คุณลักษณะทางอณูชีววิทยาของ class 1 integrons ในเชื้อซาลโมเนลลา เอนเทอริกา ที่แยกได้จากไก่และสุกร

นางสาวศิรินทิพย์ เข็มทอง

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MOLECULAR CHARACTERISTICS OF CLASS 1 INTEGRONS IN *SALMONELA ENTERICA* ISOLATED FROM POULTRY AND SWINE

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A Thesis Submitted in Partial Fulfillment of the Requirements
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ศิรินทิพย์ เข็มทอง : คุณลักษณะทางอณูชีววิทยาของ class 1 integrons ในเชื้อซาลโมเนลลา เอนเทอริกา ที่แยกได้จากไก่และสุกร (MOLECULAR CHARACTERISTICS OF CLASS 1 INTEGRONS IN *SALMONELLA ENTERICA* ISOLATED FROM POULTRY AND SWINE) อ.ที่ปรึกษา: ผศ.สพ.ญ.ดร. รุ่งทิพย์ ขวนชื่น, อ. ที่ปรึกษาร่วม : รศ.น.สพ.ดร. อลงกร อมรศิลป์, 66 หน้า.

ศึกษาลักษณะทางอณูชีววิทยาของ class 1 integrons ในเชื้อซาลโมเนลลา เอนเทอริกา 31 ซีโรวาร์ จำนวน 150 ตัวอย่างซึ่งแยกได้จากไก่และสุกร ทดสอบความไวต่อยาปฏิชีวนะและจัดรูปแบบการดื้อยา ตรวจหาการปรากฏของ class 1 integrons ในเชื้อทุกตัว ศึกษาลักษณะและจำแนกชนิดของยีนดื้อยาที่พบใน class 1 integrons ศึกษาตำแหน่งของ class 1 integrons ตรวจหา *Salmonella* Genomic Island1 (SGI1) และทดสอบความสามารถในการถ่ายทอดของเชื้อที่มี class 1 integrons ผลการวิจัยพบว่ามีเชื้อซาลโมเนลลา เอนเทอริกาดื้อต่อยาปฏิชีวนะอย่างน้อยหนึ่งชนิด 89 %และมีเชื้อที่ดื้อต่อยาปฏิชีวนะหลายชนิดพร้อมกัน 59.3% รูปแบบการดื้อยาที่พบมากที่สุดคือ SPC-STR-SUL (6.21%) และ AMP-CHP-SPC-STR-SUL-TET-TRI (6.21%) พบเชื้อที่มีการปรากฏของยีน *intI1* 25.6% ซึ่งในเชื้อจำนวนนี้มี gene cassettes 55.5% พบการปรากฏของ gene cassettes ขนาด 650 ถึง 2,300 bp จากผลการถอดรหัสพันธุกรรมสามารถจำแนก integrons profiles (IPs) ได้เป็น 10 รูปแบบ ยีนที่พบใน gene cassettes ได้แก่ bla_{PSE-1}, *dfrA12*, *dfrA1*, *aadA2*, *aadA4*, *silB* บางส่วนของยีน *sat* และ *codB* ยีนดื้อยาที่พบได้มากที่สุดคือ *dfrA1-orfC* โดยพบ class 1 integrons บนพลาสมิดซึ่งสามารถถ่ายทอดได้ในเชื้อ 7 ตัวอย่าง และพบ SGI1 variants (SGI1-A และ SGI1-F) ในเชื้อ 9 ตัวอย่างได้แก่ ซีโรวาร์ Albany, Emek, Kedougou และ Kingston

ภาควิชา สัตวแพทยสาธารณสุข ลายมือชื่อนิสิต.....ศิรินทิพย์.....เข็มทอง
สาขาวิชา สัตวแพทยสาธารณสุข ลายมือชื่ออาจารย์ที่ปรึกษา.....
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KEY WORD: *Salmonella enterica*/ multidrug resistance/ class 1 integrons

SIRINTIP KHEMTONG : MOLECULAR CHARACTERISTICS OF CLASS 1 INTEGRONS IN *SALMONELLA ENTERICA* ISOLATED FROM POULTRY AND SWINE. THESIS ADVISOR: ASST. PROF. RUNGTIP CHUANCHUEN, D.V.M., M.Sc., Ph.D., THESIS COADVISOR: ASSOC. PROF. ALONGKORN AMONSIN, D.V.M., Ph.D., 66 pp.

A total of a hundred and fifty *Salmonella enterica* isolated from poultry and swine representing 31 serovars were used in this study. Antibiotic susceptibility was examined and resistance pattern was analyzed. All the isolates were investigated for occurrence and characteristics of class 1 integrons. Localization of class 1 integrons and their transferability were assessed by conjugal experiment. The presence of *Salmonella* genomic islands were assessed. The *S. enterica* strains that were resistant to at least one of antibiotic were 86% and 59.3% was multidrug resistant. Two common resistance patterns found were the SPC-STR-SUL (6.21%) and the AMP-CHP-SPC-STR-SUL-TET-TRI (6.21%) phenotype. The *intI1* gene was present in 25.6%, of which 55.5% carried gene cassettes with size ranging from 650 to 2,300 bp. Sequence analysis revealed 10 distinct Integrons profiles (IPs), in which genes bla_{PSE-1}, *dfrA1*, *dfrA12*, *aadA2*, *aadA4*, *silB*, incompleted *sat* and incompleted *codB* were present in variable regions. The gene cassettes array *dfrA1-orfC* was most frequenly found among the isolates. Class 1 integrons were identified on conjugative plasmid in 7 *Salmonella* isolates. SGI1 variants (SGI1-A and SGI1-F) were present in 9 isolates belonging to serovars Albany, Emek, Kedougou and Kingston.

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Field of study Veterinary Public Health Advisor's signature.....Rungtip Chuanchuen

Academic year 2007 Co-advisor's signature.....Alongkorn Amonsin

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LIST OF ABBREVIATIONS

bp	base pair
°C	degree celsius
DNA	deoxynucleic acid
DW	distilled water
e.g.	exempli gratia, for example
EDTA	ethylenediamine tetraacetic acid
et al.	Et alii, and others
g	gram (s)
i.e.	id est, such as
M	molar
mg	milligram (s)
$MgCl_2$	magnesium chloride
NSS	normal saline solution
µg	microgram (s)
µl	microliter
µM	micromolar
ml	milliliter
mM	millimolar
PCR	Polymerase Chain Reaction
pH	the negative logarithm of hydrogen ion concentration
rpm	round per minutes
TAE	Tris-Acetate-EDTA
TE	Tris-EDTA
U	unit

CHAPTER I

INTRODUCTION

Salmonella enterica is one of the most common bacterial causes of foodborne diseases in humans that have an impact on the global economy and public health (Wray, 1995). The World Health Organization (WHO) estimated that about 17 million cases per year of acute gastroenteritis or diarrhea were due to Salmonellosis with 3 million cases of death (Rabsch et al., 2001). In the United States, Center for Disease Control and Prevention (CDC) estimated that there were approximately 1.4 million cases of Salmonellosis per year (Mead et al., 1999). In 2003, it was reported that *Salmonella* was classified as the second foodborne pathogen due to diarrhea in Thailand (MOPH, 2003).

Salmonella has been isolated from animals, animal products, and environment and could be transmitted to humans through food chain (Tollefson et al., 1997). As poultry and swine are common food-producing animals in many countries (Angulo et al., 2004), including Thailand, they are also common reservoirs of *S. enterica*. To date, incidence of antibiotic-resistance *Salmonella* has been increasing worldwide (Soto et al., 2003). A previous study showed that twenty three percent *S. enterica* isolates from retail meats in the United states and China were resistant to multiple drugs (Chen et al., 2004). It is now evidence that extensive use of antibiotics in different fields, including human and veterinary medicine and livestock production for disease prevention and growth promotor, has created enormous pressure for selection of antimicrobial resistance in *Salmonella*. *Salmonella* could be simultaneously resistant to combinations of several classes of antibiotics or multiple drug resistance (MDR) and can transfer resistance determinants to other bacteria intra- and interspecies (Low et al., 1997)

In Scotland, *S.* Typhimurium isolated from animals were resistant to multiple drugs (98%) (Low et al., 1997). *S.* Enteritidis and *S.* Typhimurium isolated in Korea were resistant to multiple drugs about 26.6% and 100%, respectively. In Thailand, MDR rate in *S.* Enteritidis and *S.* Anatum isolated from poultry in 1994 were significantly higher than that in 1993 ($p<0.01$) (Boonmar et al., 1998a) and *S. enterica* isolated from patients were found resistant to 6-8 antibiotics (Boonmar et al., 1998b). Recently, Padungtod and Kaneene (2006) reported *S. enterica* isolates from chicken, pigs and diarrhea children were resistant to multiple drugs at the rate of 32%, 35% and 85% respectively. Since antibiotic resistance among *S. enterica* isolates has been increasing in both humans and animals, it supports that use of antibiotics in food producing animals has an effect on increasing antibiotic resistance in humans.

As a result, the international organizations have implemented projects to discontinue the abuse use of antibiotics e.g. WHO has recommended discontinuing use of antimicrobial growth promoters that belong to an antimicrobial class used in humans (WHO, 1999). The European Union (EU) has banned all antibiotics that are related to those used in humans from use in animals for growth promoter (Angulo et al., 2004). Codex Alimentarius Commission has issued Code of Practice to Minimize and Contain Antimicrobial Resistance, of which the main idea of this code is to create the projects to reduce antibiotic resistance in bacteria with the scientific supports and promote studies of mechanisms of resistance and molecular epidemiology of antibiotic resistance in bacteria (Codex, 2005). Currently, reports regarding antibiotic resistance mechanisms of food borne pathogens including *Salmonella* have been available from several countries including those in Asia, Korea (Kim et al., 1997), Taiwan (Hsu et al., 2006) and China (Zhang et al., 2004). However, little is known about molecular mechanisms underlying antibiotic resistance in *Salmonella* isolates in Thailand.

As there are several mechanisms of antibiotic resistance have been characterized in *Salmonella*, one efficient mechanism for the acquisition and dissemination of resistance determinants is transmission through mobile genetic elements especially integrons. Nine

classes of integrons have been described so far (Lee et al., 2002). Class 1 integrons are the most extensively studied integrons and predominant in MDR *Salmonella*. Class 1 integrons contain one or more antibiotic resistance genes that can be mobilized to other bacteria. Many antibiotic resistance genes can be found in class 1 integrons and more than 100 genes that confer antibiotic resistance have been identified (Fluit and Schmitz, 1999). Class 1 integrons has been shown to be associated with conjugative plasmids therefore, play an important role in distribution of antibiotic resistance genes among bacteria. In addition, class 1 integrons can be integrated on chromosome, particularly *Salmonella* Genomic Island (SGI1), an integrative-mobilisable chromosomal element. Therefore, these antibiotic resistance genes are stably maintained on chromosome, even though antibiotic selective pressure is not present.

Currently, the information of mechanisms of antibiotic resistance transmission through the food chain and molecular epidemiology is increasingly acquired. However, this information in Thailand is still limited. The data from the other countries can not be always used or applied to situations in Thailand because of the differences of antibiotic use. Therefore, this study aimed to determine characteristic of class 1 integrons and test for their transferability in *S. enterica* isolated from poultry and swine in Thailand.

Results from this study can be used as a part of antibiotic resistance monitoring in Thailand. It can be also used to demonstrate the link of antibiotic resistance *S. enterica* between food producing animals and humans when combines with data from humans and food of animal origin. In addition, these genetic data could be applied in risk analysis of antibiotics resistance. Technique used in this study can be used in further studies of molecular characteristics of class 1 integrons in *S. enterica* from other sources and other bacterial species.

CHAPTER II

REVIEW LITERATURES

1. General characteristics and pathogenesis of *S. enterica*

Salmonella are small 0.7-1.5 µm wide and 2-5 µm long, Gram-negative, rod shape bacteria (Figure 1). Most of *Salmonella* are usually motile by flagella, except *S.* Gallinarum and *S.* Pullorum that are non motile (Murrey et al., 2002). These organisms can metabolize when oxygen is present but they are able to shift to anaerobic metabolism, that so called facultative anaerobe bacteria (Holt et al., 2000). *Salmonella* are mesophile and prefer 37°C as the optimum growth temperature. The optimum pH for growth is between 6.5 and 7.5 (Holt et al., 2000). *Salmonella* are able to catabolize glucose with production of acid and gas that can be used for biochemical identification. They are oxidase negative, catalase positive, indole negative, methyl red and simmon citrate positive (Holt et al., 2000).

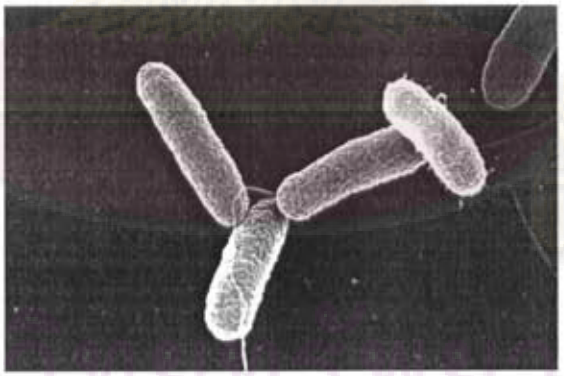

Figure 1: Electron-microscope image of *Salmonella enterica*

(Source: Brinkman and Planck, 2005)

The genus of *Salmonella* contained two species including *S. enterica* and *S. bongori*. The most common causes of food borne diseases are those in *S. enterica*, of which more than 2,500 serovars are identified (Chan et al., 2003). *Salmonella* are carried in the intestinal track of humans and animals, particularly in poultry and swine. Poultry and meat products are the most important food vehicles of *Salmonella* infection in humans (D'Aoust, 1989).

Salmonellosis is an illness caused by *Salmonella*. Most patients infected with *Salmonella* develop diarrhea, nausea, vomiting, fever, and abdominal cramps 12 to 72 hours after infection. The illness usually lasts 4 to 7 days and the majority of patients could recover without any antibiotic treatment. However, in some cases, the diarrhea may be so severe that the patient needs to be hospitalized (Baddour et al., 2007).

2. Occurrence and epidemiology of antibiotic resistance in *S. enterica*

Salmonellosis is a one of the most common human foodborne disease. According to the WHO, there are about 17 million cases of acute gastroenteritis or diarrhea due to Salmonellosis with 3 million people dead (Rabsch et al., 2001). *Salmonella* cause 68% of the outbreaks of foodborne diseases reported in Europe between 1993 and 1998 (van Pelt et al., 2003). CDC estimated the annual number of Salmonellosis cases in the USA to be approximately 1.4 million person per year (Mead et al., 1999). In Thailand, *Salmonella* was the second foodborne pathogen causing diarrhea (MOPH, 2003). The most common *Salmonella* serovars causing human Salmonellosis in Thailand between 1993 to 2002 was *S.* Weltevreden (Bangtrakulnonth et al., 2004). The most prevalent serovars in pork, chicken meat and patients of *Salmonella* isolate from Khonkaen were Rissen (61.5%), Anatum (33.3%), and Rissen (20.4%), respectively (Angkititrakul et al., 2005). The National *Salmonella* and *Shigella* Center reported that the most prevalent serovar in humans was Stanley (12.43%), followed by Enteritidis (10.98%). Among the isolates from animals Rissen

(25.34%) and Anatum (12.38%) were the most frequently identified serovars in 2005 (The National *Salmonella* and *Shigella* Center, 2005).

Use of antimicrobial agents in any fields could create selection pressures that favor the survival of antibiotic-resistant pathogens. The routine practice of giving antibiotic to food producing animals as a mean of preventing and treating diseases, as well as promoting growth, is an important factor in the emergence of antibiotic-resistant bacteria that are subsequently transferred to humans through the food chain (Tollefson et al., 1997).

Emergence of antibiotic resistance in *Salmonella* has been increasing worldwide. Most of them are multiple resistant to drugs in several classes resulting in inefficient antibiotic treatment. In Spain, *Salmonella* isolated from slaughtered pigs were MDR up to 64% (Astorga et al., 2007). Twenty three percent *Salmonella* isolated from retail meat in China and USA were found to be MDR and most of the isolates were resistant to tetracycline (55%). Twenty percent *Salmonella* isolated from dairy cattle in USA were MDR (Edrington et al., 2004). In Scotland, up to 98 % of *S.* Typhimurium isolates from animals were resistant to multiple drugs. In Korea, thirty seven percent *S.* Enteritidis and *S.* Typhimurium were isolated from animals from 1983 to 1999. *S.* Enteritidis isolates were highly resistant to sulfonamides (86.7%) and 4 (26.6%) isolates were multi-drug resistant whereas *S.* Typhimurium were extremely high (100%) multidrug resistant (Yang et al., 2001). In Thailand, resistance rates to ceftriaxone, amikacin, and kanamycin in *S.* Enteritidis and *S.* Anatum isolated from poultry in 1994 were significantly higher than those in 1993 ($p<0.01$) (Boonmar et al., 1998a). Sixty percent *Salmonella* isolates from chicken, pork and beef were multidrug resistant (Bangtrakulnonth et al., 2006). Padungtod and Kaneene (2006) reported *S. enterica* isolates from chicken, pigs, diarrhea children, farm workers and healthy adults were resistant to multiple drugs about 32%, 35%, 85%, 38% and 42%. Most of them were resistant to tetracycline and *S. enterica* isolated from farms were more resistant than those from other sources.

Antibiotic resistance in *Salmonella* from humans has been increasing as well. In the United States, incidence of MDR *S.* Typhimurium increased from 0.6% in 1979-1980 to 34% in 1996. In Thailand (Glynn et al., 1998) high level of multi-resistant *Salmonella* has been reported in Thai children with diarrhea (Moolasart et al., 1997). *S. enterica* isolates from patients were resistant to 6-8 antibiotics simultaneously (Boonmar et al., 1998b). All *Salmonella* isolates from patients of typhoid fever in Tak province were resistant to chloramphenicol, ampicillin, co-trimoxazole, tetracycline, and streptomycin but sensitive to kanamycin, ofloxacin, ciprofloxacin, and cefotaxime (Swaddiwudhipong and Kanlayanaphotporn, 2001). The isolates from children less than 5 years of age from both urban and rural environments but also included small numbers of adult USA soldiers on military maneuvers in Thailand were resistant to various drug i.e. ampicillin (28%), chloramphenicol (26%), trimethroprim-sulfamethoxazole (59%) and nalidixic acid (37%) (Isenbarger et al., 2002). Recently, all of non-typhoidal *Salmonella* isolates from patients in Chulalongkorn hospital were resistance to nalidixic acid (*n*=27) (Kulwichit et al., 2007).

3. Class 1 integrons in *S. enterica*

Integrons are mobile genetic elements that play an important role in dissemination of multi-drug resistance in Gram-negative bacteria including *Salmonella*. Nine of integrons have been described, based on the nature of integrase genes (Lee et al., 2002). The class 1 integrons have been most extensively examined. They are most commonly found in clinical isolates of Gram-negative bacteria and are strongly associated with multiple antibiotic resistances (Martinez-Freijo et al., 1998).

Organization of typical class 1 integrons is shown in Figure 2. The typical structure of class 1 integrons comprises two conserved segments, 5' conserved segment (5'-CS) and 3' conserved segment (3'-CS) and variable region located between these two conserved regions. The 5'-CS contains the *intI1* gene encoding an integrase enzyme responsible for the recombination of a gene cassette at specific *att1* site (Collis and Hall, 1992). The

recombination site (*attI1*) has been described as the last 40-70 nucleotide of the 5' conserved segment (Recchia et al., 1994). This segment also contains a promoter region that facilitates the efficient expression of integrated gene cassettes. The 3'-CS contained of variable length but typically consisting of *qacEΔ1*, truncate version of *qacE* gene encoding resistance to quaternary ammonium compound (QAC) (Gaze et al., 2005), the *sul1* gene encoding resistance to sulfonamides and open reading frame 5 (*orf5*) of unknown function (Paulsen et al., 1993).

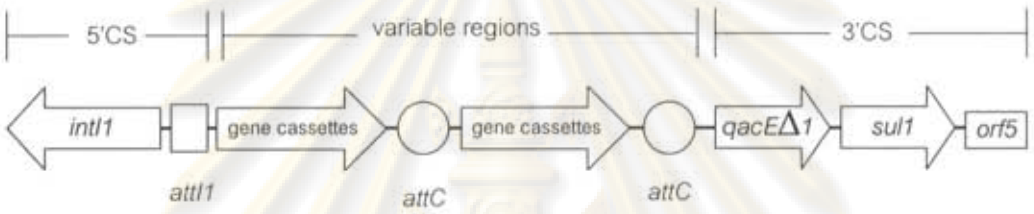

Figure 2: Typical structure of class 1 integrons. 5' CS, 5' conserved segment; 3' CS, 3' conserved segment; *intI1*, integrase gene; *attI1*, gene cassettes integration site; *attC*, gene cassette insertion sequence (59 base element); *qacEΔ1*, quaternary ammonium compound resistance; *sul1*, gene encoding resistance to sulfonamides; *orf5*, open reading frame of unknown function.

The variable regions include different combinations of inserted gene cassettes. These gene cassettes have a 59-base element called *attC* that is an integration site for other gene cassettes (Hall et al., 1991). Several gene cassettes can be present in class 1 integrons. To date about 100 different cassettes associated with resistance genes have been characterized, which allow bacterial hosts to become resistant to broad spectra of antimicrobial agents (Fluit and Schmitz, 2004). The most common resistant gene cassettes include *bla* encoding ß-lactamase that confer resistance to ß-lactams, *aad* encoding aminoglycoside adenyltransferases that confer resistance to aminoglycosides and *dfr* encoding dihydrofolate reductases that confer resistance to trimethoprim (Kim et al., 1997).

Class 1 integrons can be found on conjugative plasmids that transfer resistance gene cassettes more effectively. These plasmids can integrate into the bacterial chromosome, of which the hotspot of integration is SGI1, an integrative-mobilisable chromosomal element. SGI1 contains various resistance gene clusters. These antibiotic resistance genes are stably maintained on chromosome and contribute to dissemination of antibiotic resistance genes to other bacteria even though antibiotic selective pressure is not present.

4. *Salmonella* Genomic Island 1 (SGI1)

SGI1 is a 43-kilobase (kb) genomic island that has 44 open reading frames (*orfs*). Some of these are homologous to known genes. However, some are homologous to those with unknown functions. SGI1 is located on chromosome between the *thdf* and *int2* genes. The *thdf* gene encodes for a thiophene and furan-oxidation protein. The *int2* gene is a part of retron sequence, which has been reported to date in only *S.* Typhimurium. In all other SGI1-carrying *S. enterica* serovars, SGI1 is located between *thdf* and *yidY* genes that is the gene downstream of the retron sequence (Boyd et al., 2001). The antibiotic resistance genes have been localized to the 13-kb segment of the SGI1 termed the MDR region (Boyd et al., 2001). The MDR region of SGI1 consists of two class 1 integrons. The first class 1 integrons carries the *aadA2* gene encoding streptomycin and spectinomycin resistance. The second one carries bla_{PSE-1} encoding ampicillin resistance. *floR* encoding chloramphenicol resistance, *tetR* and *tetG* encoding tetracycline resistance. Two *orfs* are present between the two integrons. The MDR region of SGI1 seems to be an important hotspot collection of resistance genes. Not only gene cassettes in class 1 integrons can exchange, but also other antibiotic resistance genes that can integrate and be deleted and generate the SGI1 variants (Boyd et al., 2002). To date, up to 15 SGI1 variants (SGI1-A to SGI1-O) have been identified and characterized (Figure 3). In previous studies, SG1-A to SGI1-N were found only in *Salmonella*. Recently, new SGI1 variants (SGI1-O) and SGI1-L have been found in *Proteus mirabilis* (Boyd et al., 2008). The extrachromosomal circular

form of SGI1 has been additionally found (Doublet et al., 2005). SGI1 can excise from the chromosome and form circular structure by recombination between 3'end of *thdF* (*attB*) and site specific sequence (*attP*). This circular form could be horizontally transfered from *S. enterica* to non-SGI1 *S. enterica* and *E. coli* (Doublet et al., 2005).

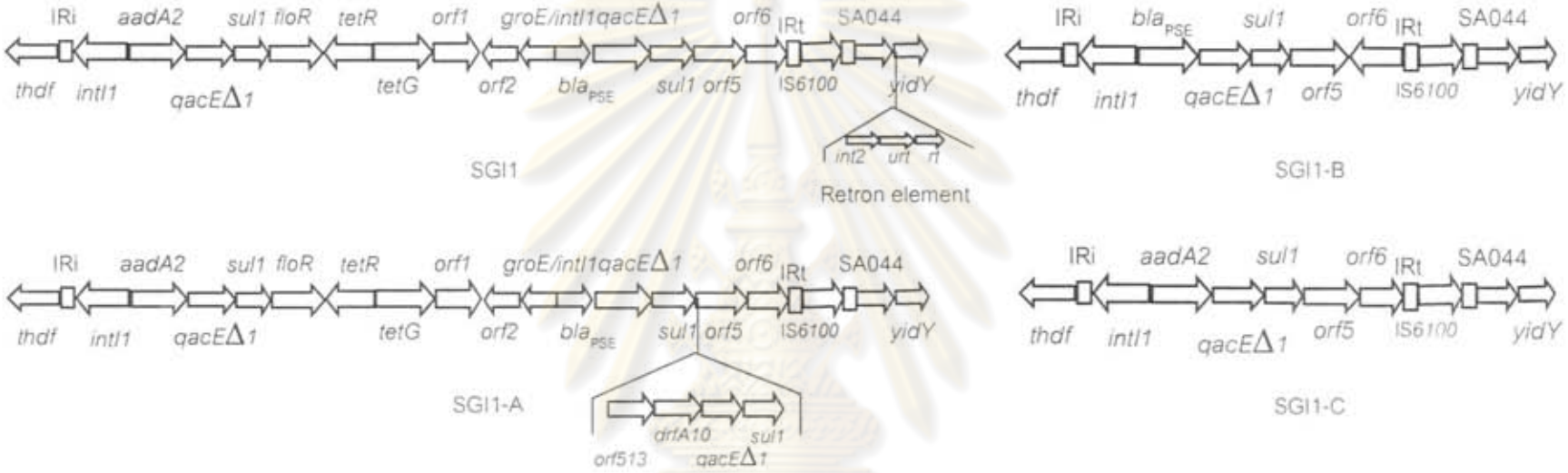

Figure 3: Structure of *Salmonella* genomic island 1 and SGI1 variants. *thdf*, thiophene oxidation gene; *intI1*, integrase gene; IRi and IRt , 25 bp imperflect insertion region; bla_{PSE-1}, gene encoding resistance to beta-lactams; *qacEΔ1*, gene encoding quaternary ammonium compound resistance; *sul1*, gene encoding resistance to sulfonamide; *floR*, gene encoding resistance to chloramphenicol; *tetR* and *tetG*, gene encoding resistance to tetracycline ; *orf1-6*, open reading frame of unknown function; *IS6100*, insertion sequence; *SO44*, open reading frame of unknown function; *yidY*, translocation gene. Retron element consist of *int2* (integrase gene), *urt* (retron phage) and *rt* (reverse transcriptase).

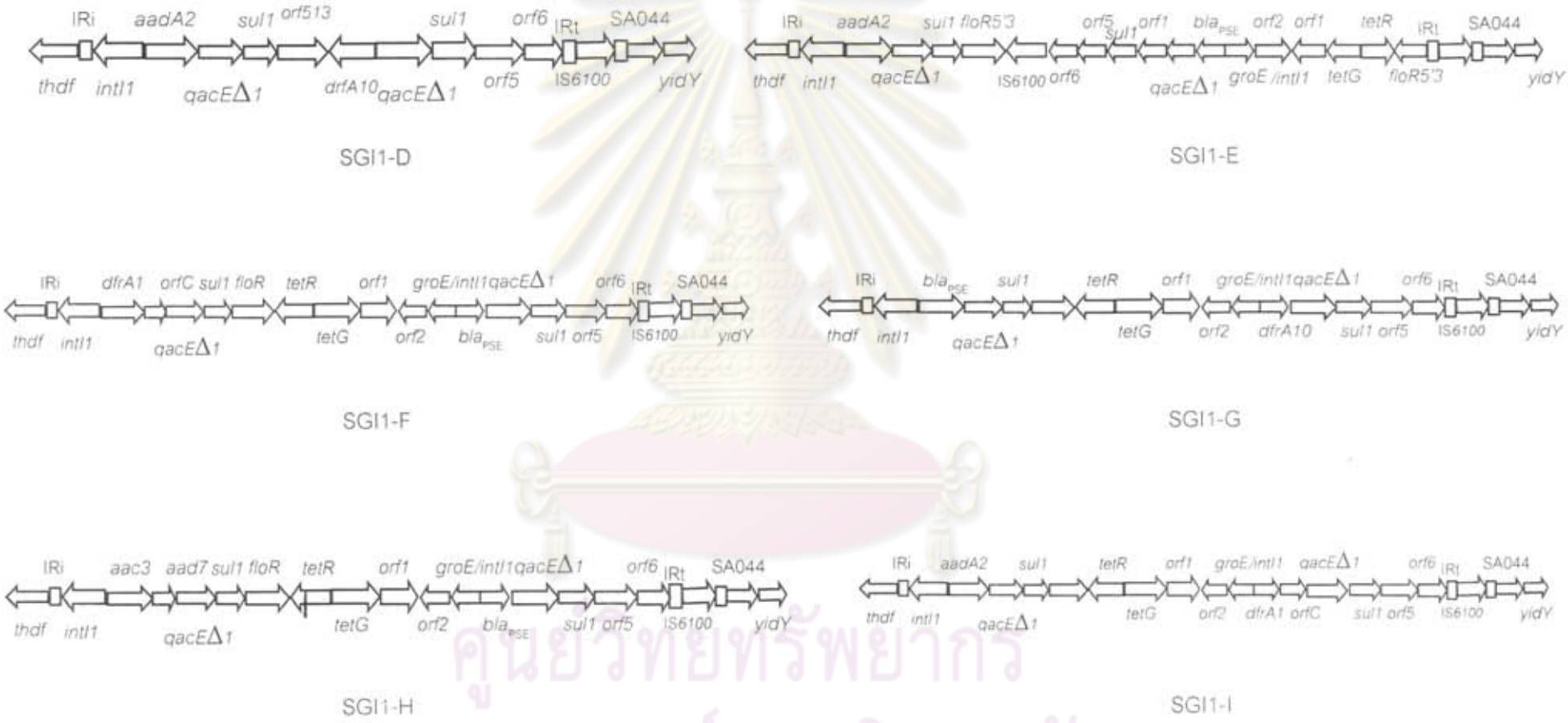

Figure 3: Structure of *Salmonella* Genomic Island 1and its variants (cont.)

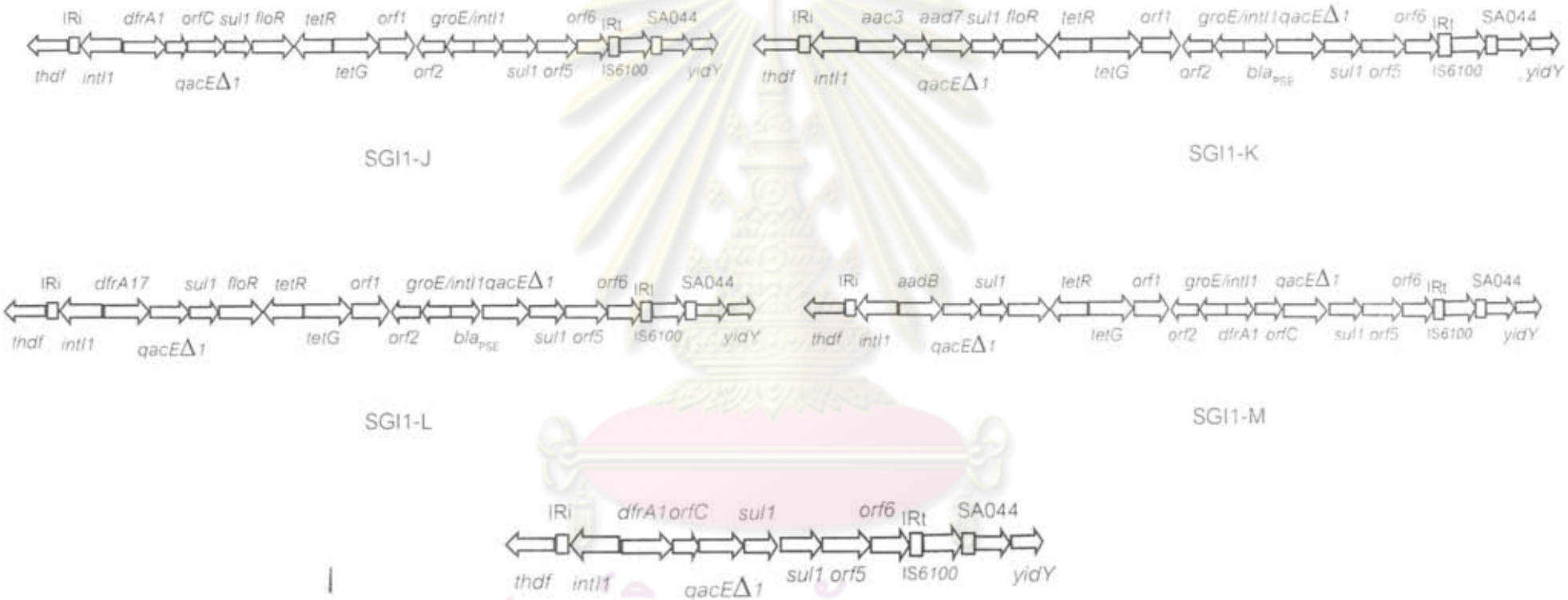

Figure 3: Structure of *Salmonella* Genomic Island 1and its variants (cont.)

4. Occurrence and epidemiology of class 1 integrons and SGI1

Class 1 integrons were first described by Stokes and Hall in 1989 (Stroke and Hall, 1989) and have been reported in clinical isolates of various Gram-negative bacteria including *Salmonella*. The integrons were first identified in MDR *S.* Typhimurium isolates (Tosini et al., 1998) and then were widely found in other *Salmonella* serovars (Guerra et al., 2000).

Salmonella isolates (34%) from farm animals in England and Wales were found to contain class 1 integrons (Liebana et al., 2002). Later, Linstedt et al (2003) dertermined class 1 integrons in *S.* Typhimurium (97 %) and *S.* Enteritidis (22.2 %) isolates from Norwegian patients. Several serovars of *Salmonella* from patient, animals and environment in UK contained class 1 integrons about 26.7% (Randall et al., 2004). In China, class 1 integrons were also found up to 17.39% in *Salmonella* from healthy humans (Zhang et al., 2004). Nogrady et al (2005) found class 1 integrons (33%) in *Salmonella* isolates in Hungary that had 10 different gene cassettes.

Different antibiotic resistance genes were identified in class 1 integrons in *Salmonella* from different countries due to different antibiotic use in geographically distinct region. The most common were *dfr* encoding trimethoprim resistance, *bla* encoding ß-lactams resistance and *aad* encoding aminoglycoside resistance. Reports from many countries including Europe i.e. Netherlands (Vo et al., 2006), Spain (Guerra et al., 2000), Portugal (Antunes et al., 2004), and Norway (Lindstedt et al., 2003) showed that *aadA1*, *aadA2*, *dfrA1*, bla_{OXA} and bla_{PSE-1} gene are most frequent presented. In US, the *aadA1* and bla_{PSE-1} genes were most commonly found (Chen et al., 2004). In Asia, there were many resistance genes reported including *dfr17* from Nepal (Tamang et al., 2007), *dfrA12* and *aadA1* from Vietnam (Ploy et al., 2003), bla_{OXA}(Lee et al., 2003), bla_{TEM} (Lee et al., 2004) and *dfrA12-aadA2* (Kim et al., 2007) from Korea, and *dfrA12-aadA2* from Taiwan (Hsu et al., 2006).

Class 1 integrons integrated into SGI1. SGI1 and SGI1 variants (SGI1-A to SGI1-N) have been described for other *Salmonella enterica* serovars (Boyd et al., 2002). As a study in France indicated that 83% of *S.* Paratyphi dt+ strains isolated between 2000 and 2003 contained SGI1 or its variants (Weill et al., 2005). Vo et al (2007) found SGI1 and SGI1 variants in 19.67 % *S.* Typhimurium from horses. A study in France reported that *S.* Albany isolated from food fish imported from Thailand harbored SGI1 variants (Doublet et al., 2003). This phenomenon suggests the worldwide distribution of SGI1 and SGI1 variants among *Salmonella*.

MATERIALS AND METHODS

In this study, the experiment was divided into 3 phases, including Phase I, antimicrobial susceptibility testing; Phase II, molecular characterization of class 1 integrons and Phase III, localization of class 1 integrons and test for their transferability.

The conceptual framework is shown in Figure 4.

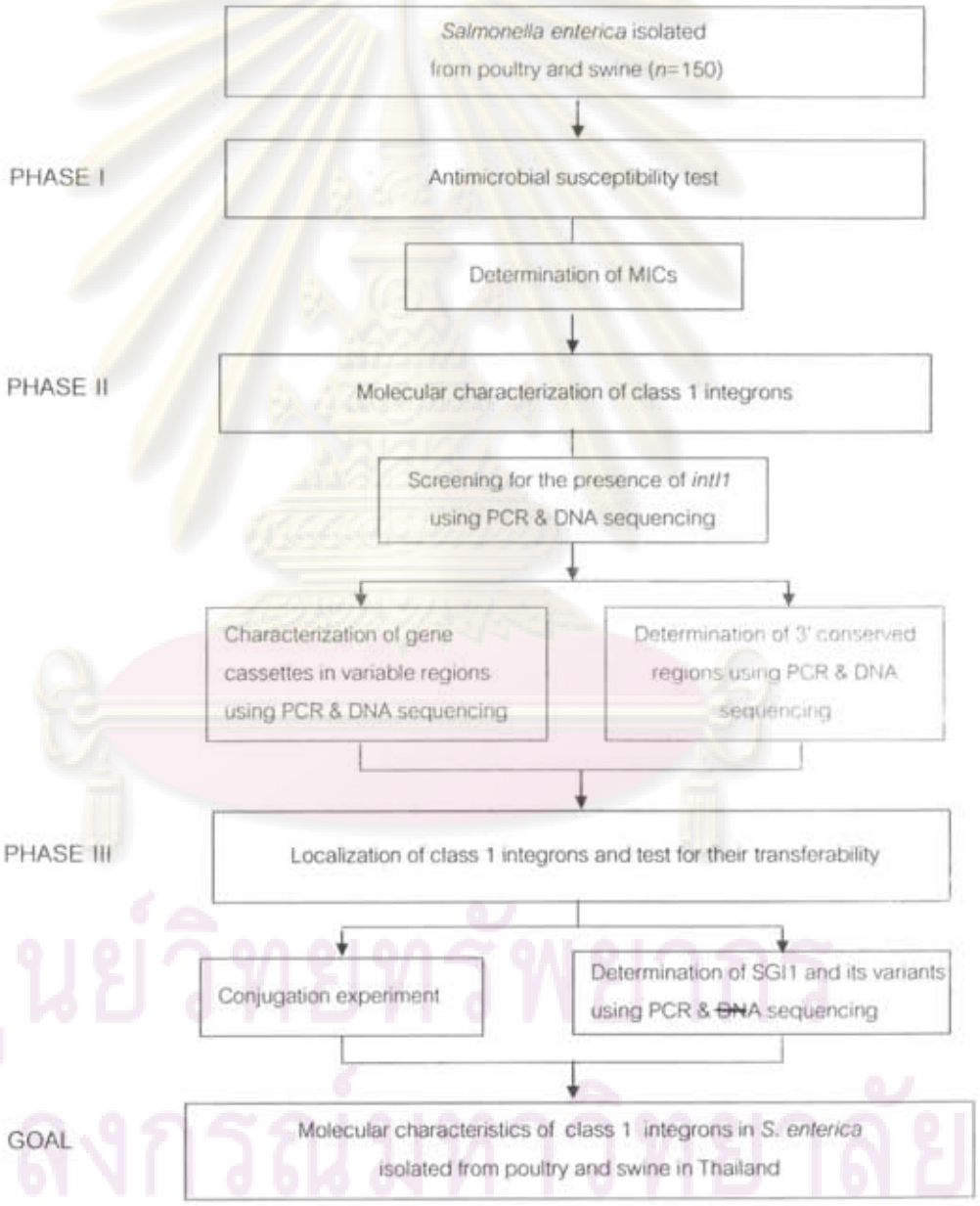

Figure 4: The conceptual framework in this study

Salmonella enterica isolates

A hundred-fifty *S. enterica* isolates were included in this study. All of the isolates were from the strain collection of National Institue of Animal Health (NIAH), Department of Livestock, Bangkok. They were isolated from samples collected from poultry and swine during 2003-2006 using methods described in International Organization for Standardization ISO 6579: 2002 (E) (ISO, 2002). The isolates were serotyped by slide agglutination using to the Kauffmann-White scheme (Popoff, 2001). All bacterial strains were stored as freeze-dried stocks in 10% skimmilk at -80°C. They were sent to Department of Veterinary of public health, Faculty of Veterinary Science, Chulalongkorn University and stored as 20% glycerol stocks at -80°C for further studies.

Phase I Antimicrobial susceptibility testing

Antimicrobial susceptibility to ampicillin (AMP), choramphenicol (CHP), ciprofloxacin (CIP), gentamicin (GEN), spectinomycin (SPC), streptomycin (STR), sulfamethoxazole (SUL), tetracycline (TET) and trimethoprim (TMP) were assessed by determining the minimum inhibitory concentration (MIC) using two-fold agar dilution technique according to Clinical and Laboratory Standards Institute (CLSI, formerly NCCLS) (NCLS, 2002). Bacteria were grown overnight at 37°C in Muller-Hinton agar (MHA); (Difco, MD, USA). Single colonies were picked to suspend in 0.85% NaCl (NSS) solution to 0.5 Mcfarland. Then, the suspension was ten fold diluted to 10^{-1} in NSS. The suspension was inoculated using a multi-point inoculator onto the MHA agar containing suitable concentrations of antibiotics. Antibiotics were dissolved in appropriate diluents and filter-sterile. The diluents and antibiotic concentrations used are shown in Table 1. Breakpoints are discriminating concentrations used in the interpretation of results of susceptibility testing to define isolates as susceptible or resistant with the interpretation guidelines established by CLSI and shown in Table 2. Multidrug resistance (MDR) was defined as isolates being resistant to 3 or more

different classes of antibiotics. *Pseudomonas aeruginosa* ATCC 27853, *E. coli* ATCC 25922 and *Staphylococus aureus* ATCC 29212 were used as control organisms.

All of *Salmonella* isolates were tested for their susceptibility to chloramphenicol, ciprofloxacin and gentamicin at the NIAH whereas the susceptibility test of ampicillin, spectinomycin, streptomycin, sulfamethoxazole, tetracycline and trimethoprim were examined at Department of Veterinary Public Health, Faculty of Veterinary Science, Chulalongkorn Univerisity.

Table1: Solvents and concentrations of antibiotic used in this study

Antibiotics	Solvents	Concentrations range (µg/ml)
ampicillin	water	0.5, 1, 2, 4, 8, 16, 32, 64,128, 256
chloramphenicol	95% ethanol	0.5, 1, 2, 4, 8, 16, 32, 64,128, 256
ciprofloxacin	0.1N NaOH	0.125, 0.25, 0.5, 1, 2, 8, 16, 32, 64,128, 256
gentamicin	water	0.125, 0.25, 0.5, 1, 2, 8, 16, 32, 64,128, 256
spectinomycin	water	0.5, 1, 2, 4, 8, 16, 32, 64,128, 256, 512,1024
streptomycin	water	0.5, 1, 2, 4, 8, 16, 32, 64,128, 256, 512,1024
sulfamethoxazole	0.1N NaOH	0.5, 1, 2, 4, 8, 16, 32, 64,128, 256, 512,1024
tetracycline	70% ethanol	0.5, 1, 2, 4, 8, 16, 32, 64,128, 256
trimethoprim	dimethylacetamide	0.5, 1, 2, 4, 8, 16, 32, 64,128, 256

Table 2: Breakpoints used in this study

Antibiotics	Breakpoint (µg/ml)
ampicillin	32
chloramphenicol	32
ciprofloxacin	4
gentamicin	8
spectinomycin	128
streptomycin	32
sulfamethoxazole	512
tetracycline	16
trimethoprim	16

Phase II Molecular characterization of class 1 integrons

The experiments included 3 steps as follows: the first step was to screen for the presence of class 1 integrase (*intI1*) gene, which was the marker for class 1 integrons. Only the *intI1*-positive isolates were used to study in the next steps. The second strep was to determine and characterize gene cassettes in variable regions. The third step was to determine a typical 3'conserved region of class 1 integrons.

All primers used in this study are listed in Table 3. Localization and specific information of each primer are described in the section where the primers were used.

1. Screening for the presence of *intI1*

Template DNA of all the *Salmonella* isolates was prepared by the whole cell boiled lysate procedure (Levesque et al., 1995). Bacteria were grown overnight at 37°C on Luria-Bertani (LB) agar (Difco, MD, USA). A single colony was emulsified in 50 µl of sterile distilled water and heated in a boiling water bath for 10 minutes. The suspension was centrifuged at 12,000 rpm for 5 minutes. The supernatant was removed to a new eppendorf tube and stored at -20°C. All DNA samples were screened for the presence of *intI1* gene using Polymerase Chain Reaction (PCR). The specific primer pair used was int1F and int1R. Localization and detail of these two primers are given in Figure 5.

All PCR assays were carried out in a final volume of 25 µl using PCR Master Mix (Eppendorf®, Hamberg, Germany) according to the manufacturer's instructions. Each PCR reaction consisted of 12.5 µl of Eppendorf® MasterMix, 5.5 µl of sterile-distilled water, 1.0 µl of each primer at 10 µM and 5 µl of DNA template. PCR amplifications were conducted on a PCR Sprint Thermocycler® (Thermo Electron Corporation®, Cambridge, UK). Thermal cycling conditions included of an initial denaturation at 94°C for 5 minutes, followed by 30 cycles of 1 min at 94°C, 1 minute at 50°C, 1 minute at 72°C and a final step of 72°C for 5 minutes The PCR products obtained were separated on gel electrophoresis in 1.5% agarose gel (Agarose, Sigma-Aldrich®, USA) Chemie GmbH, Steinheim, Germany) and

Tris-acetate/EDTA buffer and applying 100 V for 30-45 minutes. The gels were stained in ethidium bromide solution (Sigma Aldrich Inc.) and visualized by the Bio-Rad Gel-Documentation system (Bio-Rad Laboratories, Ventura, CA, USA). DNA representatives were submitted for sequencing at Macrogen Inc. (Seoul, South Korea) to confirm primer specificity. DNA from an isolate that was confirmed to contain *intI1* by DNA sequencing was used as positive control.

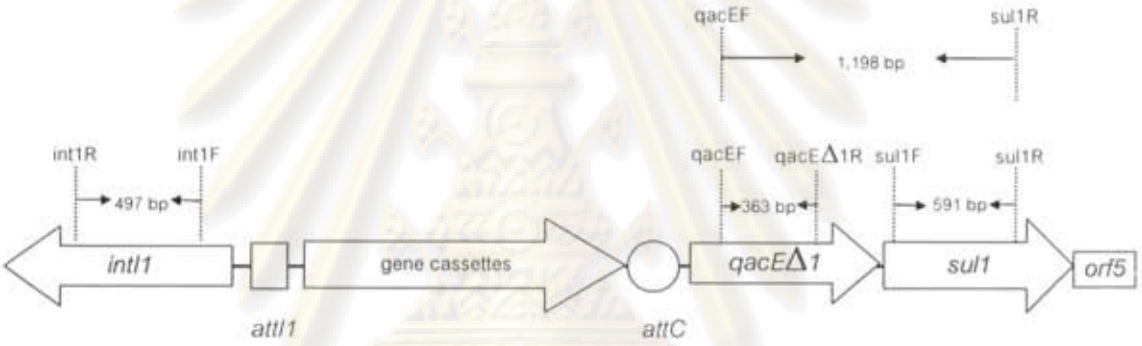

Figure 5: Localization of primers used in characterization of class 1 integrons. Primers int1F and int1R were used for amplification of the *intI1* gene. Primer pairs qacEF-qacER and sul1F-sul1R were used to amplify *qacEΔ1* and *sul1*, respectively. The fused structure *qacEΔ1-sul1* was confirmed using primer pair qacEF-sul1R. The arrows indicate the direction of primers. The vertical-dashed lines indicate location of primers.

Table 3: PCR primes used in this study for determination class 1 integrons and SGI1 in *S. enterica*

Gene	Primer	Sequence (5'-3')	Amplicon size (bp)	References
intI1	Int1F	CCT GCA CGG TTC GAA TG	497	Chuanchuen et al., 2007
	Int1R	TCG TTT GTT CGC CCA GC	497	Chuanchuen et al., 2007
variable regions	5'CS	GGC ATC CAA GCA GCA AG	variable	Levesque et al., 1995
	3'CS	AAG CAG ACT TGA CCT GA	variable	Levesque et al., 1995
qacEΔ1	qacEF	TAA GCC GTA CAC AAA TTG GGA GAT AT	363	Chuanchuen et al., 2007
	qacEΔ1R	GCC TCC GCA GCG ACT TCC ACG	363	Chuanchuen et al., 2007
sul1	sul1F	CGG ACG CGA GGC CTG TAT C	591	Chuanchuen et al., 2007
	sul1R	GGG TGC GGA CGT AGT CAG G	591	Chuanchuen et al., 2007
qacEΔ1-sul1	qacEF	TAA GCC GTA CAC AAA TTG GGA GAT AT	1,198	Chuanchuen et al., 2007
	sul1R	GGG TGC GGA CGT AGT CAG G	1,198	Chuanchuen et al., 2007
thdI	thdF1	ACA CCT TGA GCA GGG CAA G	500	Chuanchuen et al., 2007
	thdF2	AGT TCT AAA GGT TCG TAG TCG	500	Doublet et al., 2003
SA044- yidY	SO44	TGA CGA GCT GAA GCG AAT TG	500	Doublet et al., 2003
	yidY	ACC AGG GCA AAA CTA CAC AG	500	Doublet et al., 2003
SA044- int2	SO44	TGA CGA GCT GAA GCG AAT TG	515	Doublet et al., 2003
	int2	AGC AAG TGT GCG TAA TTT GG	515	Doublet et al., 2003
sul1- floR	sulTER	AAG GAT TTC CTG ACC CTG	942	Doublet et al., 2003
	F3	AAA GGA GCC ATC AGC AGC AG	942	Doublet et al., 2003
floR-tetR	F4	TTC CTC ACC TTC ATC CTA CC	598	Doublet et al., 2003
	F6	TTG GAA CAG ACG GCA TGG	598	Doublet et al., 2003
tetR- tetG	tetR	GCC GTC CCG ATA AGA GAG CA	1,559	Doublet et al., 2003
	tetG	GAA GTT GCG ATT GGT CTG CG	1,559	Doublet et al., 2003
groEL- pse1	groEL	TTC TGG TCT TCG TTG ATG CC	1,338	Doublet et al., 2003
	pse1	CAT CAT TTC GCT CTG CCA TT	1,338	Doublet et al., 2003
dfrA10	dfrA10F	ATC TAT TGG ATC ACC TAC CC	432	This study
	dfrA10R	TTC GCA GAC TCA CTG AGG G	432	This study
Circular form	SGI1circ1	AGC AAA ATC GTG AGA AGG GA	364	Doublet et al., 2005
of SGI1	SGI1circ2	TGA TGA GAC ACC TGA CGA GC	364	Doublet et al., 2005

2. Characterization of gene cassettes in variable region

All the isolates containing the *intI1* gene were assayed for the presence of resistance gene cassettes using a primer set 5' CS and 3' CS. Thermal cycling conditions consisted of an initial denaturation cycle at 94°C for 5 minutes, followed by 30 cycles of 45 second at 94°C, 1 minute at 54°C, 3 minutes at 72°C and a final step of 72°C for 5 minutes The amplicons were visualized on agarose gel and purified using QIA Quick Gel Extraction kit (Qiagen, Hilden, Germany). Then, the purified PCR products were submitted for sequencing. Nucleotide sequence analysis was performed using the Blast algorithm available at the National Center for Biotechnology Information website (www.ncbi.nlm.nih.gov).

The CS-PCR amplicons of the same size were digested with restriction endonucleases including *EcoRI and NcoI* (Fermentus, Burlington, Canada) and separated in a 1.5-2.0% agarose gel. The enzymes were chosen according to sequence results of the same size amplicons. The amplicons with the same restriction pattern were considered identical. The size of two common PCR amplicons 2,000 and 1,300 bp were expected to be *dfrA12-aadA2* and *dfrA1-orfC*, respectively. Therefore, the 2,000 bp amplicons were digested with *EcoRI* and the 1,300 bp amplicons were digested with *NcoI*.

3. Determination of typical 3'conserved regions

All the isolates containing *intI1* gene were examined for the presence of the 3' conserved regions using primers specific for *qacEΔ1* (qacEF and qacΔ1R), *sul1* genes (sul1F and sul1R) and *qacEΔ1-sul1* (qacEF and sul1). The *qacEΔ1-sul1* amplication was performed to confirm that *qacEΔ1* is located at 5' end of *sul1*. Thermal cycling conditions consisted of a hot start cycle at 94°C for 5 minutes, followed by 30 cycles of 45 second at 94°C, 1 minute at 57°C for *qacEΔ1*, 60°C for *sul1* and 57°C for *qacEΔ1-sul1*, at 72°C for 1 min and a final step of 72°C for 5 minutes.

Phase III Localization of class 1 integrons and test for their transferability

The experiments in this phase contained 2 stages including conjugation experiment and determination of *Salmonella* Genomic Island1 and its variants. Conjugation experiments elucidated if class 1 integrons were located on conjugative plasmid and could be transfered to other bacteria. Determination of *Salmonella* Genomic Island1 and its variants was to determine if class 1 integrons were located on chromosome.

1. Conjugation experiments

Conjugation experiments were performed by filter mating method as previously described (Chen et al., 2004). Twenty-two class 1 integrons-positive *Salmonella* isolates containing the resistance gene cassettes were used as donors and the spontaneous rifampicin-resistant derivatives of *E.coli* K12 strain MG1655 (MG1655 rif^r) were recipients.

1.1 Isolation of *E. coli* MG1655 rif^r

E. coli MG1655 is an *E. coli* K12 wildtype that is susceptible to all antibiotics tested in this study. It does not carry plasmid and class 1 integrons. Since all of the class 1 integrons-containing *Salmonella* isolates in this study were susceptible to rifampicin (MIC = 8 µg/ml), the rifampicin-resistance marker was chosen to counterselect the *E. coli* MG1655 recipient. Therefore, the spontaneous rifampicin-resistant derivative of *E. coli* MG1655 was made.

E. coli MG1655 was grown in 4 ml LB broth at 37°C overnight in a shaking incubator. A 100 µl portion was spreaded on LB agar containing rifampicin at different concentrations (8, 16, 32, 64 and 128 µg/ml). The inoculated LB plates were incubated at

37°C for 18-24 hours. The control plate was *E. coli* MG1655 inoculated on LB agar without rifampicin.

The rifampicin-resistant colonies were selected from LB agar supplemented with corresponding concentrations of rifampicin. These resistant colonies were repeatedly inoculated on LB without rifampicin for 10 consecutive days. The ability to grow on LB containing rifampicin was confirmed. As single colony of the spontaneous rifampicin resistant was selected and named *E. coli* MG1655 rif^r. The MICs for all antibiotics were examined.

1.2 Biparental mating

The donor and the recipient strains were cultured overnight at 37°C in 4 ml LB broth. Eighty-µl aliquots of the overnight cultures of the donors and the recipients were separately added into 4 ml fresh LB broth and incubated at 37°C for 3-4 hours to log phase of growth. Cultures of the donor and the recipient were gently mixed at 1:1 ratio in an eppendorf tube and then centrifuged at 8,000 rpm for 1 minute. The supernatant was completely removed and the cell pellets were suspended in 30 µl LB broth warmed at 37°C. The mixture was gently dropped on a sterile membrane filter (0.45 µm pore size, Millipore, Massachusetts, USA) that was placed on a LB agar plate without antibiotic. The innoculated-filter plate was incubated overnight at 37°C. Then, the filter paper was removed and put into 1 ml of NSS in a new eppendorf tube. The tube was vortexed to dislodge the cells and the empty filter paper was discarded. The suspension was centrifuged at 12,000 rpm for 1 minute and the supernatant was removed. A hundred-µl of fresh LB broth was added into the bacterial pellets. Then, the conjugation mixture was spreaded on Colimstant agar (Scharlau, Barcelona, Spain) supplemented with 32 µg/ml of rifampicin and one of the following antibiotics ampicillin (100 µg/ml), streptomycin (50 µg/ml) and trimethoprim (10 µg/ml). The inoculated plates were incubated overnight at 37°C. Transconjugants that were *E. coli* MG1655 rif^r with resistance plasmid appeared as blue colonies on the plates.

Transconjugants were confirmed to be *E. coli* by growing on MacConkey agar (Difco) or Eosin Methylene Blue agar (Difco) and subsequently tested for antibiotic susceptibility. Plasmid DNA were extracted from each transconjugant using PureLink™ Quick Plasmic Miniprep Kit (Invitrogen, Carlsbad, CA, USA) and examined for the presence of class 1 integrons, the corresponding resistance gene cassettes using PCR and restriction endonuclease analysis as described above.

2. Determination of *Salmonella* Genomic Island1 and its variants.

In all class 1 integrons-positive isolates, the presence of SGI1 and its variants and their location were determined using PCR and DNA sequencing. The isolates were first examined by PCR for the presence of the left (*thdF*) and right (*S044–yidY*) junction of SGI1 using primer pairs thdF-thdR and S044-yidY, respectively. The organization of antibiotic resistance gene cluster was determined on the basis of class 1 integrons profiles and antibiotic resistance patterns using PCR (Figure 6). Chromosomal DNA template was prepared using QIAamp DNA Mini Kit (Qiagen® Hilden, Germany). Thermal cycling conditions consisted of a hot start cycle at 94°C for 3 minutes, followed by 30 cycles of 1 minute at 94°C, 1 minute at 55°C for thdF-thdR, SO44-yidY and groEL-pse1 or 57°C for sulTER-F3, F4-F6, tetR-tetG and dfrA10F-dfrA10R, 1 minute at 72°C for all amplifications except 2 minutes for *tetG* and a final step of 72°C for 10 minutes.

The extrachromosomal circular form of SGI1 was also detected in all isolates containing SGI1 using PCR. PCR assays were performed using plasmid DNA as DNA template and primer pair SGI1circ1 and SGI1circ2. Thermal cycling conditions consisted of a hot start cycle at 94°C for 3 minutes, followed by 30 cycles of 1 minute at 94°C, 1 minute at 55 °C, 1 minute at 72 °C and a final step of 72°C for 10 minutes.

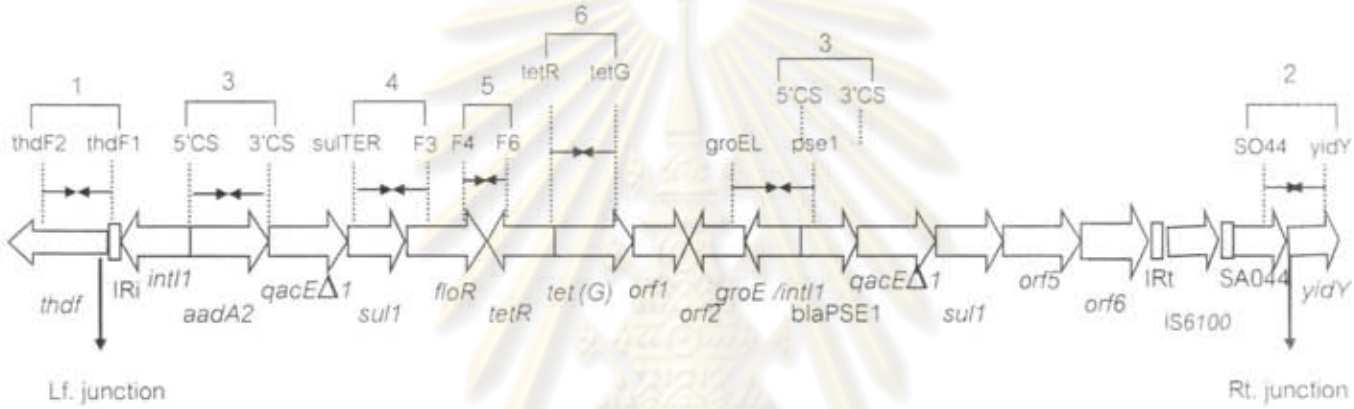

Figure 6: Localization of primer sets used in characterization of SGI1 and its variants. Primer pairs thdF1-thdF2 and SA044-yidY were used for amplification of left and right junction, respectively The organization of antibiotic resistance gene cluster was determined using primer pairs sulTER-F3, F4-F6, tetR-tetG and groEL-pse. The arrows indicate the direction of primers. The vertical-dashed lines indicate location of primers. The order of amplification was from one to six.

CHAPTER IV

RESULTS

1. *Salmonella enterica* isolates

A total of 150 *S. enterica* isolates used in this study were from poultry (76 isolates) and swine (74 isolates). Thirty-one serovars were included and shown in Table 4. The most frequent serovar in this study was Enteritidis (14%) followed by serovars Rissen (9.33%), Basilla (8.66%), Subspecies I (7.33%), and Amsterdam (6.66%). There were 6% of each Kedougou, Anatum and Corvallis and 4% of each serovars Typhimurium and Stanley and 3.33% of serovars Albany and Emek. Two percent of each Altony, Agona, Give, and Orion were investigated whereas 1.33% of each Bovismorbifican, Kentucky, Madjorio and Poona were included. For serovars Bareilly, Blockley, Eppendorf, Kingston, Lexington, Panama, Paratyphi B2, Schwarzengrund, Singapore and Virchow, only one isolate each was included.

Table 4: *Salmonella enterica* serovars isolated from poultry and swine (n=150)

Salmonella serovars	No. of isolates (%)	
	Poutry	Swine
Albany	4 (2.67)	1 (0.67)
Altony	0	3 (2.00)
Agona	3 (2.00)	0
Amsterdam	10 (6.67)	0
Anatum	0	9 (6.00)
Bovismorbifican	1 (0.67)	1 (0.67)
Bsilla	0	13 (8.67)
Bareilly	1 (0.67)	0
Blockley	1(0.67)	0
Corvallis	4 (2.67)	5 (3.34)
Enteritidis	20 (13.34)	1 (0.67)
Emek	5 (3.34)	0
Eppendorf	1 (0.67)	0
Give	2 (1.34)	1 (0.67)
Kedougou	1 (0.67)	8 (5.34)
Kingston	0	1 (0.67)
Kentucky	2 (1.34)	0
Lexington	1 (0.67)	0
Madjorio	2 (1.34)	0
Orion	1 (0.67)	2 (1.34)
Panama	1 (0.67)	0
Paratyphi B2	1 (0.67)	0
Poona	2 (1.34)	0
Rissen	1 (0.67)	13 (8.67)
Stanley	0	6 (4.00)
Subspecies I	3 (2.00)	8 (5.34)
Schwarzengrund	1 (0.67)	0
Senftenberg	2 (1.34)	0
Singapore	1 (0.67)	0
Typhimurium	3 (2.00)	3 (2.00)
Virchow	0	1 (0.67)
Total	74 (49.34)	76 (50.67)

2. Susceptibility to antibiotics

Distribution of antibiotics resistance is shown in Figure 7. In the present study, 21 isolates (14%) were susceptible to all antibiotics tested and a hundred-twenty nine (86%) isolates were resistant to at least one antibiotic.

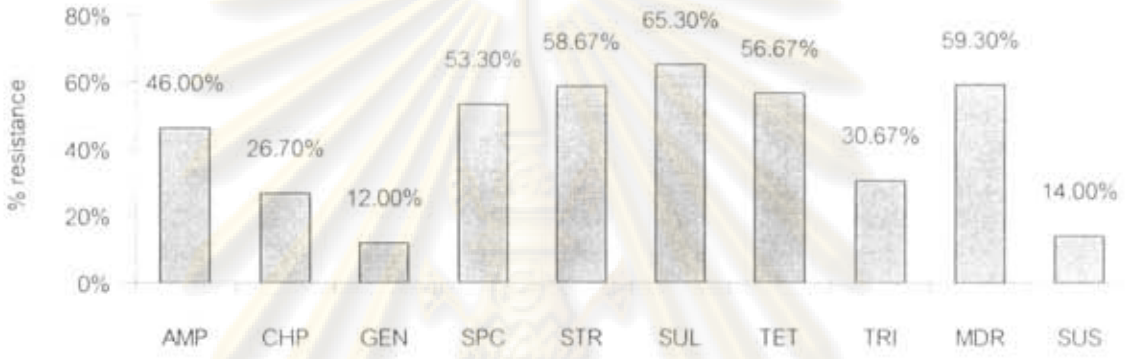

Figure 7: Distribution of antibiotics resistance in *Salmonella* isolates. AMP, ampicillin; CHP, chloramphenicol; GEN, gentamicin; SPC, spectinomycin; STR, streptomycin; SUL, sulfamethoxazole; TET, tetracycline; TRI, trimethoprim; MDR, multiple drug resistant; SUS, susceptible.

Resistance rate to ampicillin, choramphenicol, gentamicin, spectinomycin, streptomycin, sulfamethoxazole, tetracycline and trimethoprim were 46%, 26.7%, 12%, 53.3%, 58.67%, 65.3%, 56.67% and 30.67%, respectively. None of the isolates was resistant to ciprofloxacin. Ninety eight (59.3%) isolates were resistant to at least 3 different antimicrobial classes and considered MDR. Antibiotic resistance patterns were also analyzed and shown in Table 5. All *Salmonella* strains could be grouped into 56 resistance patterns. Two most common MDR phenotypes found were the SPC-STR-SUL (6.21%) and the AMP-CHP-SPC-STR-SUL-TET-TRI phenotype (6.21%).

Table 5: Antibiotic resistance patterns of *S. enterica* isolates (*n*=129)

Antibiotic resistance pattern	No. of isolates (%)
SPC	3 (2.33)
STR	1 (0.78)
SUL	4 (3.11)
TET	4 (3.11)
TRI	1 (0.78)
AMP-STR	4 (3.11)
AMP-SUL	2 (1.56)
SPC-SUL	1 (0.78)
SPC-TET	2 (1.56)
SPC-TRI	1 (0.78)
STR-SUL	1 (0.78)
STR-TET	3 (2.33)
TET-TRI	1 (0.78)
AMP-SPC-STR	1 (0.78)
AMP-SPC-SUL	4 (3.11)
AMP-STR-SUL	2 (1.56)
AMP-STR-TET	1 (0.78)
AMP-SUL-TET	1 (0.78)
AMP-SUL-TRI	1 (0.78)
SPC-STR-SUL	8 (6.21)
SPC-STR-TET	5 (3.88)
SPC-SUL-TET	1 (0.78)
STR-SUL-TET	4 (3.11)

Antibiotic resistance pattern	No. of isolates (%)
SUL-TET-TRI	3 (2.33)
AMP-SPC-STR-SUL	2 (1.56)
AMP-SPC-SUL-TET	1 (0.78)
AMP-STR-SUL-TET	2 (1.56)
AMP-SUL-TET-TRI	1 (0.78)
CHP-SPC-STR-SUL	1 (0.78)
CHP-STR-SUL-TRI	1 (0.78)
CHP-SUL-TET-TRI	2 (1.56)
SPC-STR-SUL-TET	4 (3.11)
SPC-STR-SUL-TRI	3 (2.33)
SPC-STR-TET-TRI	1 (0.78)
STR-SUL-TET-TRI	1 (0.78)
AMP-CHP-SPC-STR-TET	1 (0.78)
AMP-CHP-SUL-TET-TRI	1 (0.78)
AMP-SPC-STR-SUL-TET	4 (3.11)
AMP-SPC-STR-SUL-TRI	1 (0.78)
AMP-SPC-STR-TET-TRI	1 (0.78)
AMP-STR-SUL-TET-TRI	1 (0.78)
CHP-GEN-SPC-SUL-TET	1 (0.78)
CHP-SPC-STR-SUL-TRI	1 (0.78)
SPC-STR-SUL-TET-TRI	1 (0.78)
AMP-CHP-GEN-SPC-SUL-TET	1 (0.78)
AMP-CHP-GEN-STR-SUL-TET	1 (0.78)
AMP-CHP-SPC-STR-SUL-TET	7 (5.43)
AMP-CHP-STR-SUL-TET-TRI	4 (3.11)
AMP-CHP-GEN-SUL-TET-TRI	1 (0.78)
AMP-GEN-SPC-SUL-TET-TRI	3 (2.33)
AMP-SPC-STR-SUL-TET-TRI	2 (1.56)
AMP-CHP-GEN-SPC-STR-SUL-TET	6 (4.66)
AMP-CHP-GEN-SPC-STR-TET-TRI	1 (0.78)
AMP-CHP-SPC-STR-SUL-TET-TRI	8 (6.21)
AMP-GEN-STR-SPC-SUL-TET-TRI	1 (0.78)
AMP-CHP-GEN-SPC-STR-SUL-TET-TRI	3 (2.33)
Total	129

Resistant isolates belonged to different serovars, including *S.* Albany (5/5), *S.* Altony (3/3), *S.* Agona (3/3), *S.* Amsterdam (6/10), *S.* Anatum (9/9), *S.* Bovismorbifican (1/2), *S.* Bsilla (13/13), *S.* Bareilly (1/1), *S.* Blockley (1/1), *S.* Corvallis (9/9), *S.* Enteritidis (7/21), *S.* Emek (5/5), *S.* Eppendorf (1/1), *S.* Give (2/3), *S.* Kedougou (9/9), *S.* Kingston (1/1), *S.* Kentucky (2/2), *S.* Lexington (1/1), *S.* Madjorio (2/2), *S.* Orion (3/3), *S.* Panama (1/1), *S.* Paratyphi B2 (1/1), *S.* Poona (2/2), *S.* Rissen (14/14), *S.* Stanley (6/6), *S.* Subspecies I (11/11), *S.* Schwarzengrund (1/1), *S.* Senftenberg (2/2), *S.* Singapore (1/1), *S.* Typhimurium (6/6) and *S.* Virchow (1/1)

3. The presence of *intI1*

Forty isolates (26.67%) yielded a 497 bp amplicon when amplified with the primers specific for the *intI1* gene. Nucleotide analysis revealed that the sequence of the amplicon representative was identical to the published sequence. The PCR amplicons of *intI1* are shown in Figure 8.

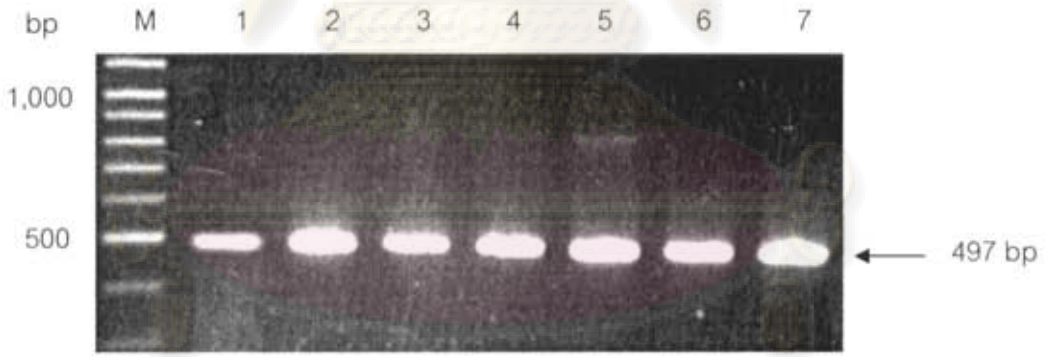

Figure 8: Detection of *intI1* in *S. enterica* isolates. DNA template was PCR amplified using primer int1F and int1R that generated the amplicon size of 497 bp Lane M, 100-bp marker; Lane 1, Positive control; Lane 2-7 the *intI1*-containing *S. enterica* strains.

4. The presence of typical 3' conserved regions

All of the *S. enterica* isolates carrying *intI1* were examined for the presence of typical 3' CS. A positive PCR result with *qacEΔ1*, *sul1* and *qacEΔ1-sul1* was indicated by the obtained PCR product of 363, 591 and 1,198 bp, respectively (Figure 9) Twenty seven isolates (67.5%) were found to carry all of *qacEΔ1*, *sul1* and *qacEΔ1-sul1*. Nucleotide sequencing results also confirmed the specificity of PCR amplification. The remaining 13 isolates (32.5%) did not yield any PCR amplicons indicating that they did not contain typical 3 'conserved regions of class 1 integrons (Table 6). From class 1 integrons analysis, distribution of 3'CS and gene cassettes among the *intI1*-positive strains is shown in Table 6.

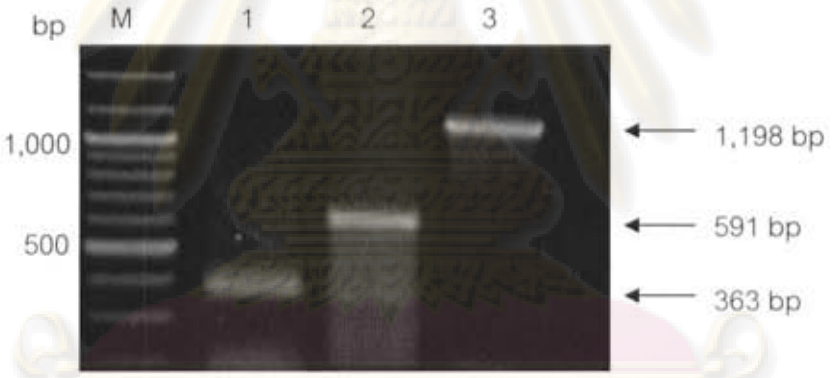

Figure 9: Typical 3'CS of class 1 integrons. DNA template from the *intI1*-positive *Salmonella* isolates was PCR amplified using primer pairs qacEF-qacEΔ1R, sul1F-sul1R and qacEF-sul1R. Amplification products of *qacEΔ1*, *sul1* and *qacEΔ1-sul1* are 363, 591 and 1,198 bp respectively. Lane M, 100-bp marker; Lane 1, *qacEΔ1*; Lane 2, *sul1* and Lane 3, *qacEΔ1-sul1*.

Table: 6 Distribution of 3'CS and gene cassettes among the *intI1*-positive strains (n=40)

Serovars	3'CS	Gene cassettes	No.
S. Kedougou	-	+	1
S. Kedougou	+	-	3
S. Kedougou	-	+	2
S. Kedougou	-	-	3
S. I	+	-	5
S. I	-	-	1
S. Anatum	+	+	1
S. Anatum	-	-	1
S. Rissen	+	+	3
S. Albany	+	+	5
S. Emek	+	+	3
S. Stanley	+	+	1
S. Stanley	-	-	2
S. Kingston	+	+	1
S. Panama	-	-	1
S. Give	+	+	1
S. Eppendorf	+	+	1
S. Kentucky	+	+	1
S. Schwarzengr	+	-	1
S. Senftenberg	+	-	1
S. Altona	-	+	1
S. Paratyphi B2	-	-	1
Total	14	12	40

5. Characterization of gene cassettes in class 1 integrons

Forty *Salmonella* isolates carrying the *intI1* gene were examined for the presence of gene cassettes in variable regions. Among these isolates, twenty-two isolates (55%) carried inserted gene cassettes with size ranging from 650 to 2,300 bp. No gene cassette regions were amplified from the remaining 18 isolates (45%). Ten integrons profiles (IPs) were defined by the number and the size of the PCR amplicons obtained and are shown in Table 7. The PCR amplicon patterns of each IPs are shown in Figure 10. Seventeen isolates carried a single class 1 integrons with the variable regions size of 2,300 bp (IP1), 2,000 bp (IP2), 1,300 bp (IP5), 1,100 bp (IP6) and 650 bp (IP10). Five isolates contained 2 class 1 integrons with the gene cassette size of 2,000 and 1,300 bp (IP3), 2,000 and 1,100 bp (IP4), 1,300 and 1,100 bp (IP7), 2,000 and 750 bp (IP8) and 2,000 and 650 bp (IP9). Seven different gene arrays were revealed by DNA sequencing including *silB*, *dfrA12-aadA2*, bla_{PSE-1}, *aadA4*, *dfrA1-orfC*, incomplete *sat* and *codB*. The complete resistance genes *dfrA12* confers resistance to trimethoprim, *aadA2* confers reistance to spectinomycin and streptomycin, bla_{PSE-1} confers resistance to β-lactams and *orfC* encodes protein of unknown function. *SilB* is membrane fusion protein and a part of an SilABC efflux system mediating resistance to cadmium, zinc and cobalt (Gupta et al., 1999). Neucleotide sequencing analysis also revealed that the variable regions with the size of 750 and 650 bp were the incomplete *codB* and *sat* gene, respectively. The *codB* gene encodes cytosine permease and the *sat* gene conferring resistance to streptothricin. Class 1 integrons lacking 3'CS were found in 3 isolates and all of these carried the incomplete *sat* gene (IP10) (Table 7).

Table 7: Different gene cassette arrays of class 1 integrons

IP	Approximate size of amplicons (bp)	Gene cassettes	No. of isolates	Transferability (No. of isolates)
1	2,300	*silB*	1	-
2	2,000	*dfrA12-aadA2*	4	1
3	1,300, 2,000	bla_{PSE-1}, *dfrA12-aadA2*	1	1 *
4	1,100, 2,000	*aadA2*, *dfrA12-aadA2*	1	1 *
5	1,300	*dfrA1-orfC*	8	3
6	1,100	*aadA4*	1	-
7	1,100, 1,300	bla_{PSE-1}, *aadA2*	1	-
8	750, 2,000	Incomplete *codB*, *dfrA12-aadA2*	1	1 *
9	650, 2,000	Incomplete *sat*, *dfrA12-aadA2*	1	-
10	650	Incomplete *sat*	3	ND

* Only class 1 integrons carrying *dfrA12-aadA2* were able to transfer.

ND Not determined

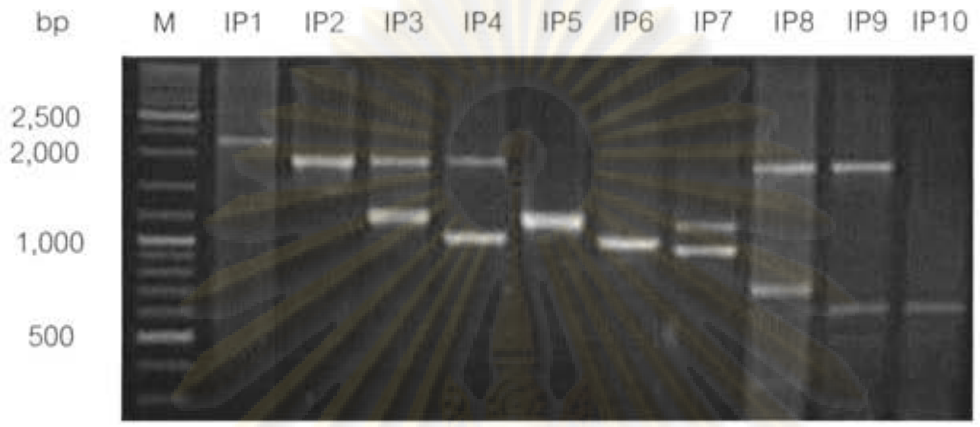

Figure 10: PCR amplifications of class 1 integrons variable regions. Template DNA was amplified using primer pair 5'CS and 3'CS. Ten integrons profile (IP1-IP10) were defined by the number and size of PCR amplicons. Lane M, 1 kb DNA marker; Lane 1,IP1: *silB*; Lane 2, IP2: *dfrA12-aadA2*; Lane 3, IP3: *dfrA12-aadA2* (upper) and bla_{PSE-1} (lower) ; Lane 4, IP4: *dfrA12-aadA2* (upper) and *aadA2* (lower) ; Lane 5, IP5: *dfrA1-orfC*; Lane 6, IP6: *aadA4*; Lane 7, IP7: bla_{PSE-1} (upper) and *aadA2* (lower) ; Lane 8, IP8: *dfrA12-aadA2* (upper) and incomplete *codB* (lower); Lane 9, IP9: dfrA12-*aadA2* (upper) and incomplete *sat* (lower) ; Lane 10, IP10: incomplete *sat*.

Base on the sequence results, the same size of PCR amplicons of 2000, and 1,300 were expected to be *dfrA12-aadA2* and *dfrA1-orfC*, respectively. All of the 2,000 bp amplicons were digested with *EcoRI*. The restriction patterns yielded three fragments of 940, 490 and 420 bp (Figure 11). The 1,300 bp amplicons were digested with *NcoI*. The restriction patterns yielded two fragments of 530 and 450 bp.

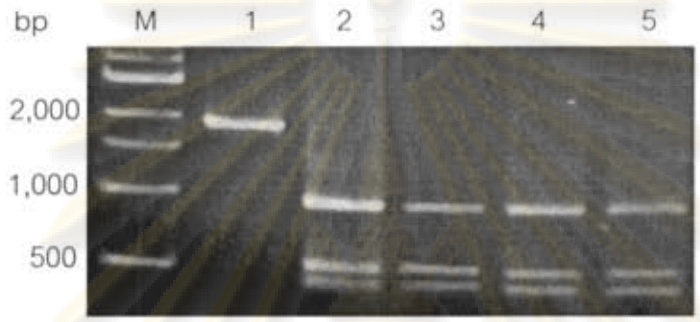

Figure 11: Restriction pattern of *dfA12-aadA2* containing amplicons digested with *EcoRI*. Lane M, 1 kb marker; Lane 1, undigested PCR amplicons of *dfA12-aadA2*; Lane 2, the *EcoRI*-digested *dfrA12-aadA2* fragment; Lane 3-5, the *EcoRI*-digested patterns of samples with 2,000 bp amplicons.

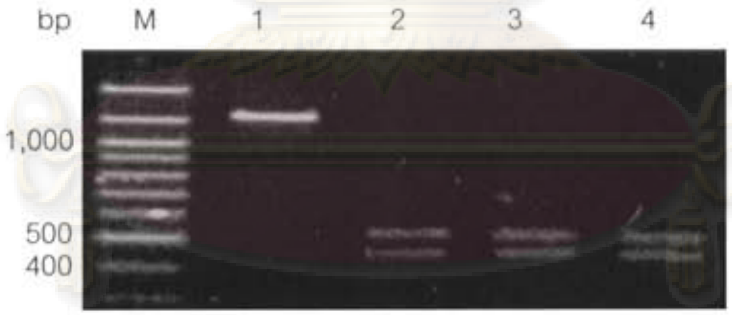

Figure 12: Restriction pattern of *dfrA1-orfC* amplicons containing digested with *NcoI*. Lane M, 1 kb marker; Lane 1, undigested PCR amplicons of *dfrA1-orfC*.; Lane 2, the *NcoI*-digested *dfrA1-orfC* fragment; Lane 3-4, the *NcoI*-digseted patterns of samples with 1,300 bp amplicons.

6. Conjugal transfer of class 1 integrons

Conjugation experiments were used to determine location of class 1 integrons. The MIC of *E.coli* MG1655 rif^r for rifampicin, ampicillin, streptomycin and trimethoprim were ≥ 256 µg/ml, 4 µg/ml, 8 µg/ml and 1 µg/ml, respectively. Transfer of class 1 integrons to *E. coli* was possible in 7 of 22 isolates carrying class 1 integrons with gene cassettes (31.81%) (Table 7). These isolates carried *dfrA12-aadA2* (IP2), *dfrA12-aadA2* and blaPSE1 (IP3), *dfrA12-aadA2* and *aadA2* (IP4), *dfrA1-orfC* genes (IP5) and *dfrA12-aadA2* and incomplete *codB* (IP7). The plasmid transfer was confirmed by observations that the transconjugants also carried plasmids and these plasmids harbored the class 1 integrons with variable regions of the same size as those in the corresponding donors.

7. Determination of *Salmonella* Genomic Island 1 and its variants

The left and right junctions of SGI1 were determined in 9 isolates of the class 1 integron positive strain (40.90%). Eight of these isolates were classified into IP5 and carried a single *dfrA1-orfC* gene array. The other was in IP7 and contained 2 class 1 integrons, of which bla_{PSE-1} and *aadA2* were identified in the variable regions. The presence of resistance gene clusters identified by PCR is shown in Table 8. The organization of the genes indicated that *Salmonella* in this study contained SGI1-A and SGI-F (see Figure3 page 12 for genetic structure). SGI1-A was present in a single S. Kingston isolate, while SGI1-F was identified in 4 Albany, 3 Emek and a Kedougou serovars. None of isolates carried SGI1 extrachromosomal circular form.

Table 8: Presence of gene clusters in SGI1 variants.

ID[a]	Serovars	Gene cassettes	IP	Lf[b]	Rt[c]	*groEL/pse1*	*sul1/floR*	*floR/tetG*	*tetR/tetG*	*dfrA10*	SGI1 variants
SA022	*S.* Albany	*dfrA1-orfC*	5	+	+	+	+	+	+	ND	SGI1-F
SA023	*S.* Albany	*dfrA1-orfC*	5	+	+	+	+	+	+	ND	SGI1-F
SA035	*S.* Albany	*dfrA1-orfC*	5	+	+	+	+	+	+	ND	SGI1-F
SA036	*S.* Albany	*dfrA1-orfC*	5	+	+	+	+	+	+	ND	SGI1-F
SA044	*S.* Kedougou	*dfrA1-orfC*	5	+	+	+	+	+	+	ND	SGI1-F
SA097	*S.* Emek	*dfrA1-orfC*	5	+	+	+	+	+	+	ND	SGI1-F
SA098	*S.* Emek	*dfrA1-orfC*	5	+	+	+	+	+	+	ND	SGI1-F
SA191	*S.* Emek	*dfrA1-orfC*	5	+	+	+	+	+	+	ND	SGI1-F
SA039	*S.* Kingston	bla_{PSE-1}, *aadA2*	7	+	+	+	+	+	+	+	SGI1-A

[a]ID The *Salmonella* strain number used in the laboratory

[b]Lf Left junction

[c]Rt Right junction

ND Not determined

CHAPTER V

DISCUSSION

Extensive use of antibiotics in different fields such as human and veterinary medicine and agriculture during the past decades has been shown to be the main cause of antibiotic resistance among food borne pathogens. Nowadays, emergence of multidrug resistance among *Salmonella* strains has been reported worldwide (Soto et al., 2003). Antibiotic resistance genes in this pathogen are often carried on mobile genetic elements, especially class 1 integrons. These integrons can be transferred to other bacteria and contribute to dissemination of antibiotic resistance in bacteria. However, class 1 integrons have not been described in *Salmonella* isolates in Thailand. So far, class 1 integrons that have ever been reported in the country were those in *E. coli* (Phongpaichit et al., 2007) and *Vibrio cholerae* (Dalsgaard et al., 2000). From our knowledge, this study is the first report of characterization of class 1 integrons in *Salmonella* isolates in Thailand.

In this study, a variety of different serovars from poultry and swine were included. The most commonly identified serovars of *Salmonella* isolated from poultry was Enteritidis and those isolates from swine were Rissen and Bsilla. *S.* Weltevereden was shown to be the most frequent serovars found from chicken at slaughter and market (Padungtod and Kaneene, 2006) but this serovars was not included in our collection. All of the *Salmonella* isolates used in this study were isolated from healthy poultry and swine. In general, only meat from healthy animals is accepted for human consumption. Therefore, these isolate are presumably associated with those in human foods.

Antibiotic resistance was found in various serovars of *Salmonella* from both poultry and swine, indicating that antibiotic resistance was not solely associated with a particular *Salmonella* serovars. High resistance level to sulfamethoxasole, streptomycin

tetracycline, spectinomycin, trimethroprim and ampicilin was observed as expected since these antibiotics have been widely used in food animal production. None of isolates tested were resistant to ciprofloxacin. These results were similar to the previous studies in Thailand (Angkititrakul et al., 2005; Bangtrakulnonth et al., 2006). The explanation could be the limited use of this class of antibiotics in food animal production. One of the main findings of this study was the prevalent multiresistance among *Salmonella* isolates from farm animals. Such high multi-drug resistance rate observed was very similar to that of a previous study in *Salmonella* isolates from retail meat in Thailand (Bangtrakulnonth et al., 2006). The most common resistance phenotypes observed in this study were SPC-STR-SUL and AMP-CHP-SPC-STR-SUL-TET-TRI. Most of these antibiotics have been commonly used in veterinary medicine and animal husbandry. Interestingly, chloramphenicol has not been used in food animals for a long period of time but the strains resistant to this antibiotic are still identified. This finding may be due to the presence of chloramphenicol resistance genes on same genetic elements with other resistant determinants. The phenomenon has been shown in previous studies in *Salmonella* (Chuanchuen et al., 2008) and *E. coli* (Antunes et al., 2007). In *Salmonella*, class 1 integrons with atypical 3'CS were demonstrated. In these integrons, *cmlA* encoding chloramphenicol resistance was found on the same cluster with *dfrA12- aadA1-aadA2* and *estx-psp-aadA2-aadA1* (Chuanchuen et al., 2008). The similar clusters were defined in *E. coli* (Antunes et al., 2007). Co-transfer of Chloramphenicol and streptomycin resistance was observed. Use of streptomycin could select for streptomycin resistance via the *aad* gene and also co-select for chloramphenicol resistance encoded by *cmlA* located in the same integrons structure. Therefore, use of other antibiotics, of which resistance genes are located on the same clusters (i.e. trimethroprim and streptothricin) could select for chloramphenicol resistance as well. However, this coselection was not investigated in this study.

Of all the *Salmonella* isolates, 26.67% had *intI1* gene and 14.67% had gene cassettes in variable regions. The prevalence of class 1 integrons among the *Salmonella* isolates in this study was comparable with that described in other studies. Class 1 integrons were detected in 11.4% in Japan (Ahmed et al., 2005), 15.2% in Netherlands

(Vo et al., 2006) and 17.36% in China (Zhang et al., 2004). These data indicate that class 1 integrons are widespread in *Salmonella* isolates worldwide. Furthermore, the empty class 1 integrons that are those without gene cassettes were also identified. Such empty integrons may be a result of the unsuccessful PCR amplification because the size of gene cassettes was too large to be amplified. There may be also class 1 integrons that lose the resistance gene cassettes in the absence of selection pressure or that exchange the resistance gene cassettes with other integrons. These empty class 1 integrons are available for new gene cassettes to insert.

Among 10 integrons profiles, class 1 integrons with gene cassettes array *dfrA1-orfC* was most commonly found. In previous studies, the *dfrA1-orfC* gene was identified in *S.* Typhimurium from humans and animals in Australia (Levings et al., 2005), S. Oslo and S. Bareily from imported seafood in USA (Khan et al., 2006). The *dfrA1-orfC* was also found in class 1 integrons in *V. cholerae* from humans in India (Shi et al., 2006; Thungapathra et al., 2002) and *P. mirabilis* from humans in China (Boyd et al., 2008). The presence of identical genes cassettes in different *Salmonella* serovar, different host, different geographic area and different bacteria indicates that antibiotic resistance genes associated with class 1 integrons may be exchanged between *Salmonella* serovars and between bacterial species (Doublet et al., 2004). This will account for the rapid dissemination of resistance among the different bacterial pathogens.

The *dfrA12-aadA2* was also common in our collection. This gene array was previously described in *S.* Choleraesuis and *E. coli* from pigs in Taiwan (Hsu et al., 2006) and *S.* Gallinarum from chickens in Korea (Kwon et al., 2002). The *dfrA12-aadA2* have been reported in *E. coli* from humans in Korea (Lee et al., 2001), from cattle, swine and poultry in Spain (Guerra et al., 2003). However, the *dfrA1* gene was previously found in combination with *aadA1* in *Salmonella* isolates from Netherlands (van Essen-Zandbergen et al., 2007), Spain (Martinez et al., 2007) and Germany (Miko et al., 2005). These data demonstrated the different combinations of resistance gene cassettes in class 1 integrons. Types of combinations and frequency of the gene cassettes in class

1 integrons may reflect the specific selective pressure to which the isolates were exposed (Chang et al., 2000b).

In this study, *aadA4* was found in a single *S.* Orion isolate. This gene cassette was found in *E. coli* in Taiwan (Chang et al., 2000a) and *Acinetobacter baumannii* in South Africa (Segal et al., 2003). The *aadA2* gene was also detected in class 1 integrons in *Salmonella* in Portuguese (Antunes et al., 2006), Hungary (Nogrady et al., 2005), *E.coli* in Norway (Sunde, 2005) and *V. cholerae* in India (Shi et al., 2006). These data reveal that different genes encoding for resistance to the same antibiotics were identified in class 1 integrons in *Salmonella* from different countries. The reason of widespread of class 1 integrons with different gene cassettes in different countries is still unclear. A previous study suggested that various use of antibiotics in each country may be the cause of selection of different resistance genes (Chang et al., 2000a).

Two new gene cassettes including incomplete *codB* gene and *silB* gene were demonstrated. The incomplete *codB* was detected in *S.* Eppendorf and could not provide any advantage for the *Salmonella* host. The *silB* gene was found in a single *S.* Give isolate. This gene encodes a membrane fusion protein of the SilABC system conferring resistance to silver compounds (Gupta et al., 1999). It is unknown whether *silB* that is not located on the same operon with *silA* and *silC* can complete its function. It may be possible that SilB functions with SilA and SilC located in other genetic elements. Further studies are required to discover the resolution.

All of the class 1 integrons-positive strains were MDR. This is in agreement with the results of previous studies, which have been reported that multi-drug resistance among *Salmonella* serovars is often associated with the presence of class 1 integrons carrying multiple resistance genes (Antunes et al., 2004). The strains with class 1 integrons carrying resistance genes exhibited the resistance phenotype corresponding to the resistance gene identified in the variable regions. Interestingly, no class 1 integrons was detected in some of multiple resistant isolates. Anitibiotic resistance in these strains may be due to other unidentified mechanisms i.e. chromosomal mutation,

the presence of antibiotic resistance genes that are not located on class 1 integrons and the presence of other resistance integrons that were not detected in this study.

The widespread of sulfamethoxazole resistance was also observed in all strains possessing *intI1*. It has been established that class 1 integrons usually contain *sul1* in their 3'CS conserved regions. Sulfonamides have been widely used in food animals. Such specific selection pressure may flavor the acquisition and maintenance of sulfonamide resistance by selective the *sul1* gene. In class 1 integrons-containing isolates most of the *sul1* were located downstream of *qacEΔ1*. It supports that these class 1 integrons are the classical-*sul*1 type. In the present study, class 1 integrons with atypical 3'CS that lacked *qacEΔ1* and *sul1* were additionally observed. Therefore, they were not the *sul1*-associated class 1 integrons. The unusual integrons were previously reported in *Pseudomonas* (Toleman et al., 2007), *Salmonella* (Antunes et al., 2005) and *E. coli* (Antunes et al., 2007). Recently, a *qacH-sul3* was found in *E. coli* (Antunes et al., 2007) and *Salmonella* (Chuanchuen et al., 2008). However, unusual 3'CS linked to f *qacH-sul3* was not examined in our isolates.

The conjugation experiments showed that *Salmonella* isolates could transfer class 1 integrons with antibiotic resistance genes to the *E. coli* recipient. Class 1 integrons-mediated plasmid has been reported in many studies (Gaind et al., 2006; Rodriguez et al., 2006). In our study, only the resistance gene arrays of *dfrA12-aadA2* and *dfrA1-orfC* carrying isolates were located on transferable-plasmid. These data support that class 1 integrons could play an important role in horizontal transfer of antimicrobial resistance among bacteria.

SGI1 and its variants are important determinants of multidrug resistance since they carry various resistance gene clusters. SGI1 was most frequently found in *S.* Typhimurium DT 104 isolates but it can be also found in other *Salmonella* serovars (Levings et al., 2005). In this study, SGI1 were found in serovars Albany, Emek, Kingston and Kedougou respectively. This indicated that the exchange of SGI1 among serovar does exist. The integration of class 1 integrons into SGI1 on chromosome contributes to

the persistence of phenotypic resistance of *Salmonella* even in the absence of selective antibiotic pressure (Doublet et al., 2005). Extrachromosomal circular form of SGI1 was reported in previous studies (Boyd et al., 2008; Doublet et al., 2005). These circular intermediate could be conjugally transferred in the presence of a helper plasmid providing the mating apparatus and may integrate into the recipient chromosome by a site specific recombination. Such mobility of SGI1-extrachromosomal circular form by conjugal transfer potentially contributes to the spread of antibiotic resistance genes between different *S. enterica* serovars and other bacteria (Doublet et al., 2005). Eventhough extrachromosomal circular form of SGI1 was not present in this study, it can not be ruled out the important role of the extrachromosomal circular form of SGI1 in all circumstance.

Conclusion and suggestion

In this study, our findings demonstrated the widespread occurrence of multiple antibiotics resistance among *Salmonella* isolated from poultry and swine in Thailand. Class 1 integrons carrying gene cassettes that confer to different classes of antibiotics i.e. aminoglycosides, ß-lactams and trimethoprim are prevalent among the MDR *Salmonella* isolates. Class 1 integrons carrying on conjugative plasmids may contribute to the horizontal dissemination of antibiotic resistance gene cassettes among the pathogens. Integration of class 1 integrons into the chromosome in several *Salmonella* serovars provide the stable multidrug resistance even in the absence of antibiotic selection pressure. In conclusion, data support that both horizontal gene transfer and clonal spread contribute to dissemination of antibiotic resistance in *Salmonella*. Therefore, guidelines for appropriate and prudent antibiotic usage in both humans and animals are mandatory.

From the results of this study, the suggestion for further studies could be as follows:

1. Some of multiple resistant isolates that did not contain class 1 integrons were found, indicating the presence of non class 1 integrons-mediating resistance. These addition resistance mechanisms could be chromosomal mutation, multidrug efflux systems, antibiotic resistance genes that are not located on class 1 integrons and the presence of other integrons i.e. class 2 and class 3 integrons. Such resistance mechanisms worth further investigations since the data will contribute to better understanding of the evolution of multidrug resistance in *Salmonella*. This will benefit the strategic program to reduce antimicrobial resistance eventually.

2. Class 1 integrons and their gene cassettes can exchange between intra- and interspecies and play important role in the dissemination of antibiotic resistance among bacteria. Therefore, studies of class 1 integrons in other bacteria particularly foodborne

pathogens should be performed. This will lead to more understanding of the effective route of resistance dissemination among bacteria.

3. Data on genetics of antibiotic resistance of *S. enterica* along the food chain are needed to create the project to reduce antibiotic resistance. However, such data is still inadequate especially in developing countries. Therefore, the studies of genetics of antibiotic resistances in *S. enterica* isolated from other sources e.g. humans, foods from animals and environment are recommended.

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APPENDICES

APPENDIX A

Distribution of the MIC values of *S. enterica*

		NO. of isolates (%)												
Antibiotic	Concentration (µg/ml)	<0.125	0.125	0.25	0.5	1	2	4	8	16	32	64	128	256
AMP	0, 0.5 - 256					1 (0.67)	41 (27.33)	23 (15.33)	8 (5.33)	8 (5.33)	1 (0.67)		7 (4.67)	61 (40.67)
CIP	0, 0.125 - 256	29 (19.33)	8 (5.33)	33 (22.00)	18 (12.00)	45 (30.00)	17 (11.33)							
CHP	0, 0.5 - 256							9 (6.00)	63 (42.00)	38 (25.33)	3 (2.00)	6 (4.00)	19 (12.67)	12 (8.00)
GEN	0, 0.125 - 256			6 (4.00)	52 (34.67)	59 (39.33)	14 (9.33)	1 (0.67)	3 (2.00)	10 (6.67)	3 (2.00)	2 (1.33)		
TET	0, 0.5-256				1 (0.67)	20 (13.33)	27 (18.00)	13 (8.67)	4 (2.67)	1 (0.67)	7 (4.67)	19 (12.67)	26 (17.33)	32 (21.33)
TRI	0, 0.5-257		3 (2.00)	40 (26.67)	14 (9.33)	30 (20.00)	1 (0.67)	2 (1.33)	14 (9.33)	4 (2.67)	5 (3.33)	3 (2.00)		34 (22.67)

Antibiotic	Concentration (µg/ml)	0.5	1	2	4	8	16	32	64	128	256	512	1024	2048
SPC	0, 0.5-1024				1 (0.67)		3 (2.00)	42 (28.00)	24 (16.00)	5 (3.33)	5 (3.33)	16 (10.67)	54 (36.00)	
STR	0, 0.5-1024			2 (1.33)	13 (8.67)	12 (8.00)	35 (23.33)	24 (16.00)	12 (8.00)	20 (13.33)	7 (4.67)	5 (3.33)	20 (13.33)	
SUL	0, 0.5-2048								35 (23.33)	14 (9.33)	3 (2.00)	4 (2.67)	14 (9.33)	80 (53.33)

APPENDIX B

Bacterial Growth Media

1. Muller Hinton Agar (MHA) (Difco™, MD, USA)

- Beef Extract Powder	2.0	g
- Acid Digest of Casien	17.5	g
- Starch	1.5	g
- Agar	17.0	g

2. Luria-Bertani Agar (LB) (Difco™, MD, USA)

- Trptone	10.0	g
- Yeast Extract	5.0	g
- Sodium chloride	5.0	g
- Agar	15.0	g

3. COLINSTANT CHROMOGENIC AGAR (Scharlau, Barcelona, Spain)

- Tryptone	10.00	g
- Yeast Extract	3.00	g
- Meat Extract	5.00	g
- Bile Salts	1.50	g
- Di-sodium phosphate	2.70	g
- Sodium phosphate	2.20	g
- Chromogenic mixture	0.40	g
- Agar	13.00	g

APPENDIX C

Preparation of antibiotics and other reagents

Reagents for PCR reaction

1. The Eppendorf MasterMix (2.5x) (Eppendorf®, Hamberg, Germany) contains

- Taq DNA Polymerase	62.5	U/ml
- KCl	125	mM
- Tris-HCl pH 8.3	75	mM
- $Mg(OAc)_2$	3.75	mM
- Igepal® -CA630	0.25	%
- each dNTP	500	µM

Reagents for agarose gel electrophoresis

1. 10 mg/ml Ethidium bromide

- Ethidium bromide	1	g
- Distilled deionized water	1,000	ml

2. 50X TAE (Tris-Acetate buffer) 1000 ml contains

- Tris	242.0	g
- Acetic acid	57.1	g
- 0.5M EDTA pH 8.0	100.0	ml
- Adjust the volume to 1,000 ml with DDW		

3. 0.5 M EDTA, pH 8.0 1000 ml contains

- Disodium ethylene diamine tetraacetate. $2H_2O$	186.1	g
- Distilled deionized water	800.0	ml

- Adjust pH to 8.0
- Adjust Volume to 1,000 ml

4. 1 M Tris HCl, pH 8.0 1000 ml contains

- Tris (ultrapure)	121.1	g
- Distilled deionized water	800.0	ml
- Adjust pH to 8.0 by adding conc. HCL	42.0	ml

Antibiotics Preparation

Antibiotic	Solvents	Stock Solution Storage
Ampicillin	Water	100 µg/ml
Chloramphenicol	Ethanol 95%	25 µg/ml
Ciprofloxacin	a minimum volume of 0.1 M NaOH to dissolve, then make up to total volume with water	10 µg/ml
Gentamicin	Water	50 µg/ml
Rifampicin	Methanol	25 µg/ml
Spectinomycin	Water	100 µg/ml
Streptomycin	Water	100 µg/ml
Sulfamethoxazole	a minimum volume of 0.1 M NaOH to dissolve, then make up to total volume with water	25 µg/ml
Tetracycline	70% Ethanol	100 µg/ml
Trimethoprim	dimethylacetamide	10 µg/ml

BIOGRAPHY

Miss. Sirintip Khemtong was born on September 5, 1982 in Bangkok, Thailand. She graduated from the Faculty of Veterinary Medicine, Chulalongkorn University, Thailand in 2006. After that, she enrolled the degree of Master of Science in the Department of Veterinary Public Health, Faculty of Veterinary Science, Chulalongkorn University since 2006.