GENOTYPIC PATTERNS OF *SALMONELLA* ISOLATED FROM VARIOUS SOURCES IN THE CHICKEN FARMS AND MARKET BY USING PULSED-FIELD GEL ELECTROPHORESIS

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ABSTRACT

This study was conducted to determine the antimicrobial resistance and genotypic patterns of a total of 112 strains of salmonellae isolated from three chicken farms in Phra Nakorn Si Ayuttaya province and Klong Toey market; which were used in a prior study in 2000. Fifty-one *S.* Braenderup, 24 *S.* Emek, 14 *S.* Weltevreden, 12 *S.* Derby, and 11 *S.* Stanley strains were examined using the antimocrobial susceptibility test and characterized by *Xba*I PFGE typing. Fifty three salmonellae strains (47.3%, 53/112) were resistant to 4 antimicrobial agents; namely nalidixic acid (NA) 37.5%, sulfamethoxazole-trimethoprim (SXT) 33.9%, tetracycline (TE) 25.9%, and ampicillin (AMP) 2.7%. All isolates were sensitive to cefotaxime (CTX) and choramphenicol (C). The prevalence of multidrug-resistant salmonellae (70%, 37/53) was significantly higher than those of single drug-resistant pattern strains (30%, 16/53, at $p < 0.05$). Moreover, 52.3% of salmonellae isolated from egg package (45/86) were antimicrobial resistant strains. *Xba*I PFGE typing was able to generate 7 patterns of *S.* Braenderup (A1-A7), 4 patterns of *S.* Emek (B1-B4), 4 patterns of *S.* Weltevreden (C1-C4), one genotype of *S.* Derby and 4 patterns of *S.* Stanley (E1-E4). The predominant clone of *S.* Braenderup was Type A2 (70.6%, 36/51) with 4 antibiotic sensitivity patterns. The most common patterns of Type A2 were sensitive to all tested antibiotics (63.9%, 23/36) and isolated from the egg packages at all three farms and the market. The SXT-NA-TE resistant strains of *S.* Emek, containing 3 PFGE types (B1-B3), were all from farm C (Phra Nakorn Si Ayuttaya province). All 12 strains of *S.* Derby isolated from farm A showed the same PFGE pattern with 3 antibiotic sensitivity patterns, and the most common pattern (83.3%, 10/12) was resistant to SXT-TE. PFGE Type E1 of *S.* Stanley was the most common clone (63.3%,7/11) and sensitive to tested antibiotics. PFGE typing was a significant method to differentiate and track down *Salmonella* strains that may cause an outbreak. The results of this study showed that the genetic related strain of *Salmonella* could be obtained from the chicken farms and the market. The same antimicrobial sensitivity pattern could be found in the different and distinct clones. Genotypic monitoring of salmonellae in the food chain could help to understand the wide spread of multidrug-resistant *Salmonella*.

KEY WORDS: *SALMONELLA*/ CHICKEN FARM/MARKET/ANTIMICROBIAL/PFGE/

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รูปแบบพันธุกรรมของเชื้อซัลโมเนลล่าที่แยกได้จากแหล่งต่างๆในฟาร์มเลี้ยงไก่และตลาดด้วยวิธี Pulsed-Field Gel Electrophoresis (GENOTYPIC PATTERNS OF *SALMONELLA* ISOLATED FROM VARIOUS SOURCES IN THE CHICKEN FARMS AND MARKET BY USING PULSED-FIELD GEL ELECTROPHORESIS)

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บทคัดย่อ

การศึกษาครั้งนี้เพื่อศึกษารูปแบบการดื้อยาและแบบแผนทางพันธุกรรมของเชื้อซัลโมเนลล่าจำนวน 112 สายพันธุ์ที่แยกได้จากแหล่งต่างๆในฟาร์มเลี้ยงไก่จำนวน 3 ฟาร์มในจังหวัดพระนครศรีอยุธยาและตลาดคลองเตยจากการศึกษาวิจัยที่ได้ดำเนินการในปี ค.ศ. 2000 โดยสายพันธุ์ที่ศึกษามาจาก *S.* Braenderup 51 สายพันธุ์, *S.* Emek 24 สายพันธุ์, *S.* Weltrvreden 14 สายพันธุ์, *S.* Derby 12 สายพันธุ์ และ *S.* Stanley 11 สายพันธุ์ โดยทดสอบการดื้อต่อยาต้านจุลชีพ 6 ชนิด และศึกษารูปแบบทางพันธุกรรมของเชื้อด้วยวิธี Pulsed-Field Gel Electrophoresis (PFGE) โดยตัดด้วยเอ็นไซม์ พบว่า 53 สายพันธุ์ (47.3%, 53/112) ดื้อต่อยาต้านจุลชีพ 4 ชนิดได้แก่ nalidixic acid (NA) ร้อยละ 37.5 รองลงมาคือ sulfamethoxazole-trimethoprim (SXT) ร้อยละ 33.9 tetracycline (TE) ร้อยละ 25.9 และ ampicillin (AMP) ร้อยละ 2.7 ไม่พบสายพันธุ์ที่ดื้อต่อยา cefotaxime (CTX), choramphenicol (C) รูปแบบของการดื้อต่อยาต้านจุลชีพหลายชนิดพบร้อยละ 70 (37/53) ซึ่งสูงกว่าสายพันธุ์ที่มีการดื้อต่อยาต้านจุลชีพชนิดเดียวร้อยละ 30 (16/53) อย่างมีนัยสำคัญสถิติ ($p< 0.05$) อย่างไรก็ตามพบว่าร้อยละ 52.3 ของเชื้อที่แยกได้จากถาดพลาสติกบรรจุไข่ดื้อต่อยาต้านจุลชีพ การจำแนกรูปแบบทางพันธุกรรมด้วยวิธี *Xba*I PFGE สามารถจำแนกรูปแบบทางพันธุกรรมของ *S.* Braenderup ได้ 7 แบบ (A1-A7), *S.* Emek 4 แบบ (B1-B4)), *S.* Weltevreden 4 แบบ (C1-C4), *S.* Derby 1 แบบ (D), and *S.* Stanley 4 แบบ (E1-E4) ด้านความสอดคล้องกันของรูปแบบทางพันธุกรรมกับการดื้อยาต้านจุลชีพพบว่า clone ที่เด่นของ *S.* Braenderup พบเป็น Type A2 ร้อยละ 70.6 (36/51) ซึ่งมีรูปแบบการดื้อยาต้านจุลชีพได้ 4 แบบ โดยรูปแบบธรรมดาที่พบมากร้อยละ 63.9 (23/36) เป็น Type A2 ที่ไม่ดื้อต่อยาต้านจุลชีพที่ใช้ทดสอบและเป็นเชื้อที่แยกได้จากถาดพลาสติกบรรจุไข่จากทุกฟาร์มและตลาด ในขณะที่เชื้อ *S.* Emek ที่ดื้อต่อยา SXT-NA-TE มีรูปแบบทางพันธุกรรม 3 ชนิดคือ B1-B3 และเป็นเชื้อที่แยกได้จากฟาร์ม C เท่านั้น *S.* Derby ทั้ง 11 สายพันธุ์ที่แยกได้จากฟาร์ม A นั้นมีรูปแบบทางพันธุกรรมแบบเดียวกันแต่มีการดื้อต่อยาต้านจุลชีพ 3 รูปแบบโดยที่ร้อยละ 83.3 (10/12) ดื้อต่อ SXT-TE ส่วนเชื้อ *S.* Stanley เป็น Type E1 ร้อยละ 63.6 (7/11) ซึ่งเป็น clone เด่นที่ไม่ดื้อยาต้านจุลชีพ การจำแนกรูปแบบทางพันธุกรรมด้วยวิธี PFGE เป็นวิธีที่เหมาะสมในการจำแนกและค้นหาสายพันธุ์ของเชื้อซัลโมเนลล่าที่อาจเป็นสาเหตุของการระบาด การศึกษาครั้งนี้แสดงให้เห็นสายพันธุ์ของเชื้อซัลโมเนลล่าที่มีความสัมพันธ์ทางพันธุกรรมสามารถพบได้จากฟาร์มเลี้ยงไก่และตลาด รูปแบบการดื้อยาที่เหมือนกันสามารถพบใน clone ที่แตกต่างและเหมือนกัน การใช้พันธุกรรมในการติดตามเชื้อซัลโมเนลล่าในห่วงโซ่อาหารสามารถช่วยให้มีความเข้าใจในการแพร่กระจายของเชื้อซัลโมเนลล่าที่ดื้ต่ออยาต้านจุลชีพหลายชนิดได้

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LIST OF ABBREVIATIONS

Bp	Base pair
CFU	Colony forming unit
SCB	Cell suspension buffer
DNA	Deoxyribonucleic acid
EDTA	Ethyylenediaminetetra-acetic acid
et al	Et alli(Latin), and other
g	Gram
h	Hour
l	Litre
Lim	Lysine indole motility medium
M	Molar
mg	Mililgram
min	Minute
ml	Millitre
M.W.	Molecular weight
MHB	Muller Hinton Broth
MHA	Muller Hinton Agar
NA	Nutrient Agar
NSS	Normal saline solution
OD	Optical density
PCR	Polymerase chain reaction
PFGE	Pulse field gel electrophoresis
RNA	Ribonucleic acid
SKG	SemKem gold agaros
SDS	Sodium dodecyle sulfate

LIST OF ABBREVIATIONS (Continued)

TSI	Triple sugar iron medium
TBE	Tris- Borate EDTA buffer
TE	Tris-EDTA buffer
µg	Microgram
µl	Microlitre
Amp	Ampcillin
C	Chloramphenicol
CTX	Cefotaxime
NA	Nalidixic acid
SXT	Sulfamethoxazone trimethoprim
TE	Tetracycline

CHAPTER 1
INTRODUCTION

Salmonella enterica is one of the most common causes of human gastroenteritis worldwide. The infection is primarily caused by improper handling and uncooked foods. A large number of different food from animal origin have been identified as reservoirs of this bacteria (1,2,3). In USA, it estimated that there are 1.4 million cases of salmonellosis patients per year (4), and the annual cost of food-borne salmonellosis is $ 0.9 to 3.5 billions (5). In Thailand, the estimated salmonellosis patients is 76-1,043 cases per 100,000 population per year (6).

More than 2,500 different serovars of *Salmonella enterica* have been identified and most of them have been described as the cause of human infections, but only a limited number of serovars are of public health importance. Most reports have mentioned *Salmonella enterica* serovar Typhimurium and *Salmonella enterica* serovar Enteritidis as the most common causes of human salmonellosis worldwide (7,8). However, other serovars have been reported to be of even greater importance in some regions. For example, *Salmonella* Saintpaul was recently implicated in a food-borne gastroenteritis epidemic in Germany, *Salmonella* Rubislaw and *Salmonella* Javiana are the most frequently isolated strains in Norway (9). In Malaysia, the relative prevalence of *Salmonella* Weltevreden isolated from infections in humans increased from 8 to10 % in the period 1973-82 to more than 30% in 1989-94 making this the most common serovar isolated from infected humans (10,11). In addition, changes in the importance of different serovars over time seem to take place (5,10).

Thus, *Salmonella* is one of the major problems of food borne diseases and in economic losts all over the world. A Global *Salmonella* Surveillance Programme has been established by WHO since 2000 for controlling *Salmonella* infection. This programme have members from 106 countries including Thailand.

In response to this concern, *Salmonella* from humans infection and other sources are continuously isolated serovars at the WHO National *Salmonella* and *Shigella* Center in Thailand every year. In 2002, the top five serovars from human were *Salmonella* Enteritidis (12.57%), *Salmonella* Rissen (8.15%), *Salmonella* Weltevreden (7.86%), *Salmonella* Anatum (7.76%), and *Salmonella* Stanley (6.42%). In addition, the most common serovars isolated from raw material food were *Salmonella* Anatum, *Salmonella* Rissen, *Salmonella* Stanley, *Salmonella* Virchow and *Salmonella* Agona 25.94, 14.85, 5.98, 5.21, 4.92 %, respectively (12).

The most 5 common serovars causing human salmonellosis in 2003 was *Salmonella* Enteritidis (8.2%), *Salmonella* Weltevreden (8.2%), *Salmonella* Stanley (6.2%), *Salmonella* Anatum (4.7%), and *Salmonella* Rissen (4.5%) (75). The most common serovars isolated from raw food were *Salmonella* Anatum, *Salmonella* Rissen, *Salmonella* Weltevreden, *Salmonella* Enteritidis and *Salmonella* Virchow, 17.55, 13.58, 6.95, 4.47, 4.30%, respectively. During 1993-2001, *Salmonella* Enteritidis was the most common serovar isolated from chicken meat (20.6%). *Salmonella* Weltevreden was frequently isolated from seafood (27%), water (15%), and duck meat (12%) and the most likely source of this serovars seems to be water-related. Among isolates from other food products, *Salmonella* Anatum (14.7%), *Salmonella* Rissen (9.3%), *Salmonella* Hadar (7.2%), and *Salmonella* Weltevreden (7.0%) were the predominant serovars and among isolates from water was *Salmonella* Weltevreden (15%), *Salmonella* Anatum (11.7%), *Salmonella* Rissen (8.7%), and *Salmonella* Derby (7.8%).

The distribution of serovars causing human infections in Thailand differs markedly from those reported in other countries and seems to be related to the occurrence of *Salmonella* serovars in the different food products and other reservoirs (13).

The occurrence of different serovars in one country can be of global importance because of travel, trade of breeding animals, and food products worldwide. Knowledge regarding the occurrence, epidemiology and importance of different serovars and different countries and geographic regions may assist in the recognition and tracing of new emerging pathogens.

The most likely sources of infection are food originated from animals such as chicken, pork, beef and other. As consumers become have better informed and perceived risks of food-borne infections increasing, feasible control and possible elimination of *Salmonella* may be of interest to animals procedures. Efficient controls to reduce *Salmonella* contamination should begin at the farms. In order to implement controls, sources of contamination must be identified. Feed, water, insects, rodents, poults, and humans are potential sources of colonization and contamination by *Salmonella*(14).

An example of the study of prevalence of *Salmonella* isolated from various sources in three chicken farms have shown that *Salmonella* isolated mostly were from plastic packages 66.2%, followed by animal feed 14.1%, feeding water 9.9%, worker hands 7.0%, and chicken feces 2.8%. The most common serovar was *Salmonella* Breanderup 36.6%, followed by *Salmonella* Emek 16.9%, *Salmonella* Bovismorbificans 7.0%, *Salmonella* Cerro and *Salmonella* Weltevreden 2.8%, etc (15). However, the primary source of *Salmonella* causing the contamination in farm has not been investigated.

Most previous studies are based on analytical epidemiological investigation and traditional phenotypic analysis such as biotyping, serotyping, antimicrobial susceptibility, and phage typing. However, the limitation associated with several of these techniques have stimulated interest in DNA-base on typing method such as plasmid analysis, ribotyping (16), arbitrarily primed-PCR (AP-PCR) and pulse-field gel electrophoresis

PFGE (17,18) Molecular typing is an invaluable epidemiological tool that can be used to determine the sources of infections and investigate the link between food animal and humans. It has previously been shown that PFGE is very discriminatory and useful in epidemiological studies (19,20). In Thailand, the epidemiological investigation has been based on the related serovars isolated from human patients, animals, food and environmental samples which are not the final conclusion. Identification of an epidemic strain is often critical to the success of epidemiological investigation aimed at preventing the spread of infection and eradicating its source (13).

Method for epidemiological investigations of bacterial diseases which rely on phenotypical properties of the pathogens often lack the necessary resolution potential for strain discrimination. They are not able to detect minor primary changes in the bacterial genome that do not influence particular features. Therefore, although it is true that phenotypic methods of microbial characterization are still useful, newer methods base on the molecular analysis of the genome have become important for the epidemiological characterization of pathogens (21).

Pulse-field gel electrophoresis (PFGE) is an established method for the analysis of large fragments generated by restriction endonuclease digestion of genomic DNA and is currently considered to be one of the most reliable typing procedures. PFGE frequently has been utilized to understand food-borne outbreaks of

Escherichia coli O157:H7 diseases, shigellosis, listeriosis, and salmonellosis (22). This method has been shown to be highly effective for epidemiological studies of some serovars of *Salmonella enterica* (23).The objective of this study was to determine the genotyping pathern among *Salmonella* previously isolated from eggs and various sources in the chicken farms by using PFGE . In addition, the occurrence of antimicrobial resistance was also determined.

CHAPTER 2

OBJECTIVES

General Objective

To study the genotypic patterns among *Salmonella* strains isolated from eggs and various sources in the chicken farms and a market by using pulsed-field gel electrophoresis.

Specific Objectives

1. To study the prevalence of antimicrobial resistance patterns of *Salmonella* strains isolated from eggs and various sources in the chicken farms and a market.
2. To study the DNA fingerprint of *Salmonella* strains isolated from eggs and various sources in the chicken farms and a market by using PFGE.
3. To study the relationship of serovars, sources, antimicrobial resistance patterns and DNA fingerprint patterns of *Salmonella* isolated from various sources in chicken farms and a market.

CHAPTER 3

LITERATURE REVIEW

Salmonella

Foodborne infection is caused by microorganism in food. If bacteria become numberous and the food is eaten, bacteria may be able to grow in intestines. *Salmonella*, *Campylobacter*, Hemorrhagic *E. coli* and *Listeria* all cause gastrointestinal infections. *Salmonella* is a bacterium which is one of the commonest cause of foodborne disease worldwide. In the USA, each year foodborne disease illnesses affected 6 to 80 million persons, caused 9,000 deaths, and cost estimated 5 billion U.S dollars.

The commonest *Salmonella* infection is due to the presence of the bacteria in food. After the contaminated food was eaten, the bacteria multiply in the intestines via the contaminated and within 12-36 hours usually cause diarrhea, stomach cramps and sometimes vomiting and fever.

However, the diarrhea can be severe, and the person may be ill enough to require hospitalization, particularly in the elderly, infants, and those with impaired immune systems. Numerous types of *Salmonella* cause disease in both animal and human. While there is much variation in the relative prevalence of different types of *Salmonella* serovar from country to country, in USA, *Salmonella* Typhimurium and *Salmonella* Enteritidis are the two commonly found. Increasing percentage of *Salmonella* Typhimurium isolates from humans in England and Wales have also been identified as type DT 104. This strain accounted for 70% of all reports of *Salmonella* Typhimurium during the first 8 months of 1992 and for 32%, and 52% of isolates in 1993, 1994, 1995, respectively. Although *Salmonella* Enteritidis phage type PT 4 continues to be the most commonly isolates of *Salmonella, Salmonella* Typhimurium

DT 104 is the second most common (27). Foodborne *Salmonella* infection are usually not severe. The very young and the very old are most susceptible to serious complications of *Salmonella* infection (29). *Salmonella* is one of the leading causes of foodborne illness and about 20% of chicken are infected with it. Incidents of foodborne illness are difficult to track and often go unreported. In 1995, *Salmonella* Enteritidis was a cause of foodborne outbreak in chain-restaurants, Maryland. *Salmonella* was isolated from scrambled eggs on a breakfast bar. This outbreak resulted in 71 illness and 17 hospitalization. *Salmonella* Enteritidis outbreaks continue to occur in the USA. The CDC estimated that 75% of the outbreaks are associated with the consumption of adequately cooked grade A whole shell egg (30).

In Denmark meat and table eggs have been the main source of these infections (24). In Norway, caused by serovar Typhimurium, have been related to infection in small passerines. In a waterborne due to serovar Typhimurium outbreak in northwestern Norway, gulls were the most likely source of infection in 1999. Moreover, during 1996 - 2000 two human outbreaks have been associated with infected hedgehog populations (29).

Foodborne transmission of DT 104 has been documented for several outbreaks; suspected vehicled roast beef, ham, pork sausage, salami sticks, cooked meats, chicken legs and unpasteuried milk (31,32). A total of 786 samples of fresh and frozen sausages in England in 1994 demonstrated that 17% were contaminated with *Salmonella* spp., including *Salmonella* Typhimurium DT 104 (33). In contrast to *Salmonella* Enteritidis, which is associated primarily with poultry and eggs, DT 104 is the most commonly detected in cattle. A majority of recent bovine salmonellosis cases investigated in England were caused by DT 104. In 1996, 15 of 16 new outbreaks of bovine salmonellosis in Scotland were caused by *Salmonella* Typhimurium DT 104 (34). These bacteria cause diarrhea in cattle and may persist in animals for up 6 months after recovery. A number of farm families appear to have acquired DT 104 infections while caring for sick farm animals (33).

Salmonella Classification and Nomenclature

Salmonella species is a member of family Enterobacteriaceae. Besides humans *Salmonella* spp. can infect many animal species and are capable of invading extraintestinal tissues and causing enteric fevers, the most severe of which is typhoid fever.

Salmonella is gram negative, facultatively anaerobic bacilli, ranging 0.7 to 1.5 x 2 to 5 µm in size. With the exception of *S.* Pullorum and *S.* Gallinarum they are motile with peritrichous flagellae. No capsule is apparent and spores are not formed. *Salmonella* are generally identified as being non-lactose fermenting organisms (NLFs). D-glucose is fermented with the production of acid and usually gas. Other carbohydrates usually fermented are L-arabinose, maltose, D-mannitol, D-mannose, L-rhamnose, D-sorbitol, trehalose and D-xylose. Other biochemical tests are shown in Table 1.

Salmonella are oxidase negative, catalase positive, indole and Voges Proskauer (VP) negative, methyl red and Simmons citrate positive, H_2S producing except *Salmonella* Paratyphi A and *Salmonella* Choleraesuis, and urea negative (although *Salmonella arizonae* appear positive on prolonged incubation). Some of these characteristics are used for biochemical confirmation of *Salmonella* (35). The biochemical reaction for identification of *Salmonella* are shown in Table 2 and 3.

The growth factor of *Salmonella* such as temperature is 37-45^{o}C, pH range is between 4.5-9.0 and water activity is 0.93-0.99. The *Salmonella* is not resistant to heat. It is killed at 55^{o}C for 1 h, 60^{o}C for 15-20 min, 62^{o}C for 4 min. It still survives in frozen for long periods and resistant to certain chemicals :- brilliant green, sodium tetrathionate and sodium deoxycholate. *Salmonella* was destroyed with phenol, formaldehyle (36).

Table 1 Differential characters of *Salmonella* species and subspecies by biochemical tests (38)

Characters	*S. enterica subspecies*						*S. bongori*
	Enterica	*salama*	*arizonae*	*diarizonae*	*houtenae*	*indica*	
Dulcitol	+	+	-	-	-	d	+
ONPG (2 h)	-	-	+	+	-	d	+
Malonate	-	+	+	+	-	-	-
Gelatinase	-	+	+	+	+	+	-
Sorbitol	+	+	+	+	+	-	+
Culture with KCN	-	-	-	-	+	-	+
L(+)-tartrate(a)	+	-	-	-	-	-	-
Galacturonate	-	+	-	+	+	+	+
γ-glutamyltransferase	+(b)	+	-	+	+	+	+
β-glucuronidase	d	d	-	+	-	d	-
Mucate	+	+	+	- (70%)	-	+	+
Salicine	-	-	-	-	+	-	-
Lactose	-	-	- (75%)	+(75%)	-	d	-
Lysis by phage O1	+	+	-	+	-	+	d
Usual habitat	Warm-blooded Animals		Cold-blooded animals and environment				

(a) = *d*-tartrate

(b) = Typhimurium d, Dublin -.+

+ = 90% or more positive reaction

Table 2 The main biochemical reaction of *Salmonella* (28)

Test or substrate	*S.* Choleraesuis	*S.* Typhi	*S.* Paratyphi A	Other *Salmonella*
Oxidase	-	-	-	-
TSI	K/A gas+	K/A gas+	K/A gas+	K/A gas+
H_2S	+	+	-	+
Motility	+	+	+	+
Indole	-	-	-	-
Urease	-	-	-	-
Citrate	d	-	-	+
Lysine	+	+	-	+
Ornithine	+	-	+	+

+ positive within one or two days incubation

- no reaction

d different reactions; + or -

Table 3 Differentiation of *Salmonella*, *Citrobacter*, *Edwardsiella tarda* and *Proteus* (28)

Test or substrate	*Salmonella*	*Citrobacter*	*E. tarda*	*Proteus*
TSI	K/A gas+ H_2S+	K/A gas+ H_2S+	K/A gas+ H_2S+	K/A gas+ H_2S+
Lysine	+	-	+	-
Urease	-	-	-	+
Mannitol	+	+	-	-

The pathogen of salmonellosis was first cultivated in 1884. The initial serodiagnostic of *Salmonella* was performed in 1896 by using the serum of salmonellosis patient to agglutinate with his isolated pathogen. In 1926, the analysis procedure of O and H antigens were initiated by White and these procedures were studied deeply by Kauffman. Thus, almost of references of *Salmonella* serotyping have been performed according to the Kauffman – White Scheme (38).

The genus *Salmonella* consists of only two species, *S. enterica*, which is divided into six subspecies: *S. enterica* subsp. *enterica*, *S. enterica* subsp. *salamae*, *S. enterica* subsp. *arizonae*, *S. enterica* subsp. *diarizonae*, *S. enterica* subsp. *houtenae*, and *S. enterica* subsp. *indica*, and *S. bongori*. This nomenclature reflects present *Salmonella* taxonomy. Serovars belonging to *Salmonella enterica* subsp. *enterica* are designated by a name usually related to the geographical place where this serovar was first isolated. This name is written in roman letters (not italicized) and the first letter is a capital letter. Serovars belonging to other subspecies are designated by their antigenic formulae, following the subspecies name (38,39).

In 1997, 1998 WHO Collaborating Center for Reference and Research on *Salmonella* Institute Pasteur published the report of Antigenic Formula of *Salmonella* serovar classified Genus *Salmonella* as following data (Table 4).

The Method of Writing *Salmonella* Serovar

Genus (italics) *Salmonella*

Species (italics): *enterica* (italics) subsp. *enterica* (italics)

Serotype (or serovar; or ser.) capitalized, not italicized

A. Serotypes of species. I subspecies. I designated by a name, for example :

- *Salmonella enterica* subsp. *enterica* serovar Typhimurium
- *Salmonella ser.* Typhimurium
- *S.* ser. Typhimurium
- *S.* Typhimurium

Table 4 Present number of serovars in each species and subspecies (38)

***Salmonella* species**	**Subspecies**	**Number of serovars in year**	
		1997	2001 [a]
Salmonella enterica	subsp. *Enterica*	1,435	1,478
	subsp. *Salamae*	485	489
	subsp. *Arizonae*	94	94
	subsp. *Diarizonae*	321	321
	subsp. *Houtenae*	69	71
	subsp. *Indica*	11	12
Salmonella bongori		20	21
Total		**2,435**	**2,501**

[a] The latest

B. Serotypes of all the rest designated by antigenic formulae, for example :

a - *Salmonella enterica* subsp. *salamae serovar* 6,8 :b : 1,5
- *Salmoella* II 6,8 :b :1,5
- *S.* II 6,8:b:1,5

b - *Salmonella enterica* subsp. *houtenae* serovar 43 :z_4 ,z_{24} :-
- *Salmonella* IV 43: z_4 ,z_{24} :-
- *S.* IV 43: z_4 ,z_{24} :-

The simplified antigenic formulae of *Salmonella* serovars are listed in a document called the Kauffmann-White Scheme (Table 5).

Identification

1. Biotyping

Biochemical variation between two organisms with the same serovar is known biotype. Biotype can be associated with differences in virulence. For example, *Salmonella* Pullorum and *Salmonella* Gallinarum are considered the same organism by some authors and are treated as such as in Bergey's Manual, as *Salmonella* Gallinarum- Pullorum (40). These historically have been treated as different bacteria that cause different disease syndrome in chicken. Eraborate tables and treat on biochemical reactions that seem to differentiate *Salmonella* Pullorum from *Salmonella* Gallinarum have been published, in the poultry disease syndromes by these two biotypes. *Salmonella* Pullorum cause pullorum disease, which is a septicemia of embryos and chicks, whereas "fowl typhoid" is caused by *Salmonella* Gallinarum and affects adults chickens (41).

Biotyping is an important component in the Kauffman-White and Minor schemes, which have been widely used to clearify the biochemical heterogeneity among *Salmonella* (36, 42), and to segregate serovars into serofermantative groups. It also helped to establish epidemiological relationship between strains that affected humans and those that did not.

Table 5 Antigenic Schema for *Salmonella* Formulas (38)

Group	Serovar	Somatic (O) Antigen	Flagella (H) Antigen	
			Phase I	phase II
O:2 (A)	*S.* Paratyphi A	1,2,12	a	(1,5)
	S. Kiel	1,2,12	g,p	-
O:4 (B)	*S.* Paratyphi B	1,4,(5),12	b	1,2
	S. Derby	1,4,(5),12	f,g	(1,2)
	S. Typhimurium	1,4,(5),12	i	1,2
O:7 (C1)	*S.* Paratyphi C	6,7,(Vi)	c	1,5
	S. Choleraesuis	6,7	c	1,5
	S. Livingstone	6,7,14	d	l,w
	S. Virchow	6,7	r	1,2
	S. Infantis	6,7,14	r	1,5
O:8 (C2-C3)	*S.* Newport	6,8,20	e,h	1,2:[z_{67}]
	S. Brunei	8,20	y	1,5
O:9 (D1)	*S.* Typhi	9,12, (Vi)	d	-
	S. Javiana	1,9,12	l,z_{28}	1,5
	S. Gallinarum	1,9,12	-	-
O:3,10 (E1)	*S.* Anatum	3,10,(15), (15,38)	e,h	1,6
	S. Weltevreden	3,10,(15)	r	z_6
	S. Lexington	3,10,(15),(15,34)	z_{10}	1,5
	S. Ratchaburi	3,10	z_{35}	1,6
O:11 (F)	*S.* Aberdeen	11	i	1,2
O:13 (G)	*S.* Poona	1,13,22	z	1,6
	S. Worthington	1,13,23	z	l,w
O: 38 (P)	*S.* Bangkok	38	z_4z_{24}	-

Biotype has been used successfully to subdivide serovar in a number of epidemiological investigation of *Salmonella*. A model for biotyping schemes is developed by Duguid et al. (40) for application to *S.* Typhimurium. This schemes have high discriminatory and recognized which are suitable for other *Salmonella* such as *S.* Agona, and *S.* Montevideo. An evaluation of the discriminatory ability of biotyping schemes for *S.* Typhimurium and *S.* Paratyphi B showed that while discrimination was high when used alone, the schemes were most effectively used in conjunction with phage typing (43).

2. Serotyping

Serotyping is a definitive typing method used for epidemiological characterisation of bacteria. Serotyping of *Salmonella enterica* strains is carried out by identification of surface antigens (LPS, O-antigens) and flagella antigens (proteins, H-antigens). Most commonly strains of *Salmonella* express two phases of H- antigens but aphasic, monophasic and triphasic variants are known. Some salmonellae have capsular (K) antigens, referred to as Vi, which may interfere with agglutination by O antisera and are associated with invasiveness. Agglutination tests with absorbed antisera for different O and H antigens forms the basis for serological classification of salmonellae in accordance with the scheme of Kauffmann and White (36).

The important antigenic characteristic of *Salmonella* for serological tests are 3 main types.

O antigen or Somatic antigen is the one of cell wall component. Main substance are polysaccharide, protein and phospholipid. O antigen can resist $100^{O}C$ for two and a half hour, endure to 95% ethanol, endure to diluted acid. The reaction of O - antigen with antisera will be the granular form. O antigen of *Salmonella* is classified as Kauffman – White Scheme that each group of O antigen will be named in Arabic starting from group A which composed of O:1, O:2, O:12 antigen, and in other group until Z which is O:50. After Z group is from O:51 to O:67 group (Table 6).

Table 6 Arrangement of *Salmonella* grouping on O antigen

Alphabetical	Present	Alphabetical	Present	Alphabetical	Present
A	2	G_1-G_2	13	Q	39
B	4	H	6,14	R	40
C_1-C_4	6,7	I	16	S	41
C_2-C_3	8	J	17	T	42
D_1	9	K	18	U	43
D_2	9,46	L	21	V	44
D_3	9,46,27	M	28	W	45
E_1-E_2-E_3	3,10	N	30	X	47
E_4	1,3,19	O	35	Y	48
F	11	P	38	Z	50

A which composed of O:1, O:2, O:12 antigen, and in other group until Z which is O:50. After Z group is from O:51 to O: 67 group.

K antigen (Vi antigen) are external to O antigens that occur as capsules or envelopes around the cells, thereby preventing agglutinating antibodies from reacting with the bacterial cell. The *Salmonella* strains with Vi antigen have an greater pathogenicity than the strains without Vi antigen. The important strains with Vi antigen are *S*. Typhi, *S*. Paratyphi, and *S*. Dublin (36).

H antigens (Flagella antigen) are proteinaceous, located on flagellar and are denatured or removed by heat or alcohol. Flagellar are associated with the motility of *Salmonella* and contribute to one of the virulence factors. Because many motile bacteria have the capacity to move towards to enter a more favorable environment and also to move from colonized sites to blood vessels. The reaction of H antigen with antisera will be the floccular form. Almost *Salmonella* possesses 2 phases of H antigen, the first phase called specific phase and second phase called non – specific phase. The first phase is named by small type letter start from a to z respectively, but nowadays many H antigen are found then are named by z_1,……., z_2 and others. In case of second phase, there are many types and some types are difficult to detect. The important groups of single phase H – antigen strains such as *S*. Paratyphi, *S*. Typhi, *S*. Derby, *S*. Enteritidis, *S*. Dublin and non - H - antigen such as *S*. Gallinarum (36).

3. Phage typing

Phage type reflects differences between two organisms with the same serovar but different susceptibilities to bacterial virus; individual isolates of a particular serovar may be differentiated further by determining the differences in susceptibility to a lytic bacteriophage, termed phage typing.

The underlying principal of phage typing is the host specificity of bacteriophage. Phage typing schemes which are in routine use in LEP include those for *S*. Typhi, *S*. Paratyphi A and B, *S*. Enteritidis, *S* Hadar, *S*. Typhimurium and *S*. Virchow (1).

The statistical association between phage type (PT) 8 and poultry is based on a small isolates. However, that PT 8 was also a common PT in human and sporadic outbreak of *S*. Enteritidis isolates, the association with poultry supports the epidemiological connection between *S*. Enteritidis infection and eggs (1). In Canada, reported for S. Enteritidis isolated froms collected between 1976-1989, found PT 8 and 13a accounted for 83% of S. Enteritidis (44). Hickman-Bremer (45), reported that the most common phages were PT 8 (48.2%), PT 13a (20.1%), PT13 (7.8%) and PT 14b (7.8%). Most of the strains were specifically collected from eggs related outbreak in the northeastern United Stated in 1988-1989. The epidemiological studies from chicken meat transmitted to humans in 1987-1997, found 302 of strains from study phage type, found 10 phage types and PT 4 was an important PT. The results were found PT 73.9% in humans and 76.2% in poultry (46).

4. Antibiotic susceptibility

In general, antibiotic resistance in *Salmonella* strains is encoded by plasmid (R factors) which have been acquired as a consequence of selective pressure generated by the use of antibiotics in human and veterinary medicine. Antibiotic resistance patterns analysis is an extremely rapid method of typing which has proved invaluable for discrimination within certain serovars (47).

Patterns of antibiotic resistance (antibiograms) have been used to sub-group *Salmonella*. The zone of growth inhibition are measured resistance assessment according to the National Committee for Clinical Laboratory Standard (NCCLS). It was reported that *S*. Enteritidis strains which are multiply resistant to antimicrobial agents were isolated from the blood of 12 patients hospitalized at the institute Pasteur of Bangui, Central African Republic, during a 4-5 months period. The lack of gas production in Kligler-Hajna medium initially suggested *S* Typhi, but isolates were confirmed as unsual *S*. Enteritidis strains. The occurrence of unique strains in an unusual site of infection may indicate an epidemic due to unusually invasive and resistant strain of *S*. Enteritidis. Some variation in plasmid profile and susceptibility to antimicrobial agents was noted, possibly reflecting antibiotic pressures exiting in the Central African Republic. All were of the same bacteriophage lysis pattern, unlike patterns documented for recent US and European isolates of *S*. Enteritidis (47).

In Thailand during 2000-2002, Aroon et al., (48) reported that antibiotic resistant of Non-Typhoidal *Salmonella* isolated from human and carrier to ampicillin (AMP), chloramphinical (C), tetracycline (TE), trimethroprim/sulfamethoxazole (SXT), and streptomycin (S) were 31.8%, 20.5%, 47.3%, 32.5%, and 47.4% respectively. According to the serovars of *Salmonella*, it was found that *S.* Weltevreden was resistant to tetracycline increased from 5% to 8.4%, ampicillin decreased from 3% to 1.4%, chloramphinical decreased from 1.3% to 0.7%, streptomycin decreased from 4.5% to 1.4%, and trimethroprim/sulfamethoxazole decreased from 2.8% to 2.1%, respectively. *Salmonella* Enteritidis was resistant to trimethroprim/sulfamethoxazole and ampicillin increased from 8% to 28% and 1.8% to 5.2%, respectively. In addition, it was decreased from 6.4% to 1.6% and 16.7% to 4.7% of chloramphinical and streptomycin. *Salmonella* Anatum was resistant to tetracycline increased from 64.4% to 74.3%, ampicillin increased from 49.4% to 71.3%, and trimethroprim/sulfamethoxazole increased from 44.6% to 58.1%, respectively. Whiles, chloramphinical and streptomycin decreased from 10.7% to 8.1% and 82.8% to 21.6%.

The occurrence of antibiotic resistant of *Salmonella* isolated from chicken meat, duck meat, seafood, water, and ready to eat food in Thailand during 2002 (13) were reported as follow: from chicken meat (43.3% to TE, 27.0% to S, 16.3% to SXT, 10.7% to C, 9.0% to AMP,and 0.6% to CTX), duck meat (5.7% to TE, 3.4% to CTX, 2.3% to AMP and SXT), seafood (20.8% to TE, 12.5% to S, 8.3 to SXT, 4.2 to AMP and C), water (40.0% to TE, 12.0% to AMP and SXT, 8.0% to S, 4% to C), and ready to eat food (61.4% to TE, 47.7% to AMP, 38.4% to SXT, 25.0% to S, and 15.9% to C) (Table 7).

Table 7 Antibiotic resistance of *Salmonella* isolated from various sources in Thailand, 2002

Source	**% of resistant to**					
	AMP	**C**	**CTX**	**S**	**TE**	**SXT**
Chicken meat	9.0	10.7	0.6	27.0	43.3	16.3
Duck meat	2.3	0	3.4	0	5.7	2.3
Seafood	4.2	4.2	0	12.5	20.8	8.3
Water	12.0	4.0	0	8.0	40.0	12.0
Ready to eat food	47.7	15.9	0	25.0	61.4	38.4

AMP = Ampicillin, C = Chloramphinical, CTX = Cefotaxime, S = Streptromycin
TE = Tetracycline, SXT = trimethroprim/sulfamethoxazole

5. Molecular techniques

5.1 Plasmid fingerprinting

Determination of the plasmid profiles or fingerprints is useful in additional to the tradition epidemiological tools of serotyping and phage typing. In the last few years, plasmids associated with virulence have been reports in several serovars of *Salmonella* such as *S.* Typhimurium (33). The minority of *S.* enteritidis strains can be differentiated by plasmid frofile typing. This plasmid corresponds to that previously designated the *S.* Enteritidis serotype-specific plasmid (34). The plasmid analysis has been show to be very limited use for subtyping of *S.* enteritidis despite the fact that less on plasmid can be found in most strains.

Brown et al. (52) reported that the plasmid in selected type strains of *S.* Enteritidis phage typing have been characterized by restriction enzyme fingerprinting and by DNA-DNA hybridization with oligonucleotide proves for *Salmonella* plasmid virulence (*spv*) genes. With one exception, the fingerprints of the 38 MDa plasmid studies were homogeneous but there was heterogeneity in the fingerprints of 59 MDa plasmid found in 4 of type strains. However, all 38 MDa and 59 MDa plasmid were related to 45 MDa plasmid identified in the type strains of phage type 19. In contrast, a 65 MDa plasmid found in the type strains of phage type 10 was not related to these three plasmids molecular weight groups and did not carry the *spv* gene.

5.2 Polymerase chain reaction (PCR)

The PCR method offers several advantages for rapid, reliable detection of microbial pathogens in food and promises of be a valuable addition DNA hybridization, the expression of genes can be effectedby environment factors and so gene probes have an inherent advantage compared with immunoassays (53). The PCR have been widely applied to a large variety of human pathogen (54), Which including caused by *Salmonella* species or collectively known as salmonellosis.

To overcome problem with non-specific PCR amplification by other non-*Salmonella* bacteria Jitrapakdee et al. (56) developed PCR method for the detection of *Salmonella* in food by using a primer set designed to amplify a 199 bp *Salmonella* specific DNA fragment derived from a repetitive DNA of *S.* Weltevreden. The assay detected all 52 most prevalent serovars found in contaminated food Thailand and no cross-reaction was reported with other non-*Salmonella* organisms. The limit of detection was 1 fg of purified target DNA or five bacteria from pure colture. The sensitivity and specificity was 100%, 69%, respectively when compared with standard microbiological culture method.

Shu et al. (57) developed a fluorogenic 5' nuclease assay for the detection of *Salmonella* in food samples. The system employs the 5' nuclease activity of *Taq* DNA polymerase to hydrolyse a *Salmonella* specific internal fluorogenic probe for monitoring the amplification of a 287 bp region of the *Salmonella invA* gene. Using the fluorogenic 5' nuclease assay, 164 *Salmonella* strains representing all the subspecies of *S. enterica* were detected while over 50 non-*Salmonella* strains were not detected. The detection limit of the assay was two colony-forming units (CFU) per PCR reaction when a pure culture of *S.* Typhimurium was used.

Waage et al. (58) developed a PCR assay with two nested pairs of primers selected from conserved sequences within 2.3 kb randomly cloned DNA fragment from the *S.* Typhimurium chromosome. The nested PCR assay correctly identified 128 of a total of 129 from the environmental water, sewage and food samples allowing a 312 bp PCR product with the sensitivity of 2 cfu/ml.

5.3 Arbitrarily primed polymerase chain reaction (AP-PCR)

The arbitrarily primed polymerase chain reaction (AP-PCR) is based on the amplification of genomic DNA with a single primer selected from an arbitrary nucleotide sequence. One advantage of AP-PCR is that it can be used without previous knowledge of the nucleotide sequence of target DNA. AP-PCR has potential as a tool in ecological studies of foodborne pathogens such as *Salmonella* nucleotide sequence of target DNA (59).

5.4 Pulsed-Field Gel Electrophoresis (PFGE)

Various phenotypic methods, such as serological typing, phage typing, and antimicrobial susceptibility testing, have traditionally been used in epidemiological studies of *Salmonella* outbreaks. However, these methods do not always give sufficient information for epidemiological purposes, even in outbreaks caused by a rare *Salmonella* serotype or phage type. More recently, molecular epidemiology-based techniques analyzing chromosomal DNA or plasmids have been shown to be useful for typing several *Salmonella* serotypes. Whole-cell DNA analysis by pulse-field gel electrophoresis (PFGE) has usually proven to be superior to other molecular methods in its discriminatory value. PFGE based on analysis of the whole genome by restriction endonuclease digestion has been shown to be a useful method for investigating the source, transmission, and spread of infectious agents and more particularly, for epidemiologic typing and determination of the genetic relatedness of strains. It has been applied to the study of the molecular epidemiology of numerous bacterial species (60). It is considered to be one of the most reliable typing procedure and to be the "gold standard" for the subtyping of *Salmonella* strains. However, the discriminatory ability of PFGE also is not optimal, and despite strenuous effects at standardization, there may be striking variability in PFGE gels (and data interpeatation) among various laboratories (61).

The basic principle of PFGE is the application of two electrical fields alternatively, at different angles defined time periods. Separation is achieved by competing large DNA molecules to continuously change direction as proceed through the gel matric. The adventages of this technique are increase in the efficiency of the separation of DNA fragments up to 9 million base pairs with good resolution and preservation of the vital integrity of DNA molecules. The whole cells could be embedded in agarose gel " inserts " which provide a natural and safe environment. The chromosomal DNAs are obtained in the inserts by subsequent digestion of all chromosomal proteins and other cell components. The inserts can be directly loaded into the well of the agarose gel to be run.

Mamuka et al. (62) found that multilocus sequence typing (MLST) was discriminatory ability was compared to those of pulsed-field gel electrophoresis (PFGE) and serotyping. PFGE differentiated several strains undifferentiable by serotyping the strains of several PFGE types were futher differentiated by MLST, which suggests that the discriminatory ability of MLST for the typing of *Salmonella* is better than that of serotyping and/or PFGE typing. It is indicated that the genes differ in their evolutionary origins and that recombination played a significant role in their evolution.

The study of Kumao et al. (61) demonstated that PFGE was less sensitive than ERIC2-PCR and ribotyping in intraserovar discrimination of *S.* Oranienburg isolates from human, as one of the river isolate which is genetically closely related, was indistinguishable from the outbreak strains by PFGE.

Shimizu et al. (63) used PFGE of chromosomal DNA digested with *Sma*I to characterize the strains of S. *aureus* coagulase type VII most frequently involved in staphylococcal food poisoning outbreaks in Tokyo, Japan. They then conducted a molecular epidemiological analysis of coagulase type VII strains by using PFGE. The results of the study suggest that PFGE might be a useful tool for subdividing coagulase types within *S. aureus* together with coagulase typing should be useful in detailed epidemiological studies.

Hampton et al. (64) demonstrate that PFGE, coupled with plasmid profile analysis, is a useful method for discriminating MDR isolates of *S.*Typhi Vi-phage type E1. The results also suggest that isolates of Vi-phage type E1 with the X2a pulsed-field profile have been derived within the Indian subcontinent from a drug-sensitive strain of Vi-phage type E1 already endemic in this area.

Nesse et al. (65) found that the combined results of PFGE and plasmid analyses showed that each factory harbored only a few *S. enterica* clones. Some of these clones persisted for at least 3 years in the factories, indicating that there was long-lasting contamination probably due to inadequate decontamination procedures.

Murase et al. (66) found that the isolates were indistinguishable by pulsed-field gel electrophoresis patterns and biotyping from the strain recovered from the roast pork that had been consumed by all of the people. This indicates that these isolates obtained from the infected people had originated in the contaminated pork.

Thorbjorn et al. (29) investigate the molecular epidemiology of *S.* Typhimurium from avian wildlife, domestic animal, and environment in Norway by PFGE, found that passerines constitute an important source of infection to human. It is suggested that gulls and pigions base on PFGE analysis, represent only a minor source of human serovar Typhimurium infections.

Alice et al. (67) used PFGE of chromosomal DNA digested with *Xba*I to characterize the strains of *S. enterica* subsp. *enterica* ser. 4,5,12: i :- that causing foodborne infection and including a large food poisoning outbreak in New York. The strains showed 11 or 12 resolvable gemonic fragments with 18 banding patterns and three PFGE profile (PFP) clusters (ie., PFP/A, PFP/B, and PFP/C). Seventy-five percent of the isolates fingerprintes were closely related ; 63% of these were indistinguishable from each other (PFP/A_1). PFP/A_1 was common to all strains from the outbreak and 11 hospital sources. Strains from six other hospitals shared clusters PFP/B and PFP/C. PFP/C_4 of the environmental isolate, was unrelated to PFP/A and PFP/B.

The PFGE subtyping method was used to assess the extent of genetic diversity and clonality of *S.* Weltevreden strains from human and environmental in Malaysia by Thong et al. (68). PFGE of *Xba*I-digested chromosomal DNA from 95 strains of *S.* Weltevreden gave 39 profiles, indicating that PFGE is very discriminative and that multiple clones of *S.* Weltevreden exist among clinical and environmental isolates. Strains of one dominant PFGE profiles (X_1 and X_2) appeared to be endemic in the region, as they were consistently recovered from humans with salmonellosis between 1996 and 2001 and from raw vegetables. The sharing of similar PFGE profiles among isolates from humans, vegetables, and beef provides indirect evidence of the possible transmission of salmonellosis from contaminated raw vegetables and meat to humans. Furthermore, the recurrence of PFGE X_{21} among isolates found in samples of vegetable from wet market indicated the persistence of this clone. The environment in the wet markets may represent a major source of cross-contamination of vegetables with *S.* Weltevreden.

Pathogenesis

The mode of transmission of *Salmonella* is ingestion of the organism in contaminated food, water, milk, meat, eggs and their products. Changing agricultural and food distribution methods, increased consumption of raw or slightly cooked food, and increase in the number of immunocompromised or chronically ill people and deterioration of the public health infrastructure are the most potential factors of epidemic salmonellosis. Pets such as dog, cat as well as poultry, swine, cattle, rodent and infected humans are potential source of reservoir.

The dose of organism in vehicle is the prime concerning factor for the salmonellosis. To be fully pathogenic, *Salmonella* must possess a variety of attributes called virulence factors such as ability to invade, lipopolysaccharides, intracellular replication and their toxins.

As the pathogen enters and move through its animal host, it encounters a series unique environment. Each host environment possesses distinct chemical and physical properties such as temperature, pH, osmolarity and nutrient availability.

Mechanism of *Salmonella* pathogenesis have not been fully eludicated, but several virulence-related factors have been associated with the ability of salmonellae to invade, reside within the lamina propria, and successfully live as intracellular parasites within a host. Virulence is defined as the ability to produce enteritis, invade the blood stream, and produce metastatic foci of infection (47). *Salmonella* are complex organism that produce a variety of virulence factors including surface antigens, factor contributing to invessiveness, endotoxin, cytotoxins and enterotoxins.

Most persons infected with *Salmonella* develop diarrhea, fever and abdominal cramps. Diarrhea appears 12 to 72 h after comsumption of contaminated food or beverage. *Salmonella* infection is usually resolve in 5 to 7 days and often do not require treatment, unless the patient becomes severely dehydrated or the infection spreads from the intestines. Person with severe diarrhea may require rehydration, often with intravenous fluids.

Most types of *Salmonella* lived in the intestinal tracts of animals and birds and transmitted to human by contaminated foods of animal origin, such as beef, poultry, milk or eggs, but other foods, including vegetables may become contaminated.

Epidemiology

Nontyphoidal salmonellosis, a disease caused by *Salmonella* other than Typhi, is an important food-borne infection with a worldwide distribution and an estimated annual incidence of 1.3 billion cases and 3 million deaths. Most of the nontyphoidal salmonellosis patients who presented with gastroenteritis (diarrhea, abdominal, pains, and fever) have self-limiting cases, and usually no treatment is required. However, some serovars can progress to sepsis, followed often times by consequential focal infections involving the gall bladder, endothelial surfaces, bone, and soft tissues (47). As it is a zoonosis, contaminated food with *Salmonella* causes gastroenteritis in man and animals, and also very often causes food poisoning through contamination of meat, milk, eggs or frozen seafood. In addition, rodents, and insects may carry *Salmonella*. More than 2,000 serovars of *Salmonella enterica* are recognized, and most capable of infecting a variety of animal species, including humans. There is considerable variation with time and with geographical location in the serovars most commonly associated with human infection. The two most common causes of nontyphoidal salmonellosis are *Salmonella* Typhimurium and *Salmonella* Enteritidis (69). Serotype Choleraesuis is also an infrequent serotype isolated from human sources in Canada and the United Kingdom. However, the epidemiological pattern differed greatly in Asian, African, European and American countries. Prevalence of salmonellosis in different regions of the world such as South-East Asia, Eastern Mediterranean, Africa, Western pacific, Europe and America was 81.80%, 69.60%, 60.00%, 55.60% and 34.30%, respectively (70). The mean proportion of all *Salmonella* isolated that were Enteritidis increased globoly from 25.6% in 1990 to 36.3% in 1995. This increase was observed in most regions ; American 11.3% in 1990/42.65 in 1995, Eastern Mediterranean 2.8%/16.0%, European 47.2%/58.6%, Western Pacific 8.9%/32.3%, African 7.1%/9.6%, but not in the South-East Asian region (11.9%/2.6%). The trends in the proportion of *Salmonella* Typhimurium was more variable. This decreased in the American (20.8%/8.0%) and European region (20.2%/17.6%) and increased in the Southeast Asia (9.5%/19.6%), Eastern Mediterranean (4.5%/4.9%), Western Pacific region (17.4%/26.0%) and American region (12.9%/15.9%) beyween 1990 and 1995. Over the same time interval, the global proportion of *Salmonella* Typhi were decreased from 15.3% in 1990 to 12.1% in 1995 and sharp decreases were observed in the American (13.4%/1.9%), Eastern Mediterranean (36.1%/18.9%) and Western Pacific regions (11.8%/2.5%) compared to the African (34.5%/34.2%), European (0.8%/1.3%) and the Southeast Asia region (37.8%/39.1%) (71).

A number of recent studies have shown that the increased incidence of *S*. Enteritidis may be related to the ingestion of raw, undercooked, or contaminated eggs or egg products (14).

In Canada, *Salmonella* Typhimurium and *Salmonella* Enteritidis have also caused a majority of cases of sporadic diseases and are responsible for most outbreaks due to *Salmonella* since 1983. While *Salmonella* Infantis outbreak led to the identified of pet treats produced from pig ears as a common source of human illness for many of outbreak cases, suggesting that there are alternate ways for these bacteria to infect the human population (50).

Sprouts have become an important cause of outbreaks of food-borne salmonellosis. In Finland, eight sprout-borne *Salmonella* outbreaks have occurred since 1980. The outbreak caused by *Salmonella enterica* Stanley the fact that the outbreak ended only after the industrial sprouting of alfalfa seeds was prohibited gave evidence that the seeds were the source (51).

The distribution of salmonellosis among humans in Denmark, *Salmonella* Typhimurium and *Salmonella* Enteritidis are the most common. Since 1984, serovar Enteritidis has been the predominant serovar, and phage type 4,6 and 8 have been the most important. The main sources of these infections have been meat and table eggs (52).

There was strong evidence that *Salmonella* Typhimurium has established reservoirs in wild-living birds and hedgehogs in Norway. Wild-living birds and hedgehogs may function as effective spreader of *Salmonella* spp. to human and to different animal species through contamination of the environmant. The sporadic indigenous cases and a national outbreak of human salmonellosis, caused by *Salmonella* Typhimurium have been related to infections in small passerines. In a water-borne, *S*. Typhimurium infection outbreak in northwestern Norway in 1999, gulls were suggested to be the most likely source of infection (29).

Salmonella Agona was first isolated from cattle in Ghana. In 1969 and 1970, serovar Agona emerged as a public health problem in the United States, the United Kingdom, the Netherlands, and Israel. Its appearance was attributed to be imported Peruvian fish meal. Subsequently, sereovar Agona was recovered from domestic animals and also from humans. Its persistence in livestock has probably resulted from the recycling of treated animal wastes as feed (76). Bartolozzi et al. (77) reported on an outbreak of serovar Agona in a ward for infants in an Italian hospital in 1972-1973.

Recently reported large serovar Agona outbreaks have been associated with a ready-to-eat savory snack and with a toasted-oats cereal.

In Malaysia, for the year 1999 the two predominant agents associated with food-borne nontyphoidal salmonellosis were also *S.* Enteritidis and *S.* Typhimurium. However, during the next decade, *Salmonella* Weltevreden become the most commonly isolated serovar, having been isolated from cattles, beef, mutton, duck, prawn, monkey, and rat. There are increasing reports of outbreaks assoiciated with fresh fruits and vegetables, including tomatoes, cantaloupes, watermelons, fresh unpasteurized orange juice, and mangoes (68).

For Thailand, in during 1993-2001 the most common serovar causing human salmonellosis was Salmonella Enteritidis (20.6%) was the most common serovar among the 12,938 Salmonella isolated from chicken meat, followed by S. Hadar (9.1%) and S. Paratyphi B var Java (7.2%). The most common serovar among the 921 isolated from sea food was S. Weltevreden (27%), among the 2,387 isolates from duck meat the most commonly isolated serovars were, S. Weltevreden (12.2%), S. Hadar (10.9%), S. Stanley (10.1%), and S. Chester (7.0%). Among the 5,620 isolates from other food products, S. Anatum (14.7%), S. Rissen (9.3%), S. Hadar (7.2%), and S. Weltevreden (7.0%) were the predominant serovars and among the 916 isolates from water it was, S. Weltevreden (15%), S. Anatum (11.7%), S. Rissen (8.7%), and S. Derby (7.8%) (4). The distribution of serovars causing human infection in Thailand differs markedly from those reported in other countries and seems to be related to the occurrence of Salmonella serovars in the different foods products and other reservoirs. Of particular interest is the frequent occurrence of S. Weltevreden and recent increase in occurrence of S. Rissen, S. Stanley, And S. Schwarzengrund. However, these aspects require further investigations (14).

According to world health Organization (WHO) forcast, foodborne disease will soon become the second largest case of morbidity in Europe including Asia and Africa. In the majority of cases, the source of infection in poultry and its products. The *Salmonella* Enteritidis is the major organism among the group of zoonotic pathogens involved in human foodborne disease around the world (14). Plasmid DNA fingerprinting and bacteriophage typing of *Salmonella* isolates are powerful epidemiological tools for surveying outbreaks of salmonellosis and tracing the spread of the organism in the environment.

USFDA has noted an overall incidence of 7.2% in imported and 1.3% in domestic seafood during a 9-year study (1990-1998) of 11,312 imported and 768 domestic seafood samples (79). *Salmonella* has been isolated from freshwater fish culture ponds in many countries. A survey in Japan showed that *Salmonella* spp. were present in 21% of eel culture ponds (80) and the incidence of *Salmonella* in freshwater cultured catfish in the USA was estimated to be 5% (81). The incidence of *Salmonella* in seafood from India has been reported by a number of investigators. They have reported the presence of *Salmonella* is 7.46% in frozen peeled and deveined shrimp, they demonstrated the presence of *Salmonella* is 12% in peeled and deveined shrimp, 10% in headless, shell-on shrimp, 14% in peeled and undeveined shrimp, 17% in lobsters, 14% in cuttlefish, 25% in catfish and 20% in seer fish. In Thailand during 1972-1993, WHO National *Salmonella* and *Shigella* center reported the incidence of *S.* Enteritidis dramatically increased since 1990. Recently, it has been revealed that 55%, 37% and 8% of *S.* Enteritidis were isolated from human, chicken meat and other sources respectively. The incidence of *S.* Enteritidis in chicken increased in parallel to human *i.e.* incidence of 1.4% in 1990 has been increased to 16.75% in 1993 (82). Thus this pathogen is a public health problem associated with poultry and its relating products.

Prevention

Salmonella infection usually results from a combination of contaminated foods, poor kitchen hygiene and inadequate cooking. Both the housewife and the chief should follow the attention to food kitchen practices, including through cooking of potentially contaminated foods especially chicken. Care must be taken to ensure that food does not become contaminated after cooking. Person to person spread is reduced by good personal hygiene and in particular by through hand washing after going to the toilet or handling clothes soiled with feces. Professional food handlers recovering from *Salmonella* infection are often screened to confirm bacteriological clearance before returning to work. There is no vaccine for non-typhoidal salmonellosis. Food handlers who are long-term *Salmonella* carriers may require treatment with amoxycillin or a quionolone.

CHAPTER 4

MATERIALS AND METHODS

The Studied Strains

The strains of *Salmonella* isolated from eggs and various sources in 3 chicken farms in Phra Nakorn Si Ayuttaya provience and from Klong Toey market in Bangkok during July-September 2000 were obtained from previously study (15). The two farms are opened system (Farm A and Farm B), and the other farm is a closed system (Farm C).

A open system has been practiced conventionally and widely in Thailand. The chicken house has been constructed openly without any wall around the house. The chicken pens are controlled 1 or 2 meters above the ground. The disadvantages of this system are uncontrolling the temperature and unprotecting the safety of chicken from snakes in the farms.

The closed system (Evaporative cooling system, EVAP) is a system consisted of turnnel ventilation system, water pump with pipe line to the cooling pad, plastic curtain covering both sides in the house, and thermostat for controlling fan and water pump. Only the assigned persons are allowed to work or enter the farm in order to control the sterilized atmosphere. The advantages of the EVAP cooling system are: exchanging air from inside to outside and from outside to inside, and decreasing the high temperature by ventilation resulted in cooling the temperature in the farm.

A total of 112 strains, 86 strains were obtained from package egg, 11 strains from laying egg, 7 strains from shell egg, 5 from worker's hand, 2 from chicken, and 1 from water samples as shown in Table 8. Five predominant serovars were included (15) such as *Salmonella* Braenderup (51/112), *Salmonella* Emek (24/112), *Salmonella*

Weltevreden (14/112), *Salmonella* Derby (12/112), and *Salmonella* Stanley (11/112) were studied (Table 9). The other serovars not included in this study were *S*. Cerro (6), *S*. Alachua (6), S. Virchow (4), *S*. Yoruba (4), *S*. Rissen (2), *S*. Java (2), *S*. Bovismorbificans (2), *S*. Senftenberg (2), *S*. Schwarzengrund (1), and *S*. Ohio (1). *Salmonella* Braenderup was isolated among the 10 strains from various sources in Farm A, 3 strains from Farm B, 33 strains from Farm C and 5 strains from Klong Toey market. *Salmonella* Emek was isolated among 1 strain from package egg in Farm B, 11 strains from package egg, 1 strain from shell egg and 1 strain from worker's hand from Farm C, and 10 strains from package egg in Klong Toey market. *Salmonella* Weltevreden isolated among 8 strains from package egg, 2 strains from shell egg and 1 strain from water in farm A, 1 strain from package egg in Farm B, 1 strain from chicken feces in from Farm C and 1 strain from Klong Toey market. All of 12 strains of S. Derby were isolated among 9 from package egg and 3 from shell egg in Farm A. *Salmonella* Stanley was isolated among 7 and 3 strains from package egg in Farm C and market, and 1 strain from laying egg in Farm C (Table 10).

Reconfirmed *Salmonella* Isolates by Biochemical and Serological Tests

The tested strains were purified and reconfirmed for *Salmonella* by biochemical and serological tests.

1. Biochemical tests

All strains were subcultured on Nutrient agar (NA; Difco, USA) and incubated at 37^{o} C for 24 h for biochemical tests with Triple Sugar Iron agar (TSI; Difco, USA) and Lysine Indole Motility agar (LIM; Difco,USA). Positive results were for TSI (K/A, H2S+), and LIM (+/-/+). Other biochemical reactions such as Urease, Mannitol, Dulcitol, Malonate, (Difco, USA) are shown in Table 1. Biochemical tests were performed as mentioned in Kauffman-White Scheme, 2001 (38).

Table 8 Source and number of *Salmonella* isolated from various sources in the chicken farms and market in Phra Nakorn Si Ayuttaya and a market in Bangkok (15)

	No. of *Salmonella* isolated from various sources						**Total**
	P	**SE**	**W**	**C**	**LE**	**H**	
Farm A	25	6	1	1	0	0	33
Farm B	5	0	0	0	0	0	5
Farm C	37	1	0	1	11	5	55
Market	19	0	0	0	0	0	19
Total	**86**	**7**	**1**	**2**	**11**	**5**	**112**

P= egg package, SE= egg shell, W= water, C= chicken feces, LE= laying egg, H= worker hand

Table 9 Number of the most 5 common *Salmonella* serovars isolated from various sources in the three chicken farms in Phra Nakorn Si Ayuttaya Province and a market in Bangkok (15)

Serovars	**No. of *Salmonella* isolated from**				**Total**
	Farm A	**Farm B**	**Farm C**	**Market**	**(%)**
S. Breanderup	10	3	33	5	51 (45.5)
S. Emek	0	1	13	10	24 (21.4)
S. Weltevreden	11	1	1	1	14 (12.5)
S. Derby	12	0	0	0	12 (10.7)
S. Stanley	0	0	8	3	11 (9.8)
Total	**33**	**5**	**55**	**19**	**112 (100)**

Table 10 Origin and number of *Salmonella* isolated from various sources in the chicken farms and market (15)

Origin	No. (%) of *Salmonella* serovar					Total
	Braenderup	Emek	Weltevreden	Derby	Stanley	
Farm A	**10**	**-**	**11**	**12**	-	**33**
Egg package	9	-	8	9	-	26
Egg shell	1	-	2	3	-	6
Water	-	-	1	-	-	1
Farm B	**3**	**1**	**1**	**-**	**-**	**5**
Egg package	3	1	1	-	-	5
Farm C	**33**	**13**	**1**	**-**	**8**	**55**
Egg package	19	11	-	-	7	37
Egg shell	-	1	-	-	-	1
Chicken feces	-	-	1	-	-	1
Laying egg	10	-	-	-	1	11
Worker hand	4	1	-	-	-	5
Market	**5**	**10**	**1**	**-**	**3**	**19**
Egg package	5	10	1	-	3	19
Total	**51**	**24**	**14**	**12**	**11**	**112**

2. *Salmonella* serotyping

2.1 Polyvalent and monovalent antisera (S&A REAGENTS LAB, Thailand)

The *Salmonella* strains confirmed by biochemical characteristic tests were then cultured on Nutrient agar (NA) plate and incubated at 37^{o} C for 24 h for serotyping with polyvalent and monovalent antisera (S&A REAGENTS LAB, Thailand) as shown in Table 11.

2.2 Slide agglutination test with O-antisera

The procedure for the slide agglutination test was as follows: one drop of normal saline solution (control) on the microscopic glass slide. Tested culture from NA were mixed with normal saline solution (NSS) and observed for auto-agglutination by rotating the slide back and forth for one min. It was indicated that it was rough strains, it could not be used to test with antisera. If there was no agglutination with NSS (control), the culture could be processed for testing with O-antisera. (S&A REAGENTS LAB, Thailand).

One drop of *Salmonella* polyvalent OMA, OMB, OMC and OMD antisera on each test area, tested culture from NA were mixed separately to each antiserum, The glass slide was rocked gently for one min. If agglutination found that the strain had that type antigen with the correspondent antisera. The tested strain was reported with correspondent positive polyvalent group (OMA or OMB or OMC, etc.) and then further tested with monovalent O antisera containing in that polyvalent group. For example, if the tested bacteria was positive with group B, monovalent O:1, O:4, O:5, O:12, O:27 antisera was then tested.

2.3 Detection of H – antigen by Sven Gard Technique (Inst Pasteur, France) Tested culture from NA were transferred to swarm agar by spotting at the center, then incubated at 37^{O}C for 18-24 h. Motility strains could spread on the surface of swarm agar. The culture from the edge of swarm agar were carefully mixed with NSS (control). The reaction was observed within 30 sec to 1 min, if there was no agglutination with NSS, the culture could be processed for testing with H-antisera

Table 11 Polyvalent and monovalent of *Salmonella* O and H antisera *

Polyvalent	**Monovalent**
O antisera	
OMA	2(A) + 4(B) + 9(D_1) + 9,46(D_2) +3,10(E_1) + 1,3,19(E_4) +
OMB	21(L)
OMC	7(C_1) +8(C_2 -C_3) + 11(F) + 13(G) + 6,14(H)
OMD	16(I) + 17(J) + 18(K) + 28(M) +30(N) + 35(O) + 38(P)
OME	39(Q) + 40(R) + 41(S) + 42(T) + 43(U) +44(V) +45(W)
OMF	47(X) +48(Y) + 50(Z) +51 + 52 + 53 + 61
OMG	54 + 55 + 56 + 57 + 58 + 59
H antisera	60 + 62 + 63 + 65 + 66 + 67
HMA	
HMB	a + b + c + d + i + z_{10} + z_{29}
E complex	E complex + G complex
G complex	e + h + nx + x + z_{15}
HMC	f + g + s + t + m + p + q
L complex	k + y + z + L complex + z_4 complex + r
z_4 complex	v + w + z_{13} + z_{28}
HMD	z_4 + z_{23} + z_{24} + z_{32}
1 complex	z_{35} + z_{36} + z_{38} + z_{35} + z_{39}+ z_{41} + z_{42} + z_{44}+ z_{60}
	1 + 2 + 5 + 6 + 7

* S&A REAGENTS LAB, Thailand

(S&A REAGENTS LAB, Thailand).

One drop of *Salmonella* polyvalent HMA, HMB, HMC, HMD, and H:1 Complex antiserum was dropped separately on a slide. The tested culture from swarm agar were mixed separately to each antiserum. The reaction was observed within 30 sec to 1 min. The agglutination reaction was positive, when certain antisera was positive to the corresponding tested culture serotyped. The tested bacteria was further tested with individual monovalent antisera including in that positive polyvalent antiserum. For example, HMA antiserum included a, b, c, d, i, z_{10}, z_{29}, HMB antiserum included H:G Complex and H:E Complex, HMC antiserum included k, r, y, z, z_4, and H:1 Complex included 1, 2, 5, 6, 7. In case of H:G Complex, H:E Complex and H:1 Complex were positive, the culture could be tested with individual monovalent antisera mentioned as above followed by f, g, s, t, m, p, q for H:G Complex, h, nx, x, z_{15} for H:E Complex, and 2, 5, 6, 7 for H:1 Complex (Table 11).

The type of H – antigen could be absorbed by dropping concentrated antiserum, at a titer of 1:800 or 1:1,600, with a volume of 0.09 ml in a small petridish, melted swarm agar was poured, vigorously shaked well to mix homogeneously. Swarm agar was allowed to be cool, culture from first plate swarm agar was inoculated and incubated at 37^{o} C for 18 – 24 h. Specific antibody in swarm agar was reacted with its homologous antigen, the tested culture which was not specific to antiserum could be spread, another phase antigen would be further tested.

Another phase H – antigen, was serotyped by following the same procedure as mentioned above. Absorbing for both 2 phase H-antigens, specific concentrate antiserum were added in another one petridish containing and swarm agar, shaked vigorously, and allowed to be cool. Culture from the second petridish were transferred into the last petridish, incubated at 37^{o} C for 18 h. The results was observed if there was not any spread strain in swarm agar, 2 phase antigens were then reported. The serovar was diagnosed by using Antigenic Formulas of the *Salmonella* serovar 2001 (Table 5).

Example for Diagnosis of *Salmonella* serovar Braenderup

The freshly grown colony of *Salmonella* on NA was tested by slide agglutination, O– antigen from tested culture was mixed with one drop of individual reaction as follow: polyvalent OMA, OMB, OMC and OMD antisera, the agglutinate occurred with polyvalent OMB. Then, the tested culture was further tested for specific antisera such as : Group C, Group F, and Group G antisera. In this case, the tested culture was agglutinated with Group C antiserum. Inaddition, the following agglutination test was reacted with monovalent in group C such as O:6,O:7,O:8,O:14,O:20 antisera, the reaction showed positive with O:6,O:7.

The tested culture from NA was transferred onto a swarm agar plate, incubated at 37 °C for 18 – 24 h. Culture from swarm agar was tested with various antisera as mentioned in Table 9. The reaction showed positive with polyvalent HMB. Then, the further reaction was mixed with H:G Complex, and H:E Complex antisera. The reaction showed positive with H:E Complex. The further test was performed with H-factor of H:E Complex, such as: h, nx, x, and z_{15}. The result showed positive with H:h antiserum. It interpreted that phase 1 H-antigen was eh. The concentrated H : eh antiserum titer at 1:800 for 0.09 ml was dropped in the second petridish and mixed well with melted swarm agar. Then, the swarm agar was allowed to be cool and culture from the first petridish was transferred onto the second swam agar, incubated at 37°C, 18 – 24 h. The second swarm agar culture showed negative for H : eh antiserum. Then, the tested culture was tested for another phase 2 H-antigens. The tested swarm culture was positive for H: E, H:nx, H:z_{15} antiserum, it meaned that H-antigen phase 2 was enz$_{15}$. The third swarm agar petridish containing H : eh and H : enz$_{15}$ antisera for absorption was allowed to be cool and transferred culture from the second swam agar onto the third swam agar. After incubation at 37°C, 18 – 24 h, the homologenous culture showed no swarming on the third swarm agar plate. Therefore, it was included that the culture was positive for O-antigen: 6, 7 and H-antigen : eh ,enz$_{15}$. The positive results of those mentioned O and H antigens were further interpreted as mentioned by using Antigenic Formula Table, the tested strain was *Salmonella* serovar Braenderup.

Microbial Susceptibility Test

3.1 Antimicrobial agents

Six antibiotics in the form of discs were employed for susceptibility test of salmonellae. The concentration of the antimicrobial agents was as follows:-

Ampicillin (Amp; Oxoid, England)	10	µg
Chloramphenicol (C; Oxoid, England)	30	µg
Cefotaxime (CTX; Oxoid, England)	30	µg
Nalidixic acid (NA; Oxoid, England)	30	µg
Sulfamethoxazole Trimethoprim (SXT; Oxoid, England)	25	µg
Tetracycline (TE; Oxoid, England)	30	µg

3.2 Preparation of bacterial inoculum

The agar disc diffusion method was used for susceptibility test of *Salmonella* to antimicrobial agents. The test was described by Bauer *et al* (59). All strains were subcultured on Tryptic Soy Agar (TSA; Oxoid, England). A few colonies of salmonellae was transferred with a loop into 2 ml of Mueller Hinton Broth (MHB; Oxoid, England) then incubated at 37˚C for 2–5 h until developed a slight to moderate turbidity. The bacterial suspension was then diluted to obtain turbidity equivalent to the McFarland standard No. 0.5 ($1x10^8$ CFU/ml) by MHB.

3.3 Antibiotic susceptibility test

The bacterial suspension was growth onto the surface of Mueller Hinton Agar (MHA; Oxoid, England) with a sterilized cotton swab. The plate was streaked within 15 min of preparing the adjusted inoculum and left the plate for 5-15 min to be allowed any excess moisture to be absorbed before applying disks. The antibiotic disks were put onto the MHA by a dispenser or forceps. Any disks after contacting to the agar was not removed. Then, it should be made sure that the disk was completely contacted to the agar surface by touching the disk with forceps. All the plates were incubated at 35-37°C for 18 h. The zone diameters were measured with a caliper. Diameters of the inhibition zone were recorded and interpreted as mentioned by NCCLS. The diameter of inhibition zone is shown in Table 12. In this test, *Escherichia coli* ATCC 25922. was used to be the quality control strain.

Pulse Field Gel Electrophoresis (PFGE)

4.1. Preparation of PFGE agarose plugs from *Salmonella* cell suspensions

All strains were subcultured on NA, and incubated at 37°C for 14-16 h. Colonies were picked and suspended into 2 ml of cell suspension buffer (CSB; 100mM Tris:100mM EDTA, pH 8.0) and adjusted concentration of the cell suspension by calculating the optical density (OD) at the wavelength 610 nm by a spectrophotometer. Then, 400 µl of each cell suspensions were transferred to microcentrifuge tubes. The microcentrifuge tubes were placed in plastic holders and incubated at 37°C in a water bath for 5 min. Then, 20 µl of Proteinase K (Biolabs, England) were added to each 400 µl of cell suspensions, mixed by closed tubes and tapped at the side of tube. Four hundred µl of melted 1% Seakem Gold agarose (SKG; Biolabs, England): 1% Sodium Dodecyle Sulfate (SDS; Biolabs, England) were added which warmed at 56°C in a water-bath, then added to each of the 400 µl of cell suspensions, mixed by gently pipetting mixture up and down for a few times. Immediately, the cell suspensions were dispensed part of mixture into wells in plug molds. All plugs were allowed to solidify for 10-15 min at room temperature (Fig. 1)

4.2 Lysis of cells in agarose plugs

The cell lysis buffer : Proteinase K (5 ml:25 µl) were prepared for one sample in a 50 ml-tube. Agarose plugs were removed from plug molds into tubes that containing cell lysis buffer : Proteinase K by spatula and the plugs were immersed under the buffer. The tubes were incubated at 54°C in a water-bath with shaking with constant agigation for 24 h. The bottle containing sterilized Milli Q water for washing warmed at 54°C in a water – bath (Fig. 2).

Table 12 Zone size and their interpretation for the antimicrobial agent*

Antimicrobial agent	**Disc content** (µg)	**Inhibition zone diameter (mm)**		
		R	I	S
Ampilicillin (AMP)	10	<13	14-16	>17
Trimethoprim-Sulfamethoxazole (SXT)	25	<10	11-15	>16
Chloramphenicol (C)	30	<12	13-17	>18
Tetracycline (TE)	30	<14	15-18	>19
Nalidixic acid (NA)	30	<13	14-18	>19
Cefotaxime (CTX)	30	<14	15-22	>23

* NCCLS (2004)

Colonies on TSA agar, 14-16 h

↓

Cell suspensions in 2 ml of CSB

↓

Adjust concentration of cell suspension with spectrophotometer at 610 nm

↓

Transfer 400 µl of cell suspension to microcentrifuge tube

↓

Incubate in the 37^{o} C water bath for 5 min

↓

Prepare 1% SKG:1% SDS agarose in 56^{o} C water bath

↓

Add 20 µl of proteinase K (20 mg/ml) of cell suspension

↓

Add 400 µl of 1%SKG: 1%SDS agarose to each 400 µl of cell suspension
mix gently by pipette

↓

Dispense part of mixture into well in plug molds,
Allow to solidify for10-15 min.

Fig. 1 Preparation of PFGE plugs

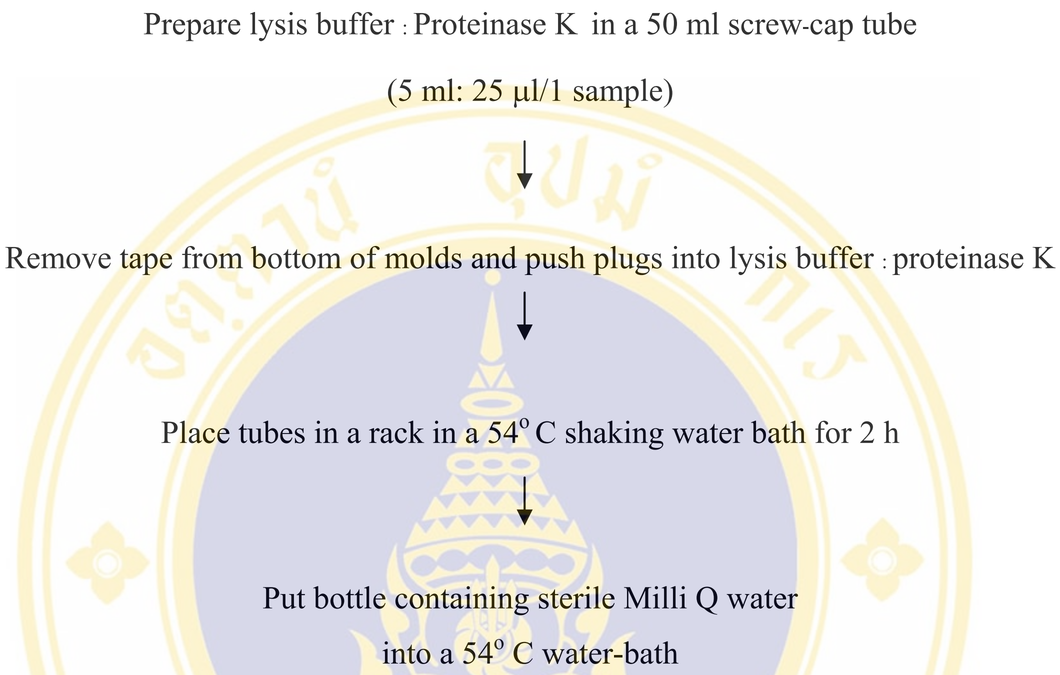
4.3 Washing of agarose plugs after cell lysis

The tubes with plugs were removed from the water-bath. Lysis buffer was carefully discarded. Bottom of tube with plugs were gently tapped against lab bench to free plugs to the bottom of tubes. Then, 15 ml of sterilized Milli Q water that was pre-warmed at 54^{o}C were added to each tube. The plugs were confirmed under water and returned for shaking at 54^{o}C in the water-bath for 10 min. Then, water was discarded and repeated washing step with pre-warmed water again for 10 mim. The bottle of Tris-EDTA buffer (TE;10. mM Tris: 1 mM EDTA, pH 8.0) was pre-warmed at 54^{o}C in a water-bath. Then, water from plugs was discarded and 15 ml of pre-warmed TE buffer was added. All tubes were incubated at 54^{o}C in a shaking water-bath for 15 min. The wash step with TE buffer were repeated more three times. Then, the agarose plugs were kept in TE buffer at 4^{o}C over night. All plugs were under in the TE buffer (Fig. 3)

4.4. Restriction digestion of DNA in agarose plugs with *Xba* I

For this step, gloves were used when handling 10x H Buffer (Biolabs, England) and it was kept in an ice box. The 10x H Buffer were prepared for 1x H Buffer by measuring sterilized Milli Q water and 10x H Buffer into a microcentrifuge tube according to the following Table 13 to make 1:10 dilution of the buffer. The volume of reagents were prepared accurately and enough amount for testing samples.

The diluted H Buffer was dispended in each label 1.5 ml microcentrifuge tubes. The agarose plugs were carefully removed from tubes containing TE buffer with spatula and placed in a sterile disposable petridish. Then, each agarose plug was cut into a 2 mm wide slice with razor blade and transferred to labeled 1.5 ml microcentrifuge tubes containing diluted H buffer. All plug slices were immersed under buffer. The tubes containing sample slices were placed in floating and incubated at 37^{o}C in a water-bath for 0-15 min. While plug slices were incubated in the buffer, diluted 10 x H Buffer 1:10 with sterilized Milli Q water according to the following (Table 14).



Prepare lysis buffer : Proteinase K in a 50 ml screw-cap tube

(5 ml: 25 µl/1 sample)

↓

Remove tape from bottom of molds and push plugs into lysis buffer : proteinase K

↓

Place tubes in a rack in a 54° C shaking water bath for 2 h

↓

Put bottle containing sterile Milli Q water
into a 54° C water-bath

Fig. 2 Step of cells lysis in agarose plugs

Carefully pour off lysis buffer

↓

Add 15 ml sterilized Milli Q water (pre-warmed) to each tubes

↓

Incubated at 54°C in a shaking water-bath for 10 min

↓

Pour off Milli Q water and repeat wash step with
pre – warmed water **one more time**

↓

Pour off water and add 15 ml pre – warmed TE,
shaking water- bath 54°C for 15 min

↓

Pour off TE and repeat wash step **more three times**

↓

Pour off TE from last wash step, add 10 ml of TE, keep at 4°C
Confirm that plugs were in the TE

Fig. 3 Step of washing of agarose plugs after cell lysis

Table 13 Preparation of 1x H Buffer

Reagent	µl /Plug slice	µl / N Plug slice
Sterile ultrapure water	180	N x 180
H buffer	20	N x 20
Total volume	200	N x 200

After plug slices were incubated, H buffer was removed by carefully avoiding to cut plug slice with a pipette tip. *Xba* I enzyme was added to the diluted buffer, mixed well by following the preparation mentioned in Table 14 and kept on ice throughout experiment. Then, 200 µl of diluted restriction enzyme mixture and added into each plug tube. The tube was closed and mixed by tapping gently on bench top, and all that plug slices were observed to be immersed under enzyme mixture. Then, sample tubes was incubated at 37^{o}C in a water-bath for 3 h (Fig. 4).

4.5. Electrophoresis of restriction digests in PFGE gel

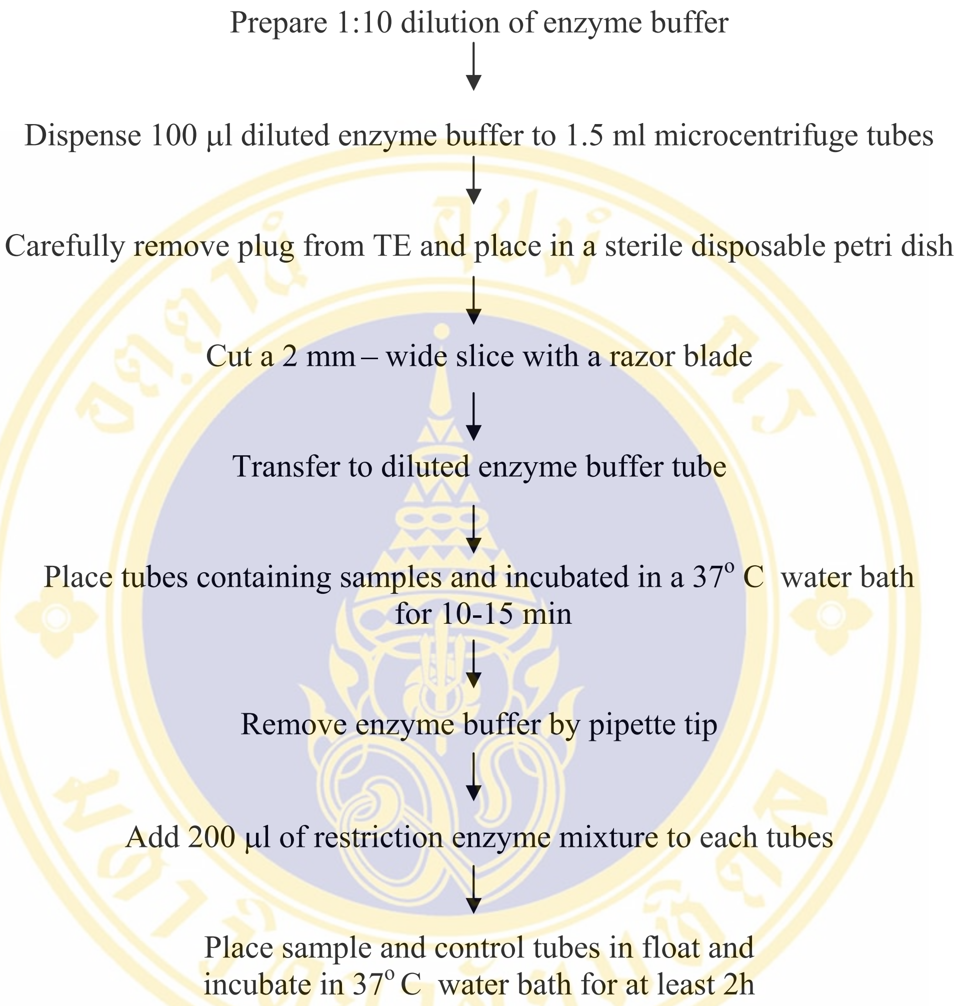
The restricted plug slice was removed from 37^{o}C water-bath, removed enzyme buffer mixture from plug slice with a pipette, and 200 µl of 0.5x TBE were added into each plug slice. For the preparation of plug in the agarose, the gel-form was set by put comb on top in its position, and loaded the plug slices on the bottom of the comb teeth. The standard marker (*S.* Braenderup H 9812) was on first, middle and last teeth, and the samples on the remaining teeth. The excess buffer of plug on the comb teeth was removed with of tissue, and the plug slices were air-dried for 3 min. The comb was set into position in the gel-form, the plug slices were correctly aligned, and 100 ml of melted 1 % Seakem Gold agarose (SKG; Biolabs, England) at 56^{o}C carefully poured into the gel-form. Any bubbles was removed with a clean pipette tip and gel was allowed to be solidified for 30 min before removing the comb.

In parallel, the preparation of electrophoresis chamber before running was set by adding 2.2 L of 0.5x TBE buffer (Tris-borate) and closed the cover of electrophoresis chamber. The power supply, pump, and cooling module were turned on, temperature of cooling module was set at 14^{o}C. Then, agarose on black casting platform was removed, and carefully placed in a electrophoresis chamber. The chamber was closed by the cover. The conditions of PFGE on the CHEF DR III electrophoresis unit were shown in Table 15.

Table 14 Preparation of 1x H Buffer containing restriction enzyme*

Reagent	µl / 1 Plug slice	µl / N Plug slices
Sterile ultrapure water	175	N x 175
H Buffer	20	N x 20
Xba I enzyme (10 U/µl.)	5	N x 5
Total volume	200	N x 200

* Pulse Net USA



Prepare 1:10 dilution of enzyme buffer

↓

Dispense 100 µl diluted enzyme buffer to 1.5 ml microcentrifuge tubes

↓

Carefully remove plug from TE and place in a sterile disposable petri dish

↓

Cut a 2 mm – wide slice with a razor blade

↓

Transfer to diluted enzyme buffer tube

↓

Place tubes containing samples and incubated in a 37° C water bath for 10-15 min

↓

Remove enzyme buffer by pipette tip

↓

Add 200 µl of restriction enzyme mixture to each tubes

↓

Place sample and control tubes in float and incubate in 37° C water bath for at least 2h

Fig. 4 Step of restriction digestion of DNA in agarose plugs with *Xba*I enzyme

4.6. Staining and documentation of PFGE agarose gel

After CHEF DR III electrophoresis run was over, chiller and pump were turned off. The gel was removed and placed in the plastic container that contain 40 µl of ethidium bromide (stock solution was 10 mg/ml) in 400 ml of distilled water. The container with gel was placed on a rocker for 25-30 min. After staining, the agarose was destained with distilled water for 60-90 min during which changing fresh distilled water was change for 2-3 times. The gel was captured image by using Gel Doc 2000 (Biolabs, England), and converted the .1sc file to a *.tif file.

4.7. Interpreting of chromosomal DNA restrictions patterns

PFGE profiles were analysed by using Bionumerics software version 3.0 (Biolabs, England) with a position tolerance of 1.0% and 0.5% optimization.

Table 15 The conditions of PFGE on the CHEF DR III electrophoresis *

Condition	
Cooling temperature	14^{o} C
Initial switch time	2.2 s
Final switch time	63.8 s
Voltage gradient	6 V/cm
Included angle	120^{o}
Actual current	0
Run time	18 h

* Pulse Net USA

CHAPTER 5
RESULTS

Reconfirmation of *Salmonella* Serovar

A total of 112 strains belonging into 5 serovars of *Salmonella* were repeatedly identified by biochemical tests and serotyping. The results reconfirmed the previous identification i.e 51 strains of *S.* Braenderup, 24 strains of *S.* Emek, 14 strains of *S.* Weltevreden, 12 strains of *S.* Derby, and 11 strains of *S.* Stanley.

Antimicrobial Susceptibility of *Salmonella*

The antibiotic resistant patterns of the 112 *Salmonella* isolated from various sources in 3 chicken farms and a market are shown in Table 16. Fifty three strains (47.3%, 53/112) of *Salmonella* were resistant to 4 of 6 tested antibiotics, namely nalidixic acid 79.2% (42/53), sulfamethoxazol trimethoprim 71.7% (38/53), tetracycline 54.7% (29/53), and ampicillin 5.7% (3/53), respectively. In this study, all (53 strains) were sensitivity to cefotaxime and chloramphinicol.

According to the serovar of *Salmonella*, it was found that *S.* Weltevreden was sensitivity to all 6 tested antibiotics (Table 16). Contrastly, all *S.* Emek strains were resistant to the tested antibiotics (100%, 24/24), followed by *S.* Derby (91.7%, 11/12), *S.* Braenderup (33.3%, 17/51), and *S.* Stanley (9.1%, 1/11). In addition, all 24 *S.* Emek strains were resistant to only 3 out of 6 tested antibiotics: nalidixic acid (100%), sulfamethoxazol trimethoprim (87.5%, 21/24), and tetracycline (54.1%, 13/24). Eleven strains of *S.* Derby were resistant to 3 antibiotics: sulfamethoxazol trimethoprim (90.9%, 10/11), tetracycline (90.9%, 10/11) and nalidixic acid (9.1%, 1/11). Seventeen strains of *S.* Braenderup were resistant to 4 antibiotics : nalidixic acid were the highest percent

(94.1%, 16/17), following by sulfamethoxazol trimethoprim (41.2%, 7/17), ampicillin (17.6%, 3/17), and tetracycline (35.3%, 6/17). Only one strain of *S*. Stanley (9.1%, 1/11) was resistant to only one antibiotic, i.e ampicillin.

Table 17 shows antimicrobial multi-drug resistance patterns, sources, farms, and serovars of isolated *Salmonella* strains. The prevalence of multi-drug resistant patterns of salmonellae (70%, 37/53) were significantly higher than those of single drug resistant pattern (30, 16/53) ($p<0.05$). In this study, there were 4 antimicrobial resistance patterns; two-antibiotic resistant pattern was the highest percent in 34% (18/53), followed by three-antibiotic resistant pattern in 32.1% (17/53), single resistant pattern (nalidixic acid) in 30.1% (16/53), and four-antibiotic resistant pattern in 3.8% (2/53).

All 16 single antibiotic resistant strains of salmonellae were resistant to nalidixic acid. Four serovars included in this pattern were *S*. Braenderup (68.8 %, 11/16), followed by *S*. Emek (18.8%, 3/16), *S*. Stanley (6.2 %, 1/16), and *S*. Derby (6.2%, 1/16). In addition, 11 *S*. Braenderup strains were detected in Farm C (6 from package and 2 from laying egg samples) and Klong Toey market (3 from package samples). Three *S*. Emek strains were detected from only package samples in Farm B (1 sample) and Klong Toey market (2 samples). One *S*. Stanley and 1 *S*. Derby strains were detected from package samples from the market, and from Farm A, respectively.

Two patterns of two-antibiotics resistance of 18 salmonellae were sulfamethoxazol trimethoprim- tetracycline pattern which were detected from 10 *S*. Derby (55.6%, 10/18) strains (7 from package and 3 shell egg samples from Farm A); and sulfamethoxazol trimethoprim- nalidixic acid pattern were detected from 8 *S*. Emek (44.4%, 8/18) strains (all from package samples in the studied market).

Two patterns of three-antibiotics resistance of 17 salmonellae were sulfamethoxazol trimethoprim-nalidixic acid- tetracycline pattern detected from 3 *S*. Braenderup strains (from a package, a laying egg and a worker hand sample in Farm C) and 13 *S*. Emek strains (12 package samples and a worker hand sample in Farm C); and sulfamethoxazol trimethoprim- ampicillin- tetracycline pattern detected from 1 *S*. Braenderup (5.9%, 1/17) strain (from a package sample in Farm A).

Four –antibiotics resistance pattern : sulfamethoxazol trimethoprim-nalidixic acid-ampicillin-tetracycline of 2 salmonellae are shown in Table 17. These 2 (100%) salmonellae were *S.* Braenderup isolated from a package sample in Farm A and C. In conclusion, 86.8% (46/53) of all resistant salmonellae were predominately detected from package samples in all studied farms and the market. It was noticed that almost half of antibiotic resistant salmonellae (43.5%, 20/46) were contaminated in package samples at Farm C which was higher than those in the other farms (Farm A 21.7%, 10/46; Farm B 2.2%, 1/46), and the market (30.4%, 14/46). However, 52.3% (45/86) of antibiotic resistant salmonellae were detected from all 86 tested salmonellae from package samples at all 3 farms and market, 47.6% (31/67) of antibiotic resistant salmonellae were detected in package samples at Farm A, B and C and 73.9% (14/19) of antibiotic resistant salmonellae were detected in package samples at market.

Pulse Field Gel Electrophoresis (PFGE)

Genotypic patterns among the 112 strains of salmonellae were analyzed by using PFGE, a DNA fingerprinting technique. In this study, PFGE patterns of 112 salmonellae including 51 strains of S. Braenderup, 24 strains of *S*. Emek, 14 strains of *S.* Weltevreden, 12 strains of *S*. Derby, and 11 strains of *S*. Stanley were generated with restriction enzyme *Xba*I resulted in 7, 4, 4, 1,and 4 different DNA patterns, respectively. The fingerprinting patterns of these strains of 5 tested serovars were reproducible and showed marked heterogeneity between member serovars (Fig.5-Fig.8).

Salmonella Braenderup

Among the 51 S. Braenderup strains showed 7 different patterns as designated as PFGE types A1-A7 (Table 18). PFGE type A2 was the most common PFGE type of *S*. Braenderup 70.6% (36/51) and found in all farms and the market, followed by type A3 15.7% (8/51), A1, and A5 3.9% each (2/51), and A4, A6, and A7 1.9% each (1/51)

Table 16 Antibiotic resistant among of 112 *Salmonella* strains isolated from the 3 chicken farms and the market

Serovar	No. of isolates	No.(%) of resistance isolates	No. (%) of *Salmonella* isolates resistance to					
			CTX	**SXT**	**NA**	**AMP**	**C**	**TE**
S. Braenderup	51	17 (33.3)	0	7 (41.2)	16 (94.1)	3 (17.6)	0	6 (35.3)
S. Emek	24	24 (100)	0	21 (87.5)	24 (100)	0	0	13 (54.1)
S. Weltevreden	14	0	0	0	0	0	0	0
S. Derby	12	11 (91.7)	0	10 (90.9)	1(9.1)	0	0	10 (90.9)
S. Stanley	11	1 (9.1)	0	0	1(100)	0	0	0
Total	**112**	**53 (47.3)**	**0**	**38 (71.7)**	**42 (79.2)**	**3 (5.7)**	**0**	**29 (54.7)**

CTX= Cefotaxime, SXT= Sulfamethoxazole trimethoprim, NA= Nalidixic acid, AMP= Ampicillin,
C= Chloramphenicol, TE = Tetracycline

Table 17 Antimicrobial resistance patterns of *Salmonella* by the sources of isolates

Resistance pattern	**No. of *Salmonella* isolated from**					**Total (%)**
	Farm A	**Farm B**	**Farm C**	**Market**	**Serovar**	**N=(53)**
Single						**16 (30.1)**
NA	-	-	6 P, 2 LE	3 P	*S*. Braenderup	11 (68.8)
	-	1 P	-	2 P	*S*. Emek	3 (18.8)
	-	-	-	1 P	*S*. Stanley	1 (6.2)
	1 P	-	-	-	*S*. Derby	1 (6.2)
Two-antibiotic						**18 (34.0)**
SXT,TE	7 P, 3 SE	-	-	-	*S*. Derby	10 (55.6)
SXT,NA	-	-	-	8 P	*S*. Emek	8 (44.4)
Three-antibiotic						**17 (32.1)**
SXT,NA,TE	-	-	1 P, 1 LE, 1 H	-	*S*. Braenderup	3 (17.6)
	-	-	12 P, 1 H	-	*S*. Emek	13 (76.5)
SXT,AMP,TE	1 P	-	-	-	*S*. Braenderup	1 (5.9)
Four-antibiotic						**2 (3.8)**
SXT,NA,AMP,TE	1 P	-	1 P	-	*S*. Braenderup	2 (100)

CTX = Cefotaxime, SXT= Sulfamethoxazole-trimethoprim, NA= Nalidixic acid, AMP = Ampicillin, C = Chloramphenicol, TE = Tetracycline

P= Package, SE= egg shell, LE= laying egg, H= worker hand, W= water

(Fig. 9). Ten strains of Farm A showed 2 different patterns of A1 (1 package sample), and A2 (8 package samples and an egg shell sample). Three strains of Farm B produced 2 different patterns of A2 (2 package samples), and A4 (a package sample). Among the 32 strains from farm C showed 5 different DNA patterns of A1 (a package sample), A2 (13 package samples, 5 laying egg samples, and 3 worker hand samples), A3 (3 package samples, and 5 laying egg), A5 (a package sample), and A7 (a package sample). Types of A2 (4 package samples), A4, and A5 (a package sample each) were found from the 6 strains of isolated from the egg package in the market.

It was noticed that there were 4 subtypes among 36 strains of *S*. Braenderup type A2 in relation to the similarity of PFGE type antimicrobial resistant patterns namely: **1)**. Type A2 with nalidixic acid resistance clone (25%, 9/36) was contaminated in egg package (5), laying egg (1), and worker hand (1) sample(s) at Farm C, and egg package (2) samples of the studied market; **2)** Type A2 with sulfamethoxazole-trimethoprim-nalidixic acid-tetracycline resistance clone (8.3%, 3/36) was detected in egg package (1), laying egg (1), and worker hand (1) samples at Farm C; **3)** Type A2 with antibiotic sensitive clone (63.9%, 23/36) was distributed in Farm A, B, C, and the studied market in 7,2,6, and 1 package sample(s), respectively; and egg shell (1) sample in Farm A, and laying egg, worker hand (3,3) samples in Farm C. **4)** Type A3 with antibiotic sensitive clone (22.2%, 8/36) was contaminated in laying egg (5), and egg package samples in Farm C only.

In addition, 2 strains (3.9%, 2/5) of *S*. Braenderup having the same multi-drug resistant pattern (sulfamethoxazole-trimethoprim-ampicillin-nalidixic acid-tetracycline) and detected from a package sample each in Farm A and C produced the same PFGE type A1 in this study. The other clones not commonly found were: Type A2 with sulfamethoxazole-ampicillin-nalidixic acid-tetracycline, Type A4 with antibiotics sensitive, Type A5 with antibiotic sensitive, Type A5 with nalidixic acid, Type A6 with nalidixic acid, and Type A7 with antibiotics sensitive clone were found in package sample in Farm A, B, C, the market, and C (1.9%, 1/51; each), respectively. It could be concluded that many different clones of *S*. Braenderup contaminated in the environment of the farm or among all farms.

***Salmonella* Emek**

For 24 *S.* Emek strains generated 4 different patterns as designated as PFGE types B1-B4 (Table 19, Fig. 10,11). PFGE type B1 showed the most common PFGE type of *S.* Emek 45.8% (11/24) found in Farm C, followed by B4 29.2% (7/24), B3 20.8% (5/24), and B2 4.2.% (1/24) (Fig. 6, 7). *S.* Emek strains were contaminated in 92.3% (12/13) of package samples and 7.7% (1/13) of a worker hand sample in Farm C and 100% (10/10) in package samples in the studied market. Only one strain of Farm B showed one pattern of Type B1. Three types of B1, B2, and B3 were observed among the 13 strains from farm C, and 2 types of B3 and B4 were observed among the 10 strains of *S.* Emek from market.

It was found that there were 5 common clones among 24 drug resistant *S.* Emek strains found in this study according to the similarity of PFGE type and antimicrobial resistant detected in package samples of Farm C and market : **1)**. Type B1 with sulfamethoxazole-trimethoprim-nalidixic acid-tetracycline resistance clone was the common clone (37.5%, 10/24) which was contaminated in package (9), and worker hand (1) sample(s) of Farm C, followed by **2)** Type B4 with sulfamethoxazole-trimethoprim-nalidixic acid resistance clone (20.8%, 5/24) was distributed in packages samples of the market, **3)** Type B3 with sulfamethoxazole-trimethoprim-nalidixic acid resistance clone (12.5%, 3/5) was detected in the market, **4)** Type B3 with sulfamethoxazole-trimethoprim-nalidixic acid-tetracycline resistance clone (8.3%, 2/24) was detected in Farm C, and **5)** Type B4 with nalidixic acid resistance clone (8.3%, 2/24) was found in the market. It was interesting that the tested drug resistant *S.* Emek strains could be detected at Farm C and the studied market, not at the other farms. Two dominant clones were separately found in Farm C (Type B1 with sulfamethoxazole-trimethoprim-nalidixic acid-tetracycline resistance clone) and the market (Type B4 with sulfamethoxazole-trimethoprim-nalidixic acid resistance clone). Type B1 with sulfamethoxazole-trimethoprim-nalidixic acid-tetracycline resistance clone was detected from a worker hand and package samples in Farm C.

***Salmonella* Weltevreden**

For all 14 antibiotic sensitive *S.* Weltevreden strains showed 4 different PFGE types designated as types C1-C4 (Table 20, Fig 12). Farm A had all 4 types C1-C4 (78.6%, 11/14) and Type C1 (57.1%, 8/14) was the most common clone and found in package (5), shell egg (2), and water (1) sample(s). Type C2 (7.1%, 1/14) was found in a package sample from Farm A. Type C3 (14.3%, 2/14) was found in package sample (1, each) from Farm A and B. Type C4 (21.4%, 3/14) was found in package sample (1, each) from Farm A and the market, and from chicken feces (1) from Fram C. In this study, Type C1 was the common clone of antibiotic sensitive *S.* Weltevreden strains occurring in Farm A (but not in Farm B,C, and the market) showing the cross contamination of this clone in the environments (package, shell egg, and water). However, Type C3 clone was found in both farms (A and B), and Type C4 clone was detected in Farm A, C, and market.

***Salmonella* Derby**

In this study, it was found that 12 strains of *S.* Derby produced only one PFGE type designated as Type D (Table 21, Fig 13). All of them were isolated from farm A. Type D with sulfamethoxazole-trimethoprim-tetracycline resistance clone (83.3%, 10/12) of *S.* Derby was the most common clone occurred in Farm A. This clone was distributed in 7 package samples, and 3 in shell egg samples in Farm A. It could be known that Type D with sulfamethoxazole-trimethoprim-tetracycline resistance clone was the common clone and contaminated in package and shell egg samples in only Farm A.

***Salmonella* Stanley**

Table 22 and Fig.14 shows 4 different DNA patterns among the 11 *S.* Stanley strains and were designated as Type E1-E4. Type E1 was the dominant clone that isolated from only Farm C in package samples (63.6%, 7/11), type E2 was found in a laying egg samples (9.1%, 1/11) in only Farm C. Type E3 isolated from package samples (18.2%, 2/11) of the market. Type E4 with nalidixic acid were detected in a package sample (9.1%, 1/11) of the market. It was concluded that Type E1 of *S.* Stanley was the common clone occurring in Farm C and could be caused the cross contamination in the environmental samples (package and laying egg) in only Farm C in this study.

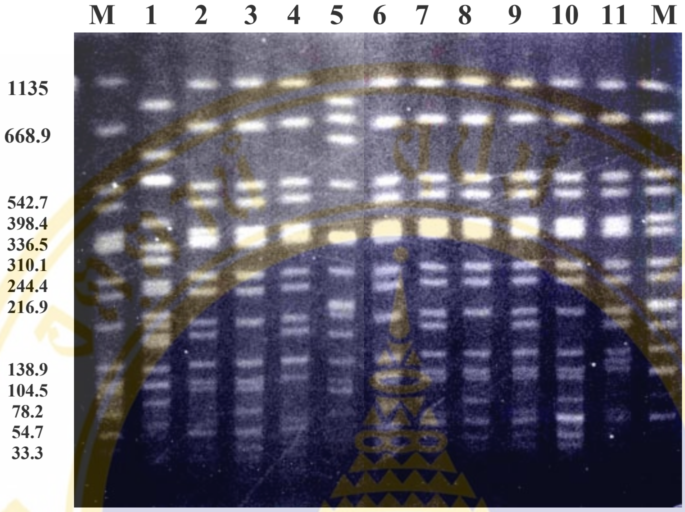


Fig. 5 Presentative PFGE profile of *S.* Braenderup isolated from various sources in the chicken farms and the market . Lane M were standard marker of *S.* Braenderup H9812. Lane 1 was *S.* Virchow and Lane 5 was *S.* Stanley. Lane 2, 3, 4, 6, 7, 8, 9, 10, 11 belong to *S.* Braenderup. Number of the left indicate the position of molecular mass marker.

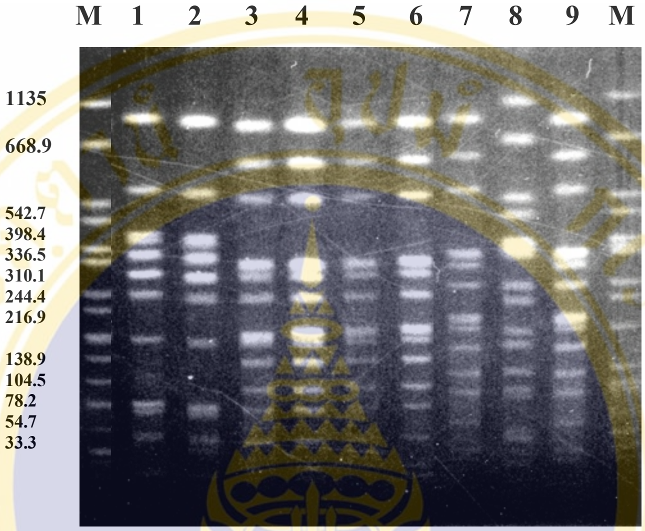


Fig. 6 Presentative PFGE profile of *S.* Stanley, *S.* Braenderup and *S.* Emek isolated from various sources in the chicken farms and the market . Lane M were standard marker of *S.* Braenderup H9812. Lane 1, 2 belong to *S.* Emek, Lane 3, 4, 5, 6, 7, 9 belong to *S.* Stanley and Lane 8 belong to *S.* Braenderup. Number of the left indicate the position of molecular mass marker.

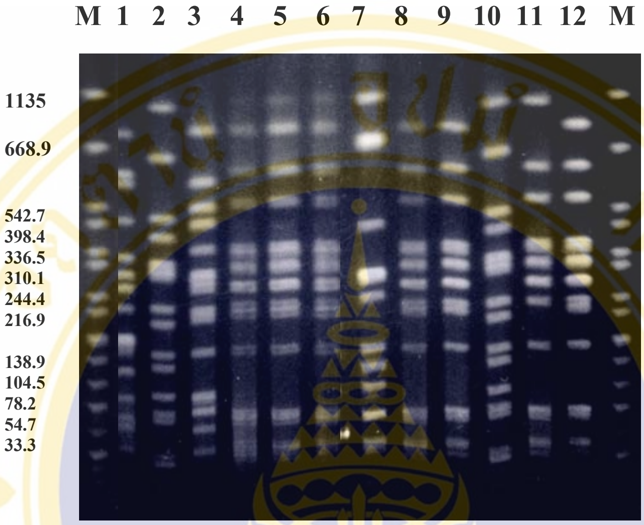


Fig.7 Presentative PFGE profile of *S.* Emek, *S.* Stanley, and *S.* Braenderup isolated from various sources in the chicken farms and the market. Lane M were standard marker of *S.* Braenderup H9812. Lane 2 belong to *S.* Stanley, Lane 4, 5, 6, 8, 9, 11, 12 belong to *S.* Emek , Lane 7 belong to *S.* Weltevreden and Lane 10 belong to *S.* Braenderup. Number of the left indicate the position of molecular mass marker.

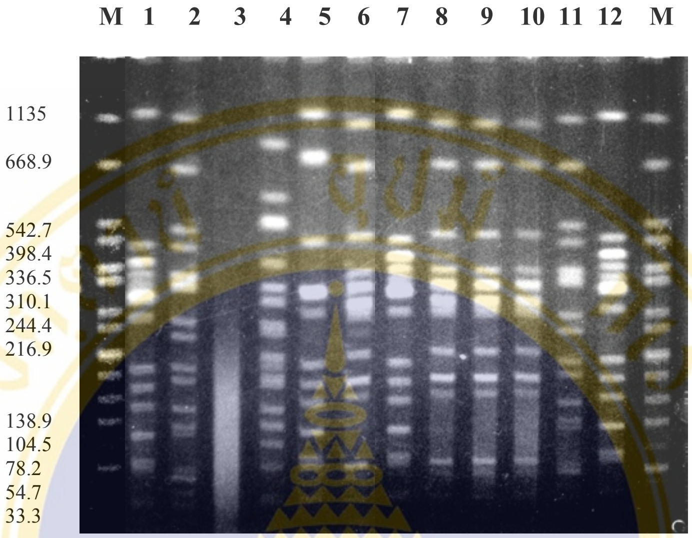


Fig 8 Presentative PFGE profile of *S.* Weltevreden, *S.* Braenderup, and *S.* Derby isolated from various sources in the chicken farms and the market. Lane M were standard marker of *S.* Braenderup H9812. Lane 1, 5, 7, 12 belong to Weltevreden, Lane 2, 11 belong to *S.* Braenderup, Lane 6, 8, 9, 10 belong to *S.* Derby Number of the left indicate the position of molecular mass marker.

Table 18 The relationship of antimicrobial resistant patterns and PFGE types of *S.* Braenderup isolated from the chicken farms and the market

PFGE type	Resistant pattern				Susceptible	Place		No. (%) of Strain
	NA	SXT, NA,TE	SXT, AMP,TE	SXT,AMP, NA,TE		Site	Sample type	
A1				+		Farm A	(P)	1(1.9)
A1				+		Farm C	(P)	1(1.9)
A2	+					Farm C	(P)	5(9.8)
A2	+					Farm C	(LE)	1(1.9)
A2	+					Farm C	(H)	1(1.9)
A2	+					Market	(P)	2(3.9)
A2		+				Farm C	(LE)	1(1.9)
A2		+				Farm C	(P)	1(1.9)
A2		+				Farm C	(H)	1(1.9)
A2			+			Farm A	(P)	1(1.9)
A2					+	Farm A	(P)	7(13.7)
A2					+	Farm A	(SE)	1(1.9)
A2					+	Farm B	(P)	2(3.9)
A2					+	Farm C	(P)	6(11.8)
A2					+	Farm C	(LE)	3(5.9)
A2					+	Farm C	(H)	3(5.9)
A2					+	Market	(P)	1(1.9)
A3					+	Farm C	(LE)	5(9.8)
A3					+	Farm C	(P)	3(5.9)
A4					+	Farm B	(P)	1(1.9)
A5					+	Farm C	(P)	1(1.9)
A5	+					Market	(P)	1(1.9)
A6	+					Market	(P)	1(1.9)
A7					+	Farm C	(P)	1(1.9)

P = Package egg, SE = egg shell, LE = Laying egg, H = worker hand

CTX = Cefotaxime, SXT= Sulfamethoxazole-trimethoprim, NA= Nalidixic acid,

AMP = Ampicillin, C = Chloramphenicol, TE = Tetracyclin

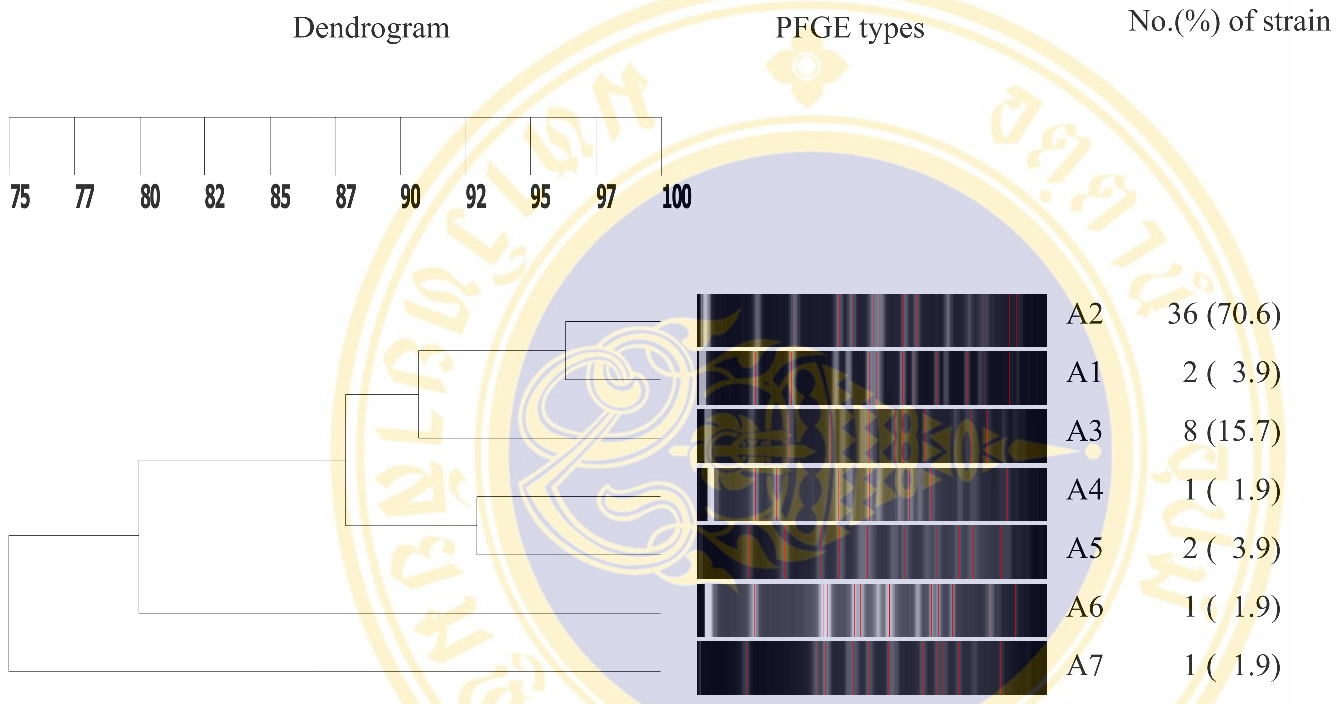


Fig. 9 Seven PFGE types of *Salmonella* Braenderup (N=51) isolated from the chicken farms and the market

Table 19 The relationship of antimicrobial resistant patterns and PFGE types of *S*. Emek strains isolated from farms and market

PFGE type	**Resistant pattern**			**Place**		
	NA	**SXT, NA**	**SXT, NA, TE**	**Site**	**Sample type**	**No. (%) of *S*. Emek**
B1	+			Farm B	(P)	1 (4.2)
			+	Farm C	(P)	9 (37.5)
			+	Farm C	(H)	1 (4.2)
B2			+	Farm C	(P)	1 (4.2)
B3			+	Farm C	(P)	2 (8.3)
		+		Market	(P)	3 (12.5)
B4		+		Market	(P)	5 (20.8)
	+			Market	(P)	2 (8.3)
N= 24	**3**	**8**	**13**			

CTX = Cefotaxime, SXT= Sulfamethoxazole-trimethoprim, NA= Nalidixic acid,
AMP= Ampicillin,C = Chloramphenicol, TE = Tetracycline
P = Egg package, H = Worker hand

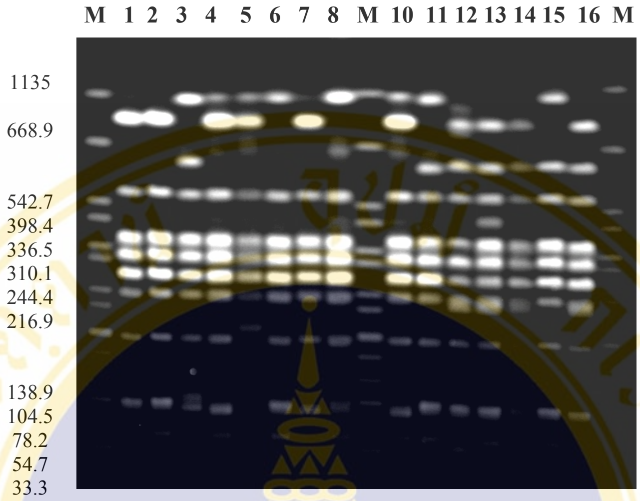


Fig.10 Presentative PFGE profile of *Salmonella* Emek isolated from various sources in chicken farms and market. Lane M were standard marker of *S.* Braenderup (H9812). Lane 2, 3, 5, 6, 8, and 11 belong to PFGE type B1. Lane 4, 7, and 16 belonging to type B2. Lane 9 was type B3 and lane 12, 13, 14, 15, and 17 belonging to type B4. Number on the right indicate the positions of molecular mass marker.

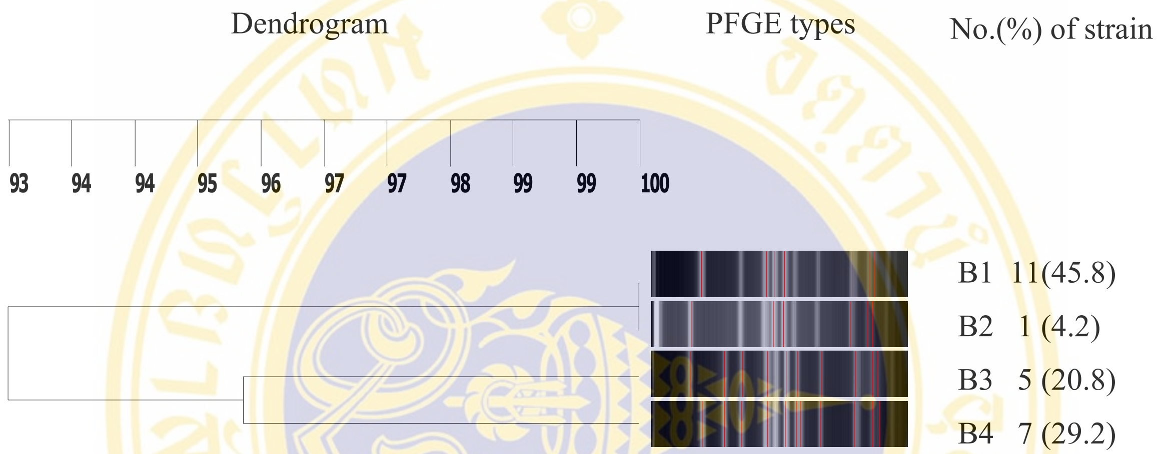


Fig. 11 PFGE types among *Salmonella* Emek (N= 24) isolated from the chicken farms and the market

Table 20 Source and distribution of *S.* Weltevreden strains by different PFGE types

PFGE types	No. of strains	No. (%) of strains detected in			
		Farm A	**Farm B**	**Farm C**	**Market**
C1	8 (57.1)	8 (5P,2SE,1W)	-	-	-
C2	1 (7.1)	1 (P)	-	-	-
C3	2 (14.3)	1 (P)	1 (P)	-	-
C4	3 (21.4)	1 (P)	-	1 (P)	1 (P)
Total	**14 (100)**	**1 (78.6) (8P,2SE,1W)**	**1 (7.1) (P)**	**1 (7.1) (P)**	**1 (17.1) (P)**

P =Egg package, SE = Egg shell, W = Water

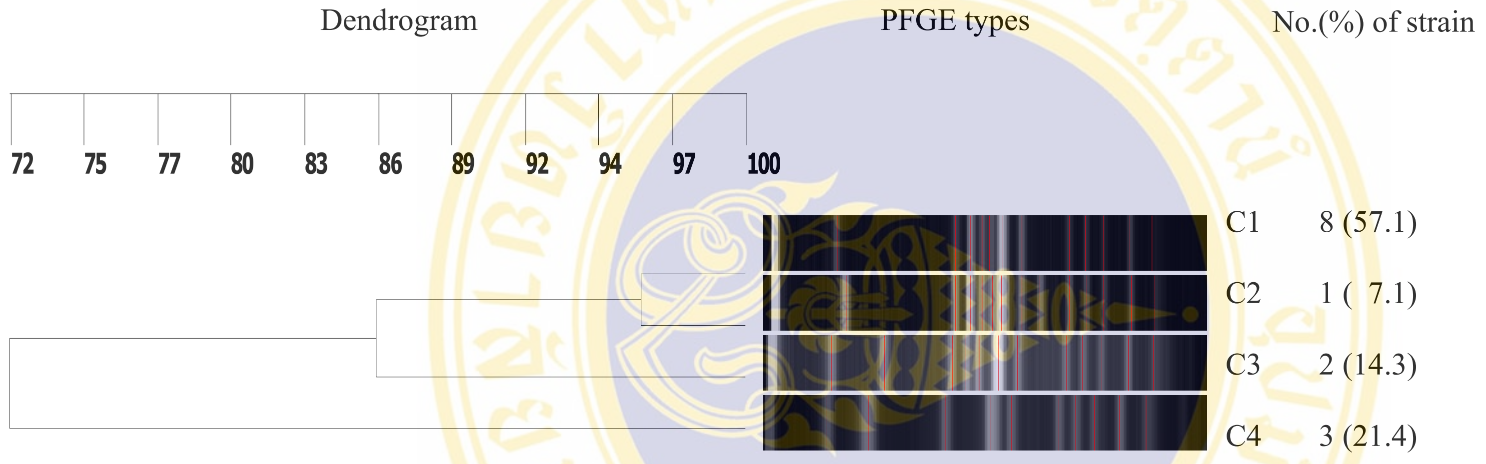


Fig. 12 PFGE types among *Salmonella* Weltevreden (N=14) isolated from the chicken farms and the market

Table 21 The relationship of antimicrobial resistant patterns and PFGE types of *S*. Derby isolated from the chicken farms and the market

PFGE Type	**Resistant pattern**			**Place (source)**	**No. (%) of *S.* Derby**
	NA	SXT, TE	Susceptible		
D			+	Farm A (P)	1 (8.3)
D	+			Farm A (SE)	1 (8.3)
D		+		Farm A (P)	7 (58.4)
D		+		Farm A (SE)	3 (25.0)
	1	**10**	**1**		**12 (100)**

P = Egg package, SE = Egg shell

NA= Nalidixic acid, SXT= Sulfamethoxazole-trimethoprim, TE = Tetracycline

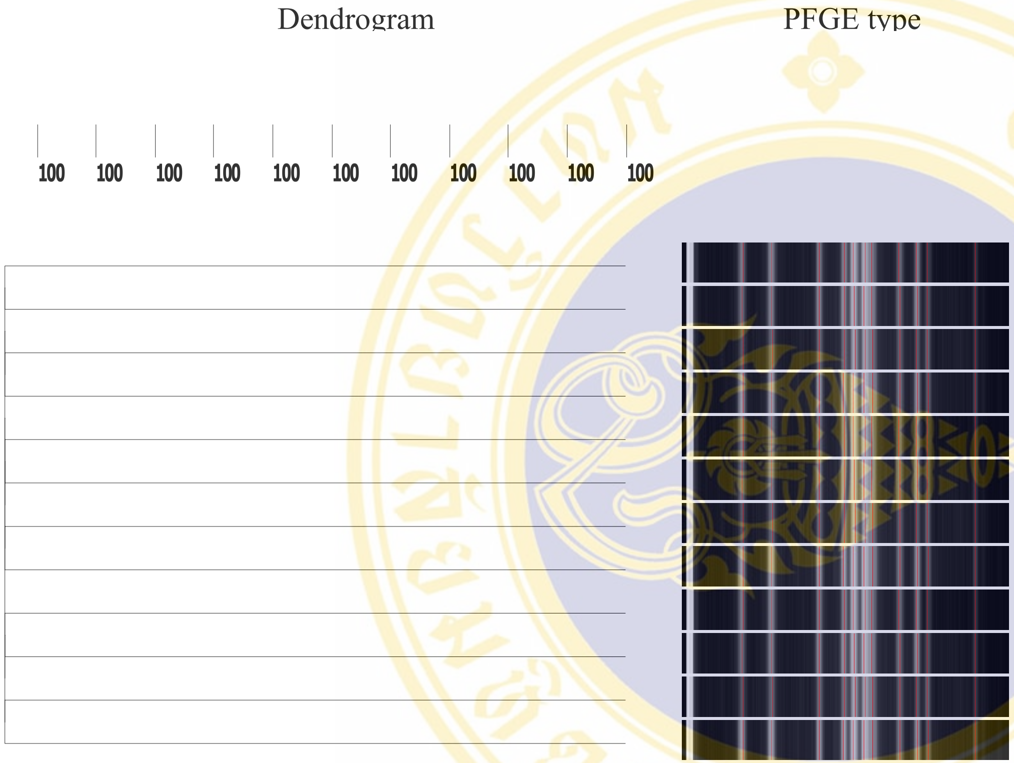


Fig. 13 PFGE types among *Salmonella* Derby (N=12) isolated from the chicken farms and the market

Table 22 The relationship of antimicrobial resistant patterns and PFGE types of *S.* Stanley isolated from farms and market

PFGE type	Sensitivity to all*	Resistant to	Place (source)		No. (%) of *S.* Stanley
		NA			
E1	+		Farm C	(P)	7 (63.6)
E2	+		Farm C	(LE)	1 (9.1)
E3	+		Market	(P)	2 (18.2)
E4		+	Market	(P)	1 (9.1)
11	10	1			

P = Egg package, LE = Laying egg

NA= Nalidixic acid

* sensitivity to all 6 tested antibiotics

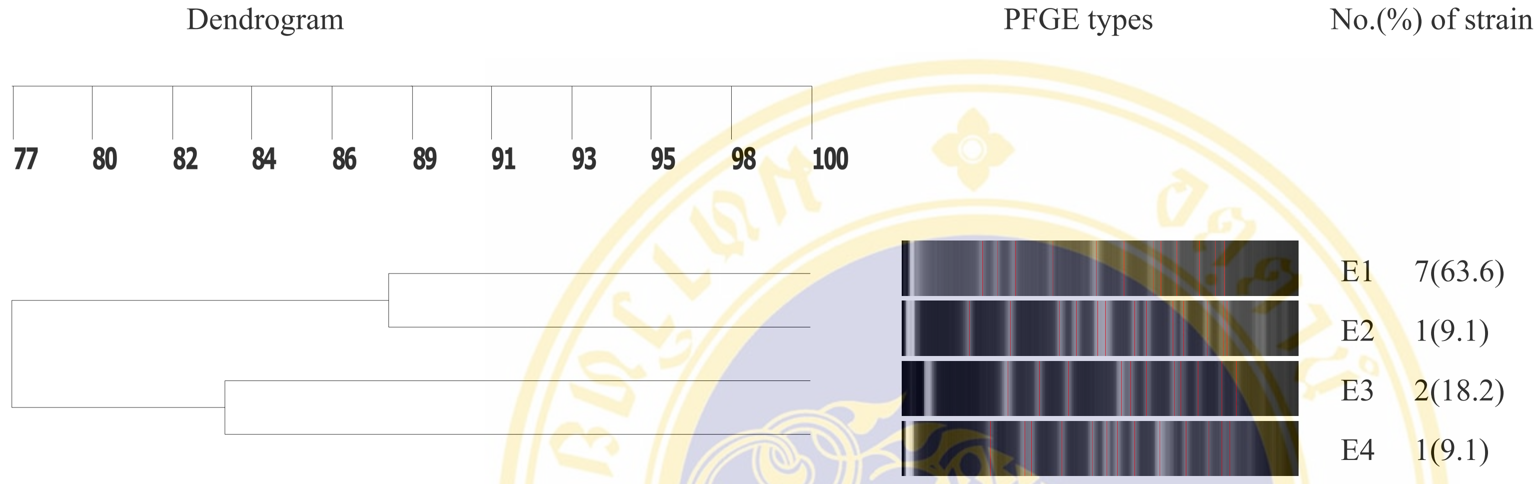


Fig. 14 PFGE types among *Salmonella* Stanley (N=11) isolated from the chicken farms and the market

CHAPTER 6
DISCUSSION

Foodborne diseases caused by nontyphoidal *Salmonella* represent an important public health problem worldwide. During the last decade *S.* Enteritidis and *S.* Typhimurium have emerged as the two predominant serovars in most western countries. However, others serovars may have a different epidemiology, which mark them from major importance in countries where conditions are optimal for spread between and persistence in certain reservoirs. In Denmark, the cost related to food borne cases of gastroenteritis was estimated to be $ 23-58 in 2001 (84) and the USA it has been estimated that nontyphoidal foodborne salmonellae are responsible for more than 1.3 million illnesses, almost 16,000 hospitalizations and more than 500 deaths every year (85). In Thailand, the estimated salmonellosis cases are 76-1,043 cases per 100,000 population per year (7).

Salmonellosis associated with contaminated chicken eggs were from being contaminated of *Salmonella* in chicken feces to the outside of egg shell (86). The prevalence of *Salmonella* in egg products, such as frozen unpasteurized eggs has ranged from 32% to 54% (87). However, the contamination of whole eggs either in retail market or in farms was relatively low. In Thailand, association of salmonellae foodborne outbeak and eggs has been reported and sporadic cases may escape from the detection.

The study of Saitanu *et al*, salmonellae were isolated from both egg, shells and egg contents(6). *Salmonella* contamination rate of eggs shells was 13.2% and 3.9% in egg content samples collected from the local markets. Detection rates were decreased in samples collected from the chicken farm. A total of 86 samples from the chicken farms, salmonellae were found on egg shells 3.5 % and egg contents 1.2%. The other studies that salmonellae contamination on egg shell and egg contents of duck eggs were collected from retail markets. A total 1,128 of eggs, salmonellae contamination on duck egg shells was 12.4%, egg content 11%, and the both 0.2% (6). Charentankul (88) reported that salmonellae contamination of eggs and their packages contamination was found 16.7% each obtained from the market in Bangkok. However, all eggs yolk samples were not contamination with *Salmonella*.

The contamination of eggs may occur through vertical (e.g. infection of the ovaries and oviduct) or horizontal routes (e.g. contamination from the environment e.g the dust, fecal material, etc.). *Salmonella* Gallinarum commonly infects the ovaries of laying hens and the organisms can be transmitted in the york of eggs (89). With the other salmonellae there is some controversy as to how often this occurs. Shivapansad *et al* (90) was able to detect *S.* Enteritidis from the eggs shell but not from egg contents delivered from experimentally infected hens. The vertical transmission of food poisoning bacteria has tended to be restricted to organisms such as *S.* Enteritidis. The horizontal routes contamination depends on phage type serovar of salmonellae. Egg pores in egg shell will not expand wider when they are freshy laid (91).

At present, the antimicrobial agents have been widely used in poultry industry. The overdose antimicrobial agents given to flocks to prevent the infections and to speed the growth of chickens increased the problems of antimicrobial resistance salmonellae (92). In USA, a government committee gave a warning of a number of strains of bacteria acquiring resistance to one or more drugs, and the ability of resistant strains to bacteria. *Salmonella* was becoming resistant to several drugs, and it was warned that people could suffer from drug resistant strains infection (93, 94). Most antibiotics are in fact used rather on animals than on human. Antibiotics of more than half of global production is used in animal farms including aquaculture. In 30 years, the use of penicillin-type drugs and tetracycline on animal farms has increased from 60 to 100% (15). For more than 40 years the ranchers and growers have been feeding low levels of penicillin, tetracycline, and other antibiotics to poultry, cattle, and pigs to speed the growth and cut cost. The USA scientists have described that using antibiotic to promote animal growth have increased the prevalence of antibiotic resistant bacteria and jeopardized human health. In this study, 47.3% (53/112) of *Salmonella* isolated from various sources in 3 chicken farms and a market were resistant to 4 of 6 tested antibiotics such as 79.2% nalidixic acid, 71.7% sulfamethoxazol trimethoprim, 54.7% tetracycline, and 5.7% ampicillin. It was found that no any salmonellae isolates (53 strains) exhibited resistance to cefotaxime and chloramphenicol as the same results reported by Kuha (15). The similar results of Bangtrakulnonth (48) reported that antibiotic resistance of salmonellae isolated from chicken meat were found resistant to tetracycline (43.3%), streptomycin (27.0%), sulfamethoxazol trimethoprim (16.3%), chloramphenicol (10.7%), ampicillin (9.0%), and cefotaxime (0.6%). Frank M. *et al* (5) reported that a large number of the isolates from human and chicken meat in Thailand were resistant to multiple antimicrobial agents. Especially the profiles with resistance to ampicillin, apramycin, gentamicin, nalidixic acid, streptomycin, sulphamethoxazole trimethoprim, and tetracycline were commonly observed. In this study, the prevalence of multi-drug resistant patterns of salmonellae (70%, 37/53) was significantly higher than those of single drug resistant pattern (30%, 16/53) ($p<0.05$). Farm C was a closed system farm and found higher contamination rate of multi-drug resistant salmonellae than those of the open farms A and B. The surrounding of the close system farm was a smaller cage in order to increase the capacity of chicken housing and with the close system atmosphere. These factors might be effected the spread of salmonellae in the chicken farms when compared to the open system. This could be noticed by observation when collected the samples; by many chicken died or were sick; some eggs contaminated with blood; and hands of workers were contaminated with blood and fluid. Chicken in the open system looked healthier than those in closed system. This might be resulted in using the over doses and more types of antibiotics used to treat chicken in closed system than those in open system farms (Table 17). The indiscriminate use of antimicrobials, in many setting but especially in food animal worldwide, therefore has international implication. It is thus essential that antimicrobials be appropriately used in food animals, including chicken farms, on a global basic to preserve the efficacy of existing drugs and to limit the risk of transfer of resistant foodborne pathogens to humans. Lastly, there is a need for increased surveillance and sharing of antimicrobial susceptibility data in bacteria of animal origin from both developed and developing countries, so that trends can be monitored and emerging resistance phenotypes identified.

Subtyping of microbiogical isolates has led to a great improvement in our ability to identify, investigate and control outbreaks of foodborne pathogens. A widely used method of molecular typing in foodborne pathogen is pulse-field gel electrophoresis (PFGE). PFGE allows the identification of clones or isolates with a similar ancestry, potentially emerging from the same source. The comparison of PFGE patterns from human and food or animal isolates allows to identify and confirm potential sources of disease. This leads to a better understanding of the means of contamination and spread and therefore to better control interventions.

In the present study, PFGE typing was found that 7 different among *S*. Braenderup, Type A2 (70.6%) was the most common and were isolated from package, shell egg, laying egg, and worker's hand in both farms and market, followed by type A3 (15.7%) from farm C, type A1 (3.9%) from farm A and farm C, type A5 (3.9%) from farm C and market. The other 3 isolates belonging to type A4, type A6, and type A7 were found in market, farm B and farm C, respectively. It was found that there are 4 common clones due to the similarity of PFGE types and antimicrobial resistant/sensitive patterns such as type A2 with nalidixic acid resistance clone, type A2 with sulfamethoxazol-ampicilin-nalidixic acid-tetracycline resistance clone, type A2 with antibiotic sensitive clone, and type A3 with antibiotic sensitive clone occurring in the chain of contamination in the environment within the farms or among 3 farms.

PFGE types of *S*. Emek, type B1 (45.8%) was the most common, followed by type B4 (29.2%), type B3 (20.8%), and B2 (4.2%) that almost contaminated from egg egg package in Farm C and market except only one isolated from worker hand. It was found that there were 5 common clones among drug resistant : type B1 (37.5%) with sulfamethoxazol- nalidixic acid –tetracycline resistant clone was the common clone that found in package and worker hand in Farm C, followed by type B4 (20.8%) with sulfamethoxazol – nalidixic resistant clone was found from package of market, type B3 (12.5%) with sulfamethoxazol- nalidixic acid resistant clone was found in package of market, type B3 (8.3%) with sulfamethoxazol- nalidixic acid - tetracycline resistant

resistant clone and type B4 with nalidixic acid resistant clone were found in Farm C and market, respectively.

All 14 *S.* Weltevreden were non resistant strains showed that type C1 (78.6%) was the most common and found from various sources and the common clone of antibiotic sensitive *S.* Weltevreden strains in Farm A only.

For PFGE type of *S.* Derby, it was belonged to only type D and the common clone with sulfamethoxazol trimethoprim-tetracycline resistant clone (83.3%) occurred in Farm A. In addition, *S.* Stanley PFGE type E1 was the dominant clone that only found from package in Farm C. While as type E2 and E4 with nalidixic acid resistant clone were detected from package in the market.

It was interesting that the common clones of 5 serovars commonly found in 3 farms and market were related to the multi-drug resistance of sulfamethoxazole-trimethoprim-nalidixicacid;sulfamethoxazole-trimethoprim-tetracyclne; sulfamethoxazole trimethoprim-nalidixic acid-tetracycline; and nalidixic acid resistant strains. In 2002, the occurrence of antibiotic resistance of salmonellae isolated from chicken meat were 43.3% to TE, 16.3% to SXT, and 9% to AMP in Thailand (49). The different percent and types of antibiotic resistant of salmonellae might be related to the different time, location, and limitation of antibiotics used in the farms. However, more number of salmonellae in serovars, farms, and studied samples should be included in the same proportion that might be resulted in knowing the source of salmonellae contamination from farm to market or from market to farm i.e. contaminated plastic package starting from farm to market or from market to farm or vice versa.

Salmonella contamination in various types of sample was found in farm C (close system) more than those in farm A and B (opened system). In Thailand, the ambient temperature is hot and moist which it is suitable for the growth of bacteria pathogens. A opened system provided naturally a good released ventilation while a closed system (Evaporative cooling system) is equipped with air conditioning cooling system (15). Recirculation of indoor-air or inadequate fresh air intake of farm C might affect the poor ventilation. In addition, hens were occupied in 3-storeys over each other in the small cubicles making more poor ventilation and overcrowded. Therefore, salmonellae might be easier contaminated in this type of setting. Nowadays, closed system farm has been used increasing for export and local needs, led to increase in number of closed farms rather than opened farms. Egg industry with evaporative cooling system cooling system should identify the risk of salmonellae contamination in the septum in order to improve the quality of eggs and prevent cross-contamination through order food items.

However, in this study, *Salmonella* contamination of plastic package in farms and market were found more than those other sources. The plastic package were the main source of salmonellae contamination in this study. The plastic package distributed in farms, and unhygienic plastic packages were the important sources of salmonellae contamination in egg from farm to market, or from market to farm. Therefore, using non-recycle package or disinfectant plastic packages before reuse could prevent salmonellae contamination of eggs. In addition, the use of antibiotic and typical setting of the closed system could facilitate the spread of drug-resistant salmonellae to eggs, consumer and the environments. The worker seemed to be an important mechanical mean of spreading salmonellae in this study. Hygienic working pratice is recommend in order to prevent transmission of the salmonellae. Finally, the information in this study might be useful to increase the awareness of salmonellae contamination in the eggs and poultry industry. The concern authorities of the government should also support an effort in improving the safety of eggs and egg products in order to prevent the outbreaks of *S.* Enteritidis and other important serovars like those occurred in developed countries, i.e. USA, Japan, etc.

CHAPTER 7
CONCLUSION

Salmonella is one of the most common infectious diseases in the world both in human and animal. Animal are the main reservoir, and the disease is usually food borne, although it can be spread from person to person.

At present, the antimicrobial agents have been widely used in poultry industry. The overdose antimicrobial agents given to flocks to prevent the infections and to speed the growth of chickens increased the problems of antimicrobial resistance salmonellae (92). In this study, 53 strains (47.3%) of salmonellae were resistant to 4 of 6 tested antibiotics. The prevalence of *S.*Braenderup Type A2 with a nalidixic acid resistance clone were the highest percent (94.1%) only found in farm C (packages and laying egg), S. Derby with a resistant clone to sulfamethoxazol trimethoprim and tetracycline were equal the highest percent 90.9% that found in farm A only (package and shell eggs), and S. Emek with resistant clone were resistant to sulfamethoxazol trimethoprim- nalidixic acid- tetracycline (54.2%) that found in farm C and the other resistant clone to sulfamethoxazol trimethoprim- nalidixic acid found in market. Thus, salmonellae contamination in the chicken farm

The used of antibiotics and typical setting of the closed system could facilitate the spread of drug-resistant salmonellae to eggs, consumer and the environmental. The worker seemed to be an important mechanical mean of spreading salmonellae in this study. Hygienic working practice is recommened in order to prevent transmission of the salmonellae.

The plastic packages were the main source of salmonellae in this study. Therefore using non-recycle package or disinfectant the plastic packages before reuse could prevent *Salmonella* contamination of eggs

Finally, this study might increase awareness of salmonellae contamination in the eggs and poultry industry and the concern authority of the government to put an effort in improving the safety of eggs and egg products.

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APPENDIX

APPENDIX

The Reagents Used for PFGE

Cell Suspention Buffer (CSB ; 100 mM Tris: 100 mM EDTA, pH 8.0)

10 ml of 1 M Tris, pH 8.0

20 ml 0.5 mM EDTA , pH 8.0

Dilute to 100 ml with sterile Type 1 water

Tris:EDTA, Buffer (TE ; 10 mM Tris: 1 mM EDTA, pH 8.0)

10 ml of 1 M Tris, pH 8.0

2 ml 0.5 mM EDTA, pH 8.0

Dilute to 1000 ml with sterile Type 1 water

1% SeaKEM gold : 1% Sodium Dodecyl Sulfate Agarose (1% SKG: 1 % SDS) inTE Buffer

a. Wight 0.50 (or 0.25) gram SKG into 250 ml screw – cap flask.

b. Add 47.0 (or 23.5) m TE Buffer; swirl gently to disperse agarose.

c. Remove cap, cover losey with clear film, and microwave for 30 – sec; mix gently and repeat for 10 sec interval until agarose is completely dissolved.

d. Place flask in 6 EC water bath for at least 5 min.

e. Add 2.5 (or 1.25 ml of 20 % SDS that has equilibrated to 56 ^{o}C and mix well.

f. Recap flask and return to 56 ^{o}C water bath until ready to use.

Cell Lysis Buffer (50 mM Tris: 50 mM EDTA, pH 8.0 + 1% Sarcosine)

25 ml of 1mM Tris, pH 8.0

50ml of 0.5 mM EDTA, pH 8.0

or

50 ml of 10 % Sarcosyl (N- Laury- Sacrosine, Sodium salt)

Dilute to 500 ml with sterile type 1 water.

Add 25 µl proteinase K stock solution (20 mg/ml) per of cell lysis buffer just before use. The final concentration of Proteinase K is 0.1 mf\ml in the buffer

1% Sea Kem Gold Agarose (1% SKG) in 0.5 X TBE Buffer)

a. Wight 1.0 gram SKG into 500 ml screw-cap bottle.

b. Add 100.0 ml 0.5 X TBE buffer; swirl to disperse agarose.

c. Remove cap, cover loosey with agarose clear film , and microwave for 60 sec; mix gently and repeat for 15 sec interval until agarose is completely dissolved.

d. Place bottle in 54-58 °C water bath for at least 15 minutes before pouring gel.

The relationship of antimicrobial resistant patterns and PFGE types of *S*. Braenderup isolated from the chicken farms and the market by code strain number

Strain no.	**PFGE type**	**Resistant pattern**				**Susceptible**	**Place**	
		NA	**SXT, NA,TE**	**SXT, AMP,TE**	**SXT,AMP, NA,TE**		**Site**	**Sample type**
A1	A1				+		Farm A	(P)
C58	A1				+		Farm C	(P)
C3	A2	+					Farm C	(P)
C19	A2	+					Farm C	(P)
C20	A2	+					Farm C	(P)
C22	A2	+					Farm C	(P)
C44	A2	+					Farm C	(P)
C46	A2	+					Farm C	(LE)
C50	A2	+					Farm C	(H)
V2	A2	+					Market	(P)
V5	A2	+					Market	(P)
C39	A2		+				Farm C	(LE)
C66	A2		+				Farm C	(P)
C72	A2		+				Farm C	(H)
A5	A2			+			Farm A	(P)
A7	A2					+	Farm A	(P)
A8	A2					+	Farm A	(P)
A10	A2					+	Farm A	(P)
A16	A2					+	Farm A	(P)
A20	A2					+	Farm A	(P)
A23	A2					+	Farm A	(P)
A26	A2					+	Farm A	(P)
A35	A2					+	Farm A	(SE)
B1	A2					+	Farm B	(P)
B6.	A2					+	Farm B	(P)
C24	A2					+	Farm C	(P)
C25	A2					+	Farm C	(P)
C31	A2					+	Farm C	(P)
C33	A2					+	Farm C	(LE)
C41	A2					+	Farm C	(LE)
C48	A2					+	Farm C	(LE)
C53	A2					+	Farm C	(P)
C54	A2					+	Farm C	(P)
C64	A2					+	Farm C	(P)
C71	A2					+	Farm C	(H)

The relationship of antimicrobial resistant patterns and PFGE types of *S.* Braenderup isolated from the chicken farms and the market by code strain number (continued)

Strain	**PFGE**	**Resistant pattern**					**Place**	
No.	**type**	**NA**	**SXT, NA, TE**	**SXT, AMP, TE**	**SXT, AMP, NA, TE**	**Susceptible**	**Site**	**Sample type**
C74	A2					+	Farm C	(H)
C75	A2					+	Farm C	(H)
V17	A2					+	Market	(P)
C37	A3					+	Farm C	(LE)
C40	A3					+	Farm C	(LE)
C43	A3					+	Farm C	(LE)
C45	A3					+	Farm C	(LE)
C47	A3					+	Farm C	(LE)
C1	A3					+	Farm C	(P)
C36	A3					+	Farm C	(P)
C51	A3					+	Farm C	(P)
B4	A4					+	Farm B	(P)
V10	A5					+	Farm C	(P)
C59	A5	+					Market	(P)
V4	A6	+					Market	(P)
C62	A7					+	Farm C	(P)

P = Package egg, SE = egg shell, LE = Laying egg, H = worker hand

CTX = Cefotaxime, SXT= Sulfamethoxazole-trimethoprim, NA= Nalidixic acid,

AMP = Ampicillin, C = Chloramphenicol, TE = Tetracycline

The relationship of antimicrobial resistant patterns and PFGE types of *S*. Emek strains isolated from farms and market by code strain number

Strain no.	**PFGE type**	**Resistant pattern**			**Place**	
		NA	**SXT, NA**	**SXT, NA, TE**	**Site**	**Sample type**
B5	B1	+			Farm B	(P)
C10				+	Farm C	(P)
C11				+	Farm C	(P)
C12				+	Farm C	(P)
C14				+	Farm C	(P)
C15				+	Farm C	(P)
C18				+	Farm C	(P)
C52				+	Farm C	(P)
C55				+	Farm C	(P)
C63				+	Farm C	(P)
C73				+	Farm C	(H)
C35	B2			+	Farm C	(P)
C61	B3			+	Farm C	(P)
C65				+	Farm C	(P)
V1			+		Market	(P)
V3			+		Market	(P)
V18			+		Market	(P)
V9	B4		+		Market	(P)
V12			+		Market	(P)
V19			+		Market	(P)
V21			+		Market	(P)
V15			+		Market	(P)
V11		+			Market	(P)
V14		+			Market	(P)
Total	**N= 24**	**3**	**8**	**13**		

CTX = Cefotaxime, SXT= Sulfamethoxazole-trimethoprim, NA= Nalidixic acid, AMP= Ampicillin,C = Chloramphenicol, TE = Tetracycline

P = Egg package, H = Worker hand

The relationship of antimicrobial resistant patterns and PFGE types of *S*. Derby isolated from the chicken farms and the market by code strain number

Strain No.	**PFGE Type**	**Resistant pattern**			**Place (source)**	
		NA	**SXT, TE**	**Susceptible**		
A14	D			+	Farm A	(P)
A34	D	+			Farm A	(SE)
A3	D		+		Farm A	(P)
A9	D		+		Farm A	(P)
A11	D		+		Farm A	(P)
A12	D		+		Farm A	(P)
A13	D		+		Farm A	(P)
A17	D		+		Farm A	(P)
A18	D		+		Farm A	(P)
A30	D		+		Farm A	(SE)
A32	D		+		Farm A	(SE)
A33	D		+		Farm A	(SE)
Total	**12**	**1**	**10**	**1**		

P = Egg package, SE = Egg shell

NA= Nalidixic acid, SXT= Sulfamethoxazole-trimethoprim, TE = Tetracycline

Relationship of antimicrobial resistant patterns and PFGE types of *S*. Stanley isolated from farms and market by code strain number

Strain no.	**PFGE type**	**Sensitivity to all***	**Resistant to NA**	**Place (source)**
C2	E1	+		Farm C (P)
C4	E1	+		Farm C (P)
C6	E1	+		Farm C (P)
C7	E1	+		Farm C (P)
C8	E1	+		Farm C (P)
C9	E1	+		Farm C (P)
C17	E1	+		Farm C (P)
C42	E2	+		Farm C (LE)
V6	E3	+		Market (P)
V16	E3	+		Market (P)
V7	E4		+	Market (P)
Total	11	10	1	

P = Egg package, LE = Laying egg

NA= Nalidixic acid

* sensitivity to all 6 tested antibiotics

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