable*: Indistinguishable patterns are designated when those isolates have the same numbers of DNA bands and their DNA bands are of the same size. The indistinguishable patterns are considered the same strain.
2. *Closely related*: Closely related patterns are considered when DNA fragments of those isolates differ from each other for 2-3 bands as a result of a single genetic event such as insertion or deletion of DNA (Figure 1).
3. *Possible related*: Possibly related patterns are considered when 4-6 bands of DNA patterns are different, which is a result of two independent genetic events occurred. The possibly related patterns are less likely to be related epidemiologically.
4. *Unrelated*: Unrelated patterns are determined when DNA fragments of those isolates differ from each other 7 or more bands, which is a result of 3 or more independent genetic events.

Table 8. Criteria to differentiate genetic relatedness of PFGE patterns

| Category | No. genetic event differences compared with other isolates | No. of different DNA fragments with other isolates |
|---|---|---|
| Indistinguishable | 0 | 0 |
| Closely related | 1 | 2-3 |
| Possibly related | 2 | 4-6 |
| Unrelated | ≥3 | ≥7 |

Modified from Tenover et al., 1995

In this study, the name of PFGE patterns contained 3 parts for example Da1. The first part is the capital letter(s) which indicates serotype such as D stands for *S.* Derby, AB stands for *S.* Albany, AT stands for *S.* Altona and W stands for *S.* Weltevreden. The second part is the small letter following the capital letter which indicates genetic relatedness, for example, if the PFGE patterns of 2 isolates showed less than or equal to 6 different DNA fragments, the small letter will be the same such as Da and Da. But if any 2 isolates showed more than 6 different DNA fragments, the small letter will be assigned differently such as Da and Db. The last part is numerical number. If the PFGE patterns of 2 isolates are indistinguishable, the last number will be the same such as Da1 and Da1. In contrary, if the PFGE patterns of 2 isolates are closely or possibly related, the last number will be different such as Da1 and Da2.

The degree of similarity between PFGE patterns were also calculated using Dice coefficient and the dendrogram was constructed using UPGMA. The Dice coefficient is used for quantifying the similarity of PFGE banding patterns between 2 isolates. The Dice coefficient is calculated as follow:

$$S_{ij} = \frac{2n_{ij}}{2n_{ij}+n_i+n_j}$$

$S_{ij}$ = similarity between 2 isolates

$n_i$ = number of bands that found only in isolate i,

$n_j$ = number of bands that found only in isolate j

$n_{ij}$ = number of bands that found in both isolates i and j

# CHAPTER IV

# RESULTS

## 1. Isolation of *Salmonella* during broiler production

1.1 First flock *Salmonella* serotypes isolated from the first broiler flock is shown in Table 9. After cleaning and disinfection, 1 of 1 water sample (100%), 2 of 2 wall swab samples (100%), 5 of 5 feeding pan swab samples (100%), 2 of 5 nipple swab samples (40%) and 2 of 5 litter before disinfection (40%) were contaminated with *Salmonella*. During rearing period, 1 of 5 water samples (20%) and 11 of 22 house lizards samples (50%) were *Salmonella* positive. There was no *Salmonella* positive from 20 samples of meconium from box-liner on chick arrival day. Later, during rearing period, 26 of 30 feces samples (86.67%) were found *Salmonella* positive. Throughout the first flock, there were 77 of 185 samples were found *Salmonella* positive.

### 1.1.1 *Salmonella* contamination in the environment of the broiler farm

Although, the broiler house environment had been already cleaned and disinfected, several isolates of *S.* Albany were found from broiler house wall, feeding pan, water and watering system. *S.* Albany was also isolated from a house lizard. In addition, *S.* Weltevreden and *S.* Havana were detected from the new litter before disinfection. However, the litter after disinfection was free from *Salmonella*. *S.* Weltevreden was isolated from house

lizards during rearing period in week 1, 2, 4, 5 and *S.* Hotutenae was isolated from house lizards in week 3 and 5. One of 15 new feed samples (6.7%) was positive for *S.* Derby. *S.* Braenderup was detected from feed in feeding pan in week 2. *S.* Derby was also isolated from water in week 3 (Table 9).

1.1.2 *Salmonella* contamination in the broiler

No *Salmonella spp*. was isolated from the box-liner on the chick arrival day, indicating that the day-old chicks were *Salmonella*-free. After one week of rearing, *Salmonella* was found from all broiler feces samples. The major serotype that was found consistently every week throughout the rearing period was *S.* Derby. The other serotypes that were periodically found during the rearing period were *S.* Caen in week 1, *S.* Weltevreden in week 2, *S.* Bovismorbificans and *S.* Albany in week 3 and *S.* Seftenberg in week 4. On slaughter day, feather around broiler vent was collected before and after transportation of the broiler to the slaughterhouse. *S.* Derby was found from both before and after the transportation. After transportation, some other *Salmonella* serotypes i.e. *S.* Orion, *S.* Stockholm, *S.* Bovismorbificans, *S.* Altona and *S.* Kentucky were also detected.

We found 3 serotypes i.e. *S.* Derby, *S.* Albany and *S.* Weltevreden that were in common to both broiler and farm environment. The environment found to be contaminated in this study were litter before disinfection, broiler house and equipment after cleaning and

disinfection, water, pest, and feed. Furthermore, several *Salmonella* serotypes that were not found during the rearing were isolated from the broiler after broiler was transported to the slaughterhouse i.e. *S*. Orion, *S*. Stockholm, *S*. Altona and *S*. Kentucky (Table 9).

Table 9. *Salmonella* serotypes found in broiler production of the first flock

| Chronology | Broiler | | | | Environment | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Box-liner | Feces | Feather | Boot swab | New feed | Feed in feeding pan | Water | Pest | Equipment |
| After C&D | NA[a] | NA | NA | NA | NA | NA | Albany (1/1) | house lizard: Albany (1/1) | floor: (0/3)<br>wall: Albany (2/2)<br>feeding pan: Albany(5/5)<br>nipple: Albany(2/5)<br>litter before disinfection :Havana (1/5)<br>Weltevreden (1/5)<br>Litter after disinfection : (0/5) |
| Chick arrival day | (0/20)[c] | NA | NA | NA | ND[b] | NA | ND | ND | NA |
| Week 1 | NA | Derby (5/5)<br>Caen (1/5) | NA | Derby (5/5) | (0/3) | (0/3) | (0/1) | house lizard :Weltevreden (1/4) | NA |
| Week 2 | NA | Derby (5/5)<br>Weltervreden (1/5) | NA | (0/5) | Derby (1/3) | Braenderup (1/3) | (0/1) | house lizard :Weltevreden (2/5) | NA |
| Week 3 | NA | Derby (4/5)<br>Albany (1/5) | NA | Derby (5/5) | (0/3) | (0/3) | (0/1) | house lizard :Derby (1/5)<br>house lizard :Hotutenae (1/5) | NA |

Table 9. *Salmonella* serotypes found in broiler production of the first flock (continued)

| Chronology | Broiler | | | | Environment | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Box-liner | Feces | Feather | Boot swab | New feed | Feed in feeding pan | Water | Pest | Equipment |
| Week 4 | NA | Derby( 4/5)<br>Seftenberg (1/5) | NA | Derby (5/5)<br>Albany (1/5)<br>Bovismorbificans (1/5)<br>Kouka (1/5) | (0/3) | (0/3) | (0/1) | house lizard<br>:Weltevreden (2/5) | NS |
| Week 5 | NA | Derby( 3/5) | NA | Derby (4/5) | (0/3) | (0/3) | Derby (1/1) | Weltevreden (1/5)<br>house lizard<br>Hotutenae (2/5)<br>house lizard | NA |
| Week 6 | NA | Derby (4/5) | Derby (1/10) | Derby (5/5)<br>Kentucky (1/5)<br>Bovismorbificans (1/5) | ND | ND | ND | ND | ND |
| Slaughterhouse | NA | NA | Derby (4/10)<br>Orion (2/10)<br>Stockholm (2/10)<br>Bovismorbificans (1/10)<br>Altona (1/10)<br>Kentucky (1/10) | NA | NA | NA | NA | NA | ND |

Remark: [a] NA means Not applicable

[b] ND means Not determined

[c] indicates number of sample that found *Salmonella* positive from all samples

1.2 Second flock *Salmonella* serotypes isolated from the second broiler flock are shown in Table 10. After cleaning and disinfection, we found 2 of 10 litter after disinfection samples (20%) were *Salmonella* positive. Throughout this flock, we collected 35 house lizards and 2 rodents which we found Salmonella positive from 11 of 35 house lizard samples (31.43) but the 2 rodent samples were not found *Salmonella*. There was no *Salmonella* positive from 10 samples of meconium from box-liner on chick arrival day and 600 samples of cloacal swab during rearing period. However, 2 of 60 samples of cloacal swab (3.33%) at slaughterhouse were *Salmonella* positive. Throughout the second flock, there were 21 of 977 samples (2.15%) were found *Salmonella* positive.

1.2.1 *Salmonella* contamination in the environment of the broiler farm

*Salmonella* contamination in the farm environment in the second flock was lower than in the first flock. *S.* Weltevreden was found from house lizards and litter after disinfection. This serotype was again found on the chick arrival day from house lizards, litter and nipple swab. During the rearing period, only *S.* Stanley was detected from boot swab sample in week 5 and *Salmonella* enterica subsp. enterica ser. 4,3:z4,z23:- was detected from house lizards in week 5 and 6 (Table 10).

Before transporting the broiler to slaughterhouse, the equipment and environment related to the transportation process were sampled. *S.* Altona, *S.* Albany and *S.*

Weltevreden were found in transport cages before used and *S.* Mbandaka was found in the transportation vehicle. No Salmonella was detected from water for spraying broiler (to prevent heat stress) and worker hands before catching broiler. But *S.* Altona was detected from worker hands after catching broiler.

1.2.2 *Salmonella* contamination in broiler

The day-old chicks were *Salmonella*-free and the birds were free from contamination throughout the rearing period. However, 3 *Salmonella* serotypes, *S.* Albany, *S.* Derby, *S.* Virginia were detected from broiler after transported to the slaughterhouse. These three serotypes have never been found before in the environment during rearing period of this flock. Notably, the *S.* Albany serotype was also isolated from the transport cage before used (Table 10).

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Table 10. *Salmonella* serotypes found in broiler production of the second flock

| Chronology | Broiler | | | Environment | | | | |
|---|---|---|---|---|---|---|---|---|
| | Box-liner | Cloacal swab | Boot swab | Feed in hopper | Feed in pan | Water | Pest | Other equipment and environment |
| After C&D | NA[a] | NA | NA | NA | NA | (0/2)[c] | house lizard: Weltevreden (3/4)<br>rodent: (0/1) | floor: (0/3)<br>wall: (0/6)<br>feeding pan: (0/20)<br>nipple: (0/20)<br>litter before disinfection: (0/10)<br>litter after disinfection<br>:Weltevreden (2/10) |
| On chick arrival day | (0/10) | NA | NA | (0/3) | (0/20) | (0/2) | houselizard: Weltevreden (3/3) | floor: Weltevreden (1/5)<br>wall: (0/3)<br>nipple : Weltevreden (1/20) |
| Day 1 | NA | (0/60) | (0/5) | (0/3) | (0/5) | (0/2) | ND[b] | NA |
| Day 2 | NA | (0/60) | (0/5) | (0/3) | (0/5) | (0/2) | ND | NA |
| Day 3 | NA | (0/60) | (0/5) | (0/3) | (0/5) | (0/2) | ND | NA |
| Day 5 | NA | (0/60) | (0/5) | (0/3) | (0/5) | (0/2) | ND | NA |
| Week 1 | NA | (0/60) | (0/5) | (0/3) | (0/5) | (0/2) | house lizard: (0/4)<br>rodent: (0/1) | NA |
| Week 2 | NA | (0/60) | (0/5) | (0/3) | (0/5) | (0/2) | house lizard: (0/4) | NA |
| Week 3 | NA | (0/60) | (0/5) | (0/3) | (0/5) | (0/2) | house lizard: (0/5) | NA |
| Week 4 | NA | (0/60) | (0/5) | (0/3) | (0/5) | (0/2) | house lizard: (0/5) | NA |
| Week 5 | NA | (0/60) | Stanley (1/5) | (0/3) | (0/5) | (0/2) | house lizard: 43:Z4Z23:- (4/5) | NA |

Table 10. *Salmonella* serotypes found in broiler production of the second flock (continued)

| Chronology | Broiler | | Environment | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Box-liner | Cloacal swab | Boot swab | Feed in hopper | Feed in pan | Water | Pest | Other equipment and environment |
| Week 6 | NA | (0/60) | (0/5) | (0/3) | (0/5) | (0/2) | house lizard: 43:Z4Z23:- (1/5) | Transport cage: Altona (3/10)<br>Albany (1/10)<br>Weltevreden (1/10)<br>Workers' hands<br>-before catching: (0/10)<br>-after catching: Altona (1/10)<br>Transportation truck: Mbandaka (1/1)<br>Spraying water: (0/2) |
| Slaughterhouse | NA | Albany (1/60)<br>Derby (1/60)<br>Virginia (1/60) | NA | NA | NA | NA | NA | NA |

Remark: [a] NA means Not applicable

[b] ND means Not determined

[c] indicates number of sample that found *Salmonella* positive from all samples

1.3 Third flock *Salmonella* serotypes isolated of the third broiler flock is shown in Table 11. After cleaning and disinfection, 3 of 10 litter before disinfection samples (30%), 5 of 10 litter after disinfection samples (50%) and 1 of 5 nipple swab samples (20%) on chick arrival day were contaminated with *Salmonella*. Throughouut this flock, 1 of 50 water samples collected from nipples (4%) and 1 of 9 water samples collected from pipe before entry the broiler house (11.11%) were *Salmonella* positive. Six of 41 house lizard samples (14.63%) and 3 of 13 centipede samples (23.08%) were *Salmonella* positive but 2 of cockroach samples were *Salmonella* negative. Eight of 10 meconium from box-liner samples on chick arrival day were found *Salmonella*. Later, during rearing period, 122 of 540 cloacal swab samples (22.59%) were found *Salmonella* positive. Throughout the third flock, there were 187 of 885 samples (21.13%) were found *Salmonella* positive.

1.3.1 Environmental *Salmonella* contamination in broiler farm

After cleaning and disinfection, *S.* Weltevreden was isolated from house lizard and litter before and after disinfection. The other serotype isolated from litter after disinfection were *S.* Cannstatt. During rearing period, *S.* Weltevreden was found from a water sample in day 5, centipedes in week 1 and from house lizards in week 5 and 6. This serotype was also found from water for spraying broiler on the slaughter day. Moreover, *S.* Corvallis was isolated from feed in feeding pan on day 3, week 1, 3, 6 and it was found from water after

spraying broiler on the slaughter day. Water sample collected from nipple on day 1 was contaminated with *S. enterica* subsp. *enterica* ser. 4,5,12:i:- (Table 11).

1.3.2 *Salmonella* contamination in broiler

*S.* Corvallis was isolated from meconium of the day-old chicks, indicating the chicks were already contaminated at this stage. After that, we consistently found the serotype *S.* Corvallis from the broiler on day 1, 3, 5 and every week until the slaughter day (Table 11).

In this flock, *S.* Corvallis was the predominant serotype that was found throughout the rearing period and it isolated from both broiler and environment samples. Interestingly, this serotype was initially identified from box-liner samples, which might suggest that the day-old chicks weren't *Salmonella* free in the first place. PFGE was used to disclose the genetic relatedness of *S.* Corvallis from broiler and that from the meconium on box-liner.

Table 11. *Salmonella* serotypes found in broiler production of the third flock

| Chronology | Broiler | | | Environment | | | | |
|---|---|---|---|---|---|---|---|---|
| | Box-liner | Cloacal swab | Boot swab | Feed in hopper | Feed in pan | Water | Pest | Other equipment and environment |
| After C&D | NA[a] | NA | NA | NA | NA | (0/2) | house lizard: Weltevreden (2/3)<br>cockroach: (0/1) | floor: (0/3)<br>wall: (0/3)<br>feeding pan: (0/5)<br>nipple: (0/5)<br>litter before disinfection: Weltevreden (3/10)<br>litter after disinfection: Weltevreden (2/10)<br>Cannstatt (3/10) |
| Chick arrival day | Corvallis (8/10) | NA | NA | (0/2) | (0/5) | (0/6) | House lizard: (0/3)<br>rodent: (0/1)<br>cockroach: (0/1) | floor: (0/3)<br>wall: (0/6)<br>nipple: (1/5) |
| Day 1 | NA | Corvallis (2/60) | Corvallis (4/5) | (0/2) | (0/5) | water from nipple: 4,5,12:i:- (1/5)<br>water entry house (0/1) | house lizard: (0/4) | NA |
| Day 3 | NA | Corvallis (9/60) | 0/5 | (0/2) | Corvallis (1/5) | water from nipple (0/5)<br>water entry house (0/1) | house lizard: (0/2) | NA |

Table 11. *Salmonella* serotypes found in broiler production of the third flock (continued)

| Chronology | Broiler | | Environment | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Box-liner | Cloacal swab | Boot swab | Feed in hopper | Feed in pan | Water | Pest | Other equipment and environment |
| Day 5 | NA | Corvallis 3/60 | 0/5 | (0/2) | (0/5) | water from nipple (0/5) Water entry house Weltevreden (1/1) | houselizard: (0/3) centipede: Corvallis (1/2) | NA |
| Week 1 | NA | Corvallis (51/60) | Corvallis (5/5) | (0/2) | Corvallis (4/5) | (0/6) | house lizard: (0/2) centipede: Weltevreden (2/11) | NA |
| Week 2 | NA | Corvallis (46/60) | Corvallis (4/5) | (0/2) | Corvallis (5/5) | (0/6) | house lizard: Corvallis (1/4) | NA |
| Week 3 | NA | Corvallis (2/60) | (0/5) | (0/2) | Corvallis (1/5) | (0/6) | house lizard: (0/5) | NA |
| Week 4 | NA | Corvallis (6/60) | (0/5) | (0/2) | (0/5) | (0/6) | house lizard: (0/5) | NA |
| Week 5 | NA | Corvallis (1/60) | Eastbourne (1/5) | (0/2) | (0/5) | (0/6) | house lizard: Weltevreden (2/5) | NA |

Table 11. *Salmonella* serotypes found in broiler production of the third flock (continued)

| Chronology | Broiler | | Environment | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Box-liner | Cloacal swab | Boot swab | Feed in hopper | Feed in pan | Water | Pest | Other equipment and environment |
| Week 6 | NA | Corvallis (2/60) | Corvallis (5/5) | (0/2) | Corvallis (2/5) | (0/6) | house lizard: Weltevreden (2/5) | Transport cage before use: (0/15)<br>Workers' hands: -before catching: (0/10)<br>-after catching: (0/10)<br>Spraying water-before use: Weltevreden (1/1)<br>-after use: Weltevreden (1/3)<br>: Corvallis (2/3) |
| Slaughterhouse | NA | Corvalis (15/60) | NA | NA | NA | NA | NA | NA |

Remark: [a] NA means Not applicable

[b] ND means Not determined

[c] indicates number of sample that found *Salmonella* positive from all samples

**2. Genetic relatedness of each *Salmonella* serotypes**

2.1 First flock

There were 3 serotypes, S. Derby, *S.* Albany and *S.* Weltevreden that were in common between broiler and environmental samples. The sample details and PFGE patterns of the tested isolates were shown in Table 12.

Forty-one isolates of *S.* Derby from selected broiler and environmental samples were identified by PFGE and all were shown to have an identical PFGE patterns (Da1) regardless of restriction enzyme, *Xba*I or *Bln*I used (Figure 3).

The Da1 pattern of *S.* Derby was found from broiler feces from week 1-6 to slaughter day and also found from new feed in week 2, house lizard in week 3 and water in week 5. Seven isolates of *S.* Albany from new litter before disinfection, house lizard, environment after cleaning and disinfection and broiler in week 3 were characterized and showed only one PFGE pattern and designated as ABa1 (Figure 3). In contrast, 8 isolates of *S.* Weltevreden from broiler feces in week 3 and house lizards in week 1, week 4 and week 5 showed 6 different PFGE patterns. An identical pattern (Wb1) were obtained between isolates from the new litter (before the litter was disinfected) and the house lizard in the second week. The PFGE patterns of S. Weltevreden isolated from house lizards from different sampling times were of different patterns indicating dynamics of the serotypes in

this host. Notably, the PFGE patterns of the isolates from house lizards in week 1 (Wa1) are closely related to those of the isolates obtained from feces of the broiler in subsequence week (Wa2) with only 1 band different.

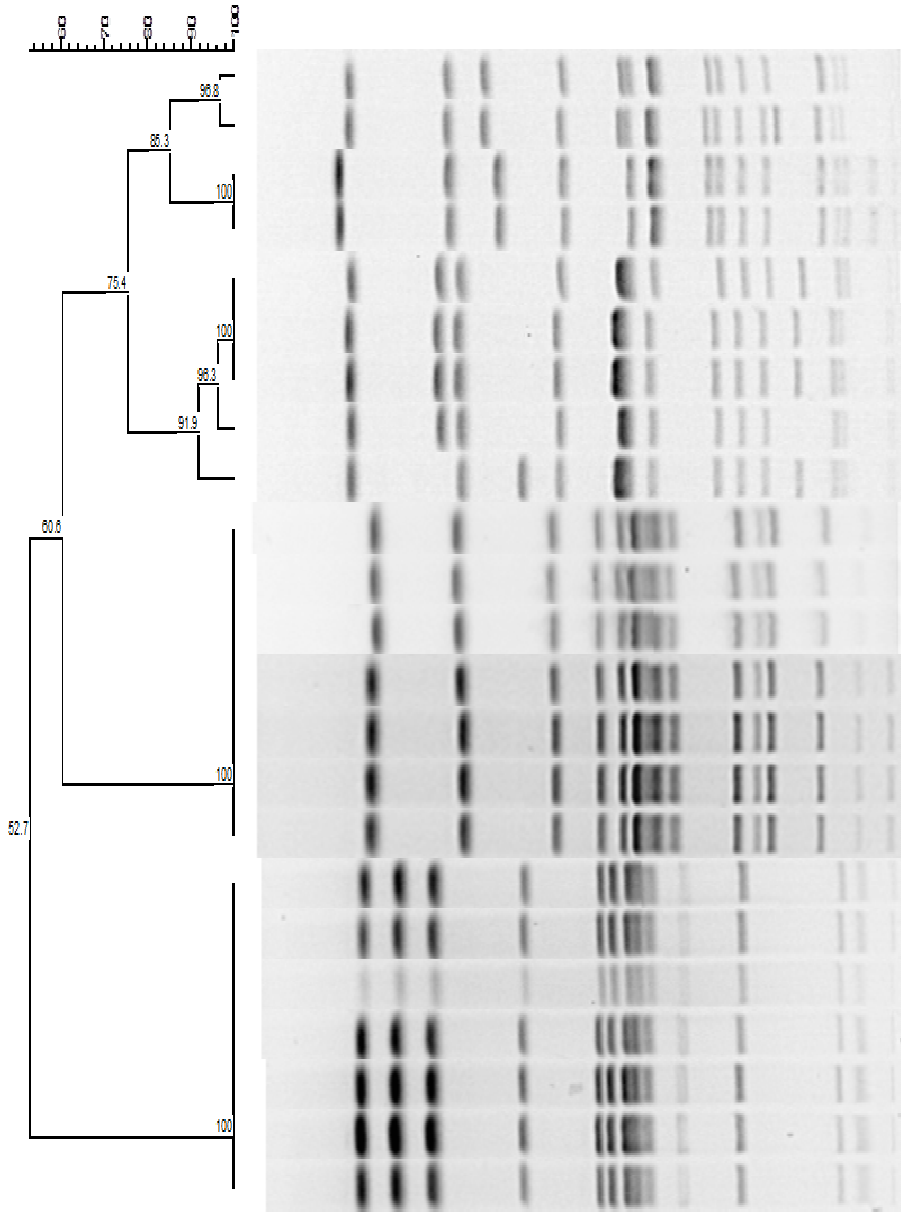
Table 12. PFGE subtypes of *Salmonella* isolates from broiler and environmental samples of the first flock.

| Serotype | Chronology | Type of sample | Number of *Salmonella* positive sample | Number of selected isolates for PFGE | PFGE subtype |
|---|---|---|---|---|---|
| *S.* Derby | Week 1 | Feces | 5 | 3 | Da1 |
| | | Boot swab | 5 | 3 | Da1 |
| | Week 2 | Feces | 5 | 3 | Da1 |
| | | New feed | 1 | 1 | Da1 |
| | Week 3 | Feces | 4 | 3 | Da1 |
| | | Boot swab | 5 | 3 | Da1 |
| | | House lizard | 1 | 1 | Da1 |
| | Week 4 | Feces | 4 | 3 | Da1 |
| | | Boot swab | 5 | 3 | Da1 |
| | Week 5 | Feces | 5 | 3 | Da1 |
| | | Boot swab | 4 | 3 | Da1 |
| | | Water | 1 | 1 | Da1 |
| | Week 6 - at farm | Feces | 5 | 3 | Da1 |
| | | Boot swab | 5 | 3 | Da1 |
| | | Feather | 1 | 1 | Da1 |
| | - at slaughterhouse | Feather | 4 | 4 | Da1 |

Table 12. PFGE subtypes of *Salmonella* isolates from broiler and environmental samples of the first flock (continue).

| Serotype | Chronology | Type of sample | Number of *Salmonella* positive sample | Number of selected isolates for PFGE | PFGE subtype |
|---|---|---|---|---|---|
| *S.* Weltevreden | After disinfection | New litter | 1 | 1 | Wb1 |
| | Week 1 | House lizard | 1 | 1 | Wa1 |
| | | Feces | 1 | 1 | Wa2 |
| | Week 2 | House lizards | 2 | 2 | Wb1 |
| | Week 4 | House lizards | 2 | 2 | Wb2 |
| | Week 5 | House lizard | 1 | 1 | Wa3 |
| *S.* Albany | After disinfection | Wall | 2 | 1 | ABa1 |
| | | Nipple | 2 | 1 | ABa1 |
| | | Feeding pan | 5 | 1 | ABa1 |
| | | Water | 1 | 1 | ABa1 |
| | | House lizard | 1 | 1 | ABa1 |
| | Week 3 | Feces | 1 | 1 | ABa1 |
| | Week 4 | Boot swab | 1 | 1 | ABa1 |

*




| Serotype | PFGE subtype |
|---|---|
| *S.* Weltevreden | Wa1 |
| | Wa2 |
| | Wa3 |
| | Wa3 |
| | Wb1 |
| | Wb1 |
| | Wb1 |
| | Wb2 |
| | Wb3 |
| *S.* Derby | Da1 |
| | Da1 |
| | Da1 |
| | Da1 |
| | Da1 |
| | Da1 |
| | Da1 |
| *S.* Albany | ABa1 |
| | ABa1 |
| | ABa1 |
| | ABa1 |
| | ABa1 |
| | ABa1 |
| | ABa1 |

Figure 3. PFGE patterns of *S.* Weltevreden, *S.* Derby and *S.* Albany with XbaI restriction enzyme. Remark: * indicates similarity index

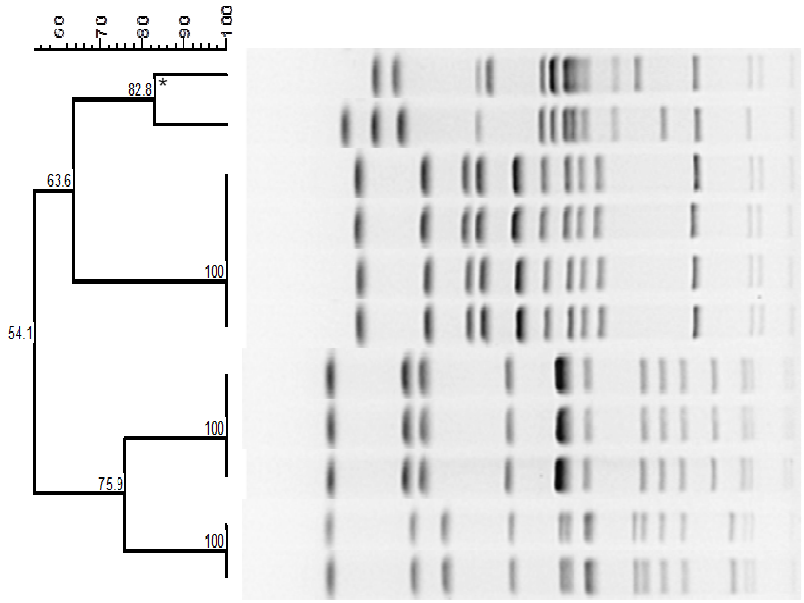
An identical PFGE pattern of *S.* Derby from water, new feed, house lizard and broiler could infer that new feed and water were the possible sources of *Salmonella* to this broiler flock. After some broiler was infected, it could transmit and spread the microorganism to the entire flock via the environment and pest. In addition, an identical PFGE pattern of *S.* Albany from water, house lizard and house and equipment after cleaning and disinfection indicated that the contaminated water may be the cause of the contaminated house and equipment. The house lizards in the broiler house were highly contaminated and may be considered as a reservoir or a vehicle for transmission of the *Salmonella* throughout the broiler house.

2.2 Second flock

There was only one serotype, *S.* Albany that was in common between the broiler and the environment at the step of live broiler at slaughterhouse and the transport cage before used. However, the PFGE patterns revealed that these isolates were not related since, as much as 7 band positions are different (Table 13 and Figure 4). The PFGE patterns of *S.* Albany from broiler at slaughterhouse and from transport cage were designated as ABa2 and ABb1, respectively (Figure 4). This evidence indicated that *S.* Albany from broiler after transporting to slaughterhouse was not derived from *S.* Albany isolated from transport cage.

Table 13. PFGE subtypes of *S*. Albany, *S*. Altona and *S*. Weltevreden of the second flock.

| Serotype | Chronology | Type of sample | Number of *Salmonella* positive sample | Number of selected isolates for PFGE | PFGE subtype |
|---|---|---|---|---|---|
| *S*. Albany | On Slaughter day | | | | |
| | -at farm | -Transport cage before use | 1 | 1 | ABb1 |
| | -at slaughterhouse | -Cloacal swab | 1 | 1 | ABa2 |
| S. Altona | On Slaughter day | -Transporting cage before used | 3 | 3 | ATa1 |
| | | -Worker hands after worked | 1 | 1 | ATa1 |
| *S*. Weltevreden | After cleaning and disinfection | -House lizards | 3 | 3 | Wa1, |
| | | -Litter after disinfection | 1 | 1 | Wb1 |




| Serotype | PFGE Subtype |
|---|---|
| *S.* Albany | ABb1 |
| | ABa2 |
| *S.* Altona | ATa1 |
| | ATa1 |
| | ATa1 |
| | ATa1 |
| *S.* Weltevreden | Wb1 |
| | Wb1 |
| | Wb1 |
| | Wa1 |
| | Wa1 |

Figure 4. PFGE patterns of *S.* Albany, *S.* Altona and *S.* Weltevreden with *Xba*I restriction enzyme

Remark: * indicates similarity index

In addition, we found a serotype, *S.* Altona, from before-used transport cages and worker hands after catching the birds. After subtyping these *S.* Altona isolates by PFGE with both *Xba*I and *Bln*I, we found that their PFGE patterns were indistinguishable. The result indicated that the pathogen may transfer from the contaminated cages to the worker hands which may in turn contaminate the broiler.

Interestingly, we also found *Salmonella* isolates of the same serotype with an indistinguishable PFGE pattern in house lizards from different flocks that were collected at different time (Table 14), indicating that this pest may play a significant role as a continuous reservoir for *Salmonella* in the broiler farm.

Table 14. PFGE subtypes of *S*. Weltevreden from the first and the second flock.

| Serotype | Flock No. | Chronology | Type | PFGE subtype |
|---|---|---|---|---|
| *S*. Weltevreden | 1 | After cleaning and disinfection | New litter | Wb1 |
| | 1 | Week 1 | House lizard | Wa1 |
| | 1 | Week 4 | House lizards | Wb1 |
| | 2 | After cleaning and disinfection | House lizards | Wa1, Wb1 |
| | 2 | After cleaning and disinfection | Litter after disinfection | Wb1 |

2.3 Third flock

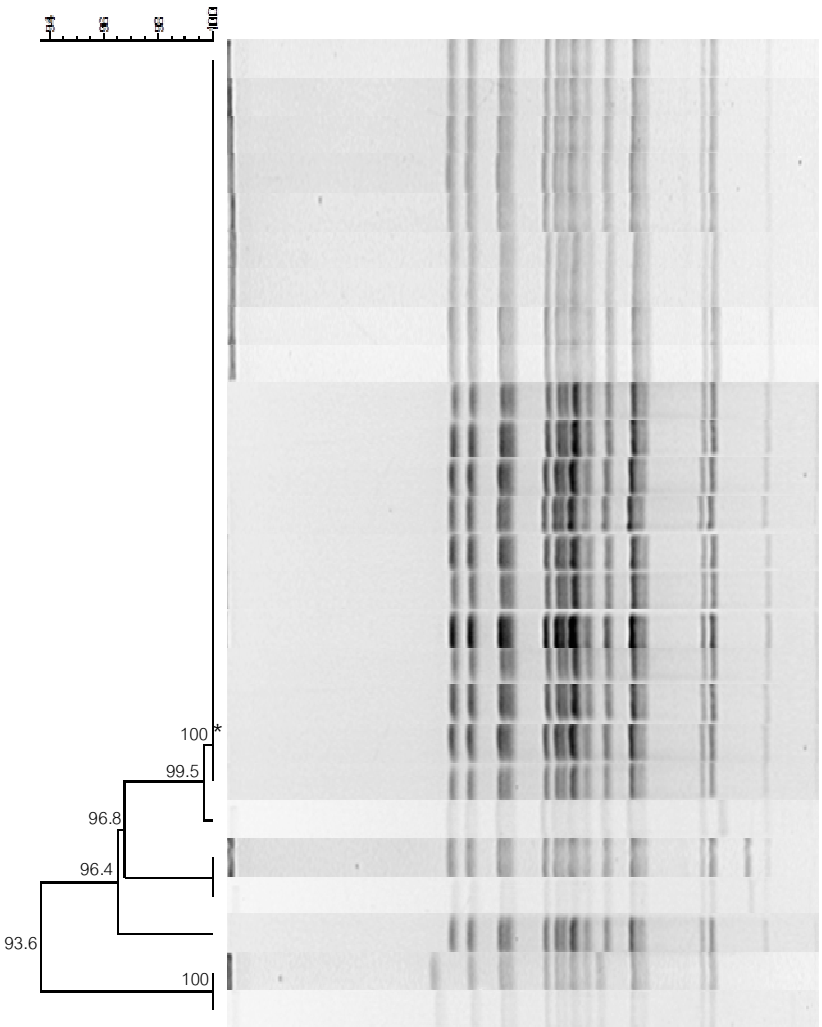
*S.* Corvallis was the only serotype that was found in common between broiler and environment in this flock. Fifty isolates of *S.* Corvallis from both broiler and environmental samples including feed in pan feeder, pest and water after spraying broiler (before they were sent to slaughterhouse) were subtyped by PFGE. The PFGE revealed that there were 5 different PFGE patterns of *S.* Corvallis in this flock. They were arbitrary termed as Ca1, Ca2, Ca3, Ca4 and Cb1 (Table 15 and Figure 5). Majority of the isolates (44 of 50 isolates) exhibited Ca1 pattern, which was found from meconium samples and cloacal swab of broiler on day 1, 3, 5 and every week until prior to slaughter. The Ca1 pattern was also found in pest on day 5 and week 2 and feed samples from feeding pan on day 3 and week 1, 2, 3, 6. The PFGE result apparently showed that the source of *S.* Corvallis in this flock was from day-old chick and the strain persisted throughout the rearing period until slaughter. Moreover, the result obviously showed that infected broiler can transmit the pathogen to the environment especially feed in feeder pan and pest. Then, the contaminated environment can enhance dissemination and circulation of the pathogen in the broiler farm. Other closely related patterns (with 1-2 genetic events) were sporadically found in cloacal swab and boot swab samples as shown in Table 15.

Table 15. PFGE subtypes of *S*. Corvallis of the third flock

| Serotype | Chronology | Type of samples | Number of *Salmonella* positive sample | Number of selected isolates for PFGE | PFGE subtype |
|---|---|---|---|---|---|
| *S*. Corvallis | Chick arrival day | Meconium on box-liner | 8 | 8 | Ca1 |
| | Day 1 | Cloacal swab | 2 | 2 | Ca1 |
| | | Boot swab | 4 | 3 | Ca1 |
| | Day 3 | Cloacal swab | 9 | 2 | Ca1 (1/2) Cb1 (1/2) |
| | | Feed from pan | 1 | 1 | Ca1 |
| | Day 5 | Cloacal swab | 3 | 2 | Ca1 |
| | | Centipede | 1 | 1 | Ca1 |
| | Week 1 | Cloacal swab | 51 | 5 | Ca1 (4/5), Ca3 (1/5) |
| | | Boot swab | 5 | 2 | Ca1 (1/2), Ca3 (1/2) |
| | | Feed from pan | 4 | 2 | Ca1 |
| | Week 2 | Cloacal swab | 44 | 4 | Ca1 (3/4), Ca2 (1/4) |
| | | Boot swab | 5 | 2 | Ca3 |
| | | Feed from pan | 5 | 2 | Ca1 |
| | | House lizard | 1 | 1 | Ca1 |
| | Week 3 | Cloacal swab | 2 | 2 | Ca1 (1/2), Cb1 (1/2) |
| | | Feed from pan | 1 | 1 | Ca1 |
| | Week 4 | Cloacal swab | 6 | 2 | Ca1 (1/2), Ca4 (1/2) |
| | Week 5 | Cloacal swab | 1 | 1 | Ca1 |
| | Week 6 | Cloacal swab | 2 | 2 | Ca1 |
| | | Boot swab | 5 | 3 | Ca1 |
| | | Feed from pan | 2 | 1 | Ca1 |

Table 15. PFGE subtypes of *S*. Corvallis of the third flock

| Serotype | Chronology | Type of samples | Number of *Salmonella* positive sample | Number of selected isolates for PFGE | PFGE subtype |
|---|---|---|---|---|---|
| | Slaughter day | | | | |
| | -at farm | Water after spraying to broiler | 2 | 1 | Ca1 |
| | -at slaughterhouse | Cloacal swab | 15 | 3 | Ca1 |




| Serotype | PFGE subtype |
|---|---|
| *S.* | Ca1 |
| Corvallis | Ca1 |
| | Ca1 |
| | Ca1 |
| | Ca1 |
| | Ca1 |
| | Ca1 |
| | Ca1 |
| | Ca1 |
| | Ca1 |
| | Ca1 |
| | Ca1 |
| | Ca1 |
| | Ca1 |
| | Ca1 |
| | Ca1 |
| | Ca2 |
| | Ca3 |
| | Ca3 |
| | Ca4 |
| | Cb1 |
| | Cb1 |

Figure 5. PFGE patterns of *S.* Corvallis with *Xba*I restriction enzyme

Remark: * indicates similarity index

# CHAPTER V

# DISCUSSION

This study collected samples from the same broiler house of a commercial broiler farm in the northeastern part of Thailand for 3 of 13 cycle productions during 2010-2012. The total number of samples from broiler and environment were 1,350 and 697, respectively. In this study, PFGE showed more discrimination power than serotyping method, which revealed the genetic relationship of *Salmonella* isolates among same serotype.

In the first flock of this study, PFGE showed an identical pattern of *S.* Derby between environment i.e. new feed in week 2, house lizard in week 3, water in week 5 and broiler in week 1-6 and until prior to slaughter (Table 12 and Figure 4). Though, the broiler samples were positive since the first week, the feed and water in the second and fifth week, respectively. It is highly possible that some of the feed may be sporadically contaminated at the earlier stage before being detected. Other possible explanation is that the serotype may derive from the vertical transmission of the day-old chicks from the parental stock. However, this possibility was ruled out because the meconium on the box-liner (an equivalent of 2,040

bird samples) were *Salmonella* negative (Table 9), indicating that the day-old chicks were free from *Salmonella*. After some broiler in the first flock was infected via contaminated feed or water, they could spread the microorganism to the other birds by contaminating the house environment and pest such as house lizards, which can enhance and continuously disseminate and circulate *Salmonella* to the entire flock. It was previously reported in conjunction with the result of the present study that feed is one of significant sources of *Salmonella* introduction to poultry flocks (Davies et al., 1997). Feed contamination less than 1 *Salmonella*/gram could infect young chicks (Hinton, 1988).

Moreover, we also found an identical PFGE pattern of *S.* Albany between the environment i.e. water and house lizard during downtime period, broiler house and equipment including feeding pan and nipple after disinfection, and broiler in week 3 (Table 12). Therefore, it could be inferred that the contaminated water may be another important source of *Salmonella* contamination to the broiler house and equipment. The house lizards, again, were suspected to play a role in circulating this *Salmonella serotypes* throughout the broiler house. However, previous studies suggested that the broiler house and equipment contamination could be associated with the inappropriate cleaning and disinfection process or introduction of portable equipment into the disinfected house (Heyndrickx et al., 2002). Inappropriate cleaning and disinfection, for instance, insufficient amount of disinfectant,

existence of organic material after cleaning which can reduce the efficiency of disinfectant (Marin et al., 2011), the persist of microbial carrier in the farm such as rodent, flies, etc. (Nogrady et al., 2008). In order to prevent broiler house and equipment from recontamination after cleaning and disinfection, the houses and equipment should be thoroughly cleaned to eliminate organic material. The mobile equipment such as feeding pan, nipple etc. should be dismantled to facilitate cleaning and disinfection process (Le Bouquin et al., 2010). Disinfectant should be approved by the Veterinary Authorities (Marin et al., 2011). Conducting pest control and periodically monitoring and restricting the entry of personnel to the disinfected house should be administered (Myint, 2004).

For another serotype, *S.* Weltevreden, the PFGE pattern of the isolates found in the broiler differenced from those found in the environment (new litter before disinfection). We found identical PFGE pattern between the new litter and the house lizards in week 2, indicating possible route of transmission from the litter to the house lizards. Hence, the quality of the litter should be concerned because the pest in the broiler farm especially, house lizards, can enhance the circulation of *Salmonella.* Furthermore, after transportation of the broiler to the slaughterhouse, several *Salmonella* serotypes that have never been found before were isolated from the broiler. This result indicated that the transportation process may have another risk factor of the *Salmonella* contamination to the broiler.

Therefore, in the later sampling period (flock 2 and 3) additional samples were collected during transportation, i.e., the bird catcher personnel, transport cages, water for spray the birds on transport trucks, and the trucks were examined.

In the second flock, the new feed, water, and day-old chicks are all free from *Salmonella*, and the farm environments were mostly negative indicating effective farm management for this flock. The result showed that the broiler were also *Salmonella*-free until the end of the rearing period. At transporting step, three isolates of *S.* Altona were detected from the transport cages and these isolates showed identical PFGE pattern with the worker hands after catching the birds (Table 13). Thus, this study indicates possibility of *Salmonella* transmission from contaminated transport cages to worker hands and may in turn to contaminate the broiler. Similar to this study, Heyndrickx et al. (2002) suggested that improving hygiene management during transportation of broiler could reduce the risk of *Salmonella* contamination of poultry meat significantly.

Furthermore, this study found that house lizards may play a significant role as a continuous reservoir for the *Salmonella* in the broiler farm. Because we found that *S.* Weltevreden isolated from some house lizards from the different flocks that were collected at different time (the first two flocks) shared an indistinguishable PFGE pattern (Table 14). House lizard was previously considered as a reservoir of *Salmonella* (Bockemuhl and

Moldenhauer, 1970). It is worth to note that for this study the other kinds of pest including rodent or litter beetle were not found because these pest are well controlled in this farm. However, rodent and insect are generally known as a source of *Salmonella* contamination to poultry flock and they should be controlled (Gast, 2003; Lapuz et al., 2007).

In the third flock, the common PFGE pattern of *S.* Corvallis from the day-old chicks samples and broiler throughout the rearing period (Table 15) strongly indicated that the day-old chicks were the main source of the *Salmonella* contamination in this broiler flock. This result also showed that the *Salmonella* infection in the day old chicks can persist until the broiler was slaughtered. Previous studies also found that *Salmonella* infection in day-old chicks was one of the risk factors for *Salmonella* infection in broiler flock (Rose et al., 1999; Cardinale et al., 2004; Namata et al., 2009; Marin et al., 2011). Day-old chicks can be infected with *Salmonella* by vertical transmission which can also be controlled by vaccination of the parent stock with *Salmonella* vaccine (Namata et al., 2009). However, other investigator found that horizontal transmission at hatchery is a major route of *Salmonella* contamination to day-old chicks (Rose et al., 1999). Kim et al. (2007) also found that both hatcheries and breeder farms played an important role in *Salmonella* contamination in broiler production. Several factors have been associated with higher probability of *Salmonella* contamination in day old chicks, for examples, mixing eggs from

various parent stocks in the hatchery, higher average egg hatchability, manual separation of eggshell and bird, and the greater amount of feces and fluff in day old chick transportation boxes. The amount of feces and fluff in transport boxes is likely to occur when either transportation distance or duration is extended (Volkova et al., 2011).

In addition, we found that only 3 days after the infected day-old chicks were delivered to the farm, the feed in feeding pans was found contaminated with the same pattern of *S*. Corvallis (Table 15). The contamination of the feed in feeding pans could happen for the fact that the infected chicks can shed the pathogen in their feces then some droppings could certainly be found in the feeding pans. The contamination of the feed in the feeding pan rapidly enhanced wide spread of *Salmonella* throughout the broiler house.

In conclusion, the possible primary sources of *Salmonella* contamination found in this study were the contaminated day-old chicks, contaminated feed and water. In addition, the pest especially house lizards that were prevalent on this farm might play an important role as a reservoir and spreading of the salmonella pathogen in the housing environments.

## Conclusions and Suggestions

This study provided the information of *Salmonella* introduction to broiler production in a chronological order and revealed possible sources of *Salmonella* contamination in a commercial broiler farm by using PFGE. The possible primary or main sources of *Salmonella* contamination in broiler production were identified as the infected day-old chicks, contaminated water and new feed. Whereas the pest, especially house lizard, could be the secondary source of *Salmonella* contamination derived from the primary sources. The result from the current study can be applied to establish risk management options for *Salmonella* control in broiler production. The suggested risk management measures are as the following.

1. Breeder flock

   - *Salmonella* vaccination in parent stocks to minimizing vertical transmission to broiler flock.

2. Hatchery

   - Separate eggs from different breeding flocks.

   - Improve personal hygiene for workers especially at egg shell and bird separation step and bird processing step.

   - Reduce distance and/or duration of transportation.

3. Broiler farm

- Improve water treatment and routinely monitor the water quality.

- Improve cleaning and disinfection process of broiler house and equipment such as the houses and equipment are thoroughly eliminated organic material, the mobile equipment such as feeding pan, nipple etc. should be dismantled to facilitate cleaning and disinfection process, adequate amount of approved disinfectant should be employed.

- Prevent broiler house and equipment from *Salmonella* recontamination after disinfection by conducting pest control and periodically monitoring, restrict the entry of personnel, prohibit the introduction of non-disinfected equipment, improve the disinfection process of litter.

- Use pellet feed from reliable feedmill and keep feed at farm in good containment or storage room.

- Control and monitor of pest routinely

- Improve hygiene of transportation related equipment such as transport cage, and cleaning water.

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# APPENDICES

## APPENDIX A Chemical substances used for PFGE

1. Tris- EDTA buffer (TE) 1,000 ml. contains

   - 1 M Tris-HCl, pH 8.0 10 ml.
   - 0.5 M EDTA, pH 8.0 2 ml.
   - Dilute with sterile Ultrapure water to 1,000 ml.

2. 0.5X Tris-Borate EDTA buffer (TBE) 2,000 ml. contains

   - 5X TBE buffer 200ml.
   - Dilute to sterile Ultrapure water 2,000 ml.

3. Cell suspension buffer 100 ml contains

   - 1 M Tris-HCl, pH 8.0 10 ml.
   - 0.5 M EDTA, pH 8.0 20 ml.
   - Dilute with sterile Ultrapure water to 100 ml.

4. Cell lysis buffer

   - 1 M Tris-HCl, pH 8.0 25 ml.
   - 0.5 M EDTA, pH 8.0 50 ml.
   - 10% Sarcosyl 50 ml.
   - Dilute with sterile Ultrapure water to 500 ml.

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

5. Ethidium bromide 10mg/ml

- Ethidium bromide 1: 10,000 Ultrapure water

6. Proteinase K (USBiological, USA)

7. *XbaI* restriction enzyme and buffer (Toyobo, Japan)

8. *BlnI* restriction enzyme and buffer (Sibenzyme, USA)

9. SeaKem® gold agarose gel (Lonza, Switzerland)

10. Pulsed field certified agarose (Biorad, Canada)

11. Mueller Hinton agar (Difco, USA)

## APPENDIX B Samples collection

1. Broiler sample

Table B-1. Broiler sample collection procedure

| Sample | 1st flock | | 2nd flock | | 3rd flock | |
|---|---|---|---|---|---|---|
| | Description | Number of samples/ sampling time | Description | Number of sample/ sampling time | Description | Number of sample/ sampling time |
| Day-old chick | meconium from box liner; 1 box (containing 102 birds) /sample | 20 | meconium from box liner; 1 box (containing 102 birds) /sample | 10 | meconium from box liner; 1 box (containing 102 birds) /sample | 10 |
| Broiler | pool feces 60 gram/sample | 5 | cloacal swab 1 broiler/ sample | 60 | cloacal swab 1 broiler/ sample | 60 |

Figure B-1. Meconium on box-liner samples

Figure B-2. Feces sample collection

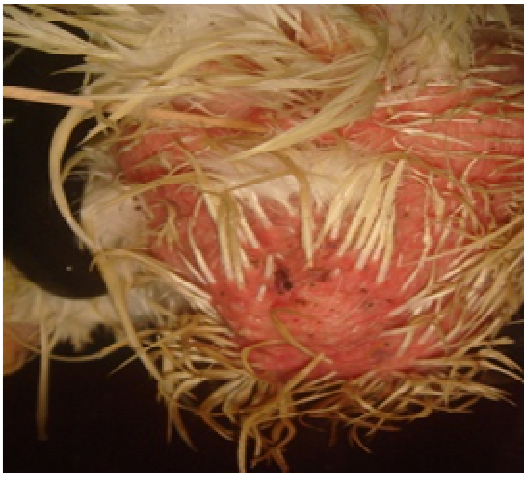

Figure B-3. Cloacal swab collection

2. Environmental sample

Table B-2.Broiler farm environmental sample collection procedure

| Sample | 1st flock | | 2nd flock | | 3rd flock | |
|---|---|---|---|---|---|---|
| | Description | Number of samples/ sampling time | Description | Number of sample/ sampling time | Description | Number of sample/ sampling time |
| Floor swab | Swab 1 sq.m./sample | 3 | Swab 1 sq.m./sample | 3 | Swab 1sq.m./sample | 3 |
| Wall swab | Swab 1 sq.m./sample | 2 | Swab 1 sq.m./sample | 6 | Swab 1sq.m./sample | 6 |
| Feeding pan swab | Swab 5 feeding pans/sample | 5 | Swab 1 feeding pans/sample | 20 | Swab 5 pans/sample | 5 |
| Nipple swab | Swab 5 nipples/sample | 5 | Swab 1 nipple/sample | 20 | Swab 5 nipples/sample | 5 |
| Litter | Collect 300 gram/sample | 5 | Collect 300 gram/sample | 10 | Collect 300 gram/sample | 10 |
| Boot swab | 1 pair of boot swab/sample | 5 | 1 pair of boot swab/sample | 5 | 1 pair of boot swab/sample | 5 |
| New feed | Collect feed in new package and/or hopper 300 gram/sample | 3 | Collect feed from hopper 300 gram/sample | 3 | Collect feed from hopper 300 gram/sample | 2 |

Table B-2.Broiler farm environmental sample collection procedure (continued)

| Sample | 1st flock | | 2nd flock | | 3rd flock | |
|---|---|---|---|---|---|---|
| | Description | Number of samples/ sampling time | Description | Number of sample/ sampling time | Description | Number of sample/ sampling time |
| Feed from Feeding pan | Collect 300 gram of feed from 5 feeding pan/sample | 3 | Collect 300 gram of feed from 5 feeding pans/sample | 5 | Collect 300 gram of feed from 5 feeding pans/sample | 5 |
| Water entry the house | - | - | Collect 100 ml of water Before entry the house | 1 | Collect 100 ml of water Before entry the house | 1 |
| Water from nipples | Collect 100 ml of water from nipple/sample | 1 | Collect 200 ml of water from 10 nipples/sample | 5 | Collect 200 ml of water from 10 nipples/sample | 5 |
| Pest | 1 pest/sample | 5 | 1 pest/sample | 5 | 1 pest/sample | 5 |
| Transport cage Swab | - | - | Swab 1 cage/sample | 10 | Swab 1 cage/sample | 15 |
| Transport vehicle Swab | - | - | Swab 1000 $cm^2$ of transport vehicle | 1 | Swab 1000 $cm^2$ of transport vehicle | 1 |
| Spraying water (before used) | - | - | Collect 200 ml of water | 1 | | 1 |

Table B-2.Broiler farm environmental sample collection procedure (continued)

| Sample | 1st flock | | 2nd flock | | 3rd flock | |
|---|---|---|---|---|---|---|
| | Description | Number of samples/ sampling time | Description | Number of sample/ sampling time | Description | Number of sample/ sampling time |
| Spraying water (after used) | - | - | Collect 200 ml of water | 1 | Collect 200 ml of water | 3 |
| Bird catcher's hands swab | - | - | Swab 1 person/sample | 10 | Swab 1 person/sample | 10 |

Figure B-4. Floor swab collecting

Figure B-5. Wall swab collecting

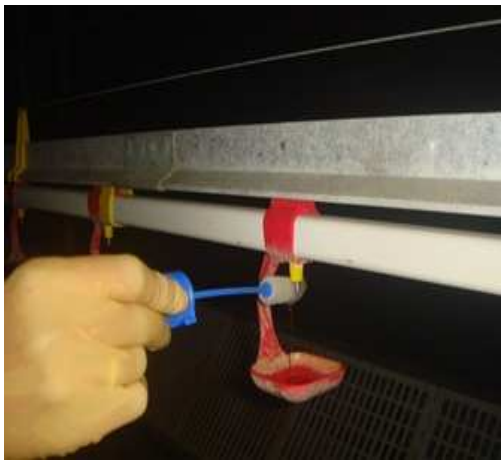

Figure B-6. Nipple swab collecting

Figure B-7. Feeding pan swab collecting

Figure B-8. Boot swab collecting

Figure B-9. Feed sample

Figure B-10. Transport cage swab collecting

## APPENDIX C Pulsed-Field Gel Electrophoresis (PFGE) process

1. Culture *Salmonella* isolates onto Mueller Hinton agar (MHA) and incubate at 37 °C for 14-18 hours.
2. Remove some of *Salmonella* colonies from the MHA by sterile cotton swab. Suspend cells into 2 ml. of Cell Suspension Buffer (CSB) (Appendix A) by spinning the swab gently for evenly dispersion. Adjust concentration of cell suspensions to optical density of 0.8-1.0 at 610 nm wavelength.
3. Transfer 200 µl adjusted cell suspensions to microcentrifuge tubes. Add 10 µl of 20 mg/ml stock Proteinase K (USBiological, USA) to adjusted cell suspensions. Then, add 200 µl melted 1% SeaKem® Gold agarose (Lonza, Switzerland) to cell suspensions. Dispense some of the mixture into plug mold immediately. Allow plugs to solidify at room temperature for 10-15 minutes.
4. Dispense master mix of 5 ml Cell Lysis Buffer (Appendix A) and 25 µl Proteinase K (20 mg/ml stock) per tube.
5. Push out agarose plugs into each appropriate Cell Lysis/Proteinase K Buffer tube. Incubate in a 55°C shaker water bath for 2 hours with constant and vigorous agitation (150-175 rpm).

6. Prepare sterile Ultrapure water for each sample for washing the plugs 2 times and prepare Tris-EDTA (TE) buffer (Appendix A) 10-15 ml/ tube for washing the plugs 4 times. Pre-heat sterile Ultrapure water and TE to 55 °C.
7. Remove tubes from water bath and pour off lysis buffer. The plugs can be held in tubes with CHEF® screened caps (Biorad,Canada).
8. Add 10-15 ml sterile Ultrapure water that has been pre-heated to 55°C to each tube and shake the tubes in 55 °C water bath for 10-15 minutes. Then pour-off water from the plugs and repeat wash step with pre-heated water one more times.
9. Pour off water, add 10-15 ml pre-heated TE, and shake the tubes in 55 °C water bath for 10-15 minutes. Then, pour off TE and repeat wash step with pre-heated TE 3 more times. Decant last wash and add 5-10 ml TE.
10. Prepare a master mix of pre-restriction buffer by diluting 10X restriction buffer 1:10 with sterile Ultrapure water (20 µl M buffer and 180 µl sterile Ultrapure water per plug slide). Add 200 µl diluted restriction buffer to each microcentriuge tube.

11. Remove plug from TE and place on Petri dish, cut a 2.0 mm-wide slice from each samples with a razor blade. Then, transfer to each diluted restriction buffer tube. Incubate plug slices in 37°C water bath for 5-10 minutes. After incubation, remove buffer from plug slice with a pipet.
12. Prepare a master mix of restriction enzyme by diluting 10X restriction buffer 1:10 with sterile Ultrapure water and adding restriction enzyme (50 U/sample). Add 200 µl restriction enzyme mixture to each plug slices tube. Incubate plug slices in 37°C water bath for 2 hours.
13. Remove enzyme mixture and add 200 µl of 0.5 XTBE. Incubate at room temperature for 5 minutes. After that, remove plug slices from tubes and load plug slices on the bottom of the comb teeth. Remove excess buffer with tissue and allow plug slices to air dry on the comb for 3-5 minutes.
14. Position comb in gel form. Pour the 1% Pulsed-field certified agarose (Biorad, Cananda) (Appendix A) which is already cooled to 55°C into the gel form. Allow gel to solidify for 30-45 minutes.
15. Put gel frame in electrophoresis chamber and add 2 L 0.5X TBE (Appendix A). Turn on cooling module (14 °C), power supply, and pump approximately 30

minutes before gel is to be run. After gel solidifies, place gel inside gel frame in electrophoresis chamber.

16. When electrophoresis run is over (18 hours run). Remove and stain gel with ethidium bromide (Appendix A). Stain gel for 20 minutes in covered container. Then, destain gel in 500 ml distilled water for 3 times (each time approximately 20 minutes). Next, capture image under UV light with Gel Doc (Synoptics, Ltd., UK).

# BIOGRAPHY

Miss Roikhwan Soontravanich was born on October 25, 1983 in Bangkok, Thailand. She got the Degree of Veterinary Sciences (D.V.M.) (the 2nd class honour) from the Faculty of Veterinary Sciences, Chulalongkorn University, Bangkok, Thailand in 2007. After that, she enrolled the Master of Science Program in the Department of Veterinary Public Health, Faculty of Veterinary Science, Chulalongkorn University since academic year 2010.