ลักษณะทางอณูชีววิทยาของเชื้อซาลโมเนลลา เอนเทอริกาที่ดื้อยาหลายชนิดพร้อมกัน
ที่แยกได้จากมนุษย์และเนื้อสุกร

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MOLECULAR CHARACTERISTICS OF MULTIDRUG-RESISTANT *SALMONELLA ENTERICA* ISOLATED FROM HUMANS AND PORK

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A Dissertation Submitted in Partial Fulfillment of the Requirements
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เวชสิริ วรรณประสาท : ลักษณะทางอณูชีววิทยาของเชื้อซาลโมเนลลา เอนเทอริกาที่ดื้อยาหลายชนิดพร้อมกันที่แยกได้จากมนุษย์และเนื้อสุกร. (MOLECULAR CHARACTERISTICS OF MULTIDRUG RESISTANT *SALMONELLA ENTERICA* ISOLATED FROM HUMANS AND PORK) อ.ที่ปรึกษาวิทยานิพนธ์หลัก : รศ.สพ.ญ.ดร.รุ่งทิพย์ ชวนชื่น , อ.ที่ปรึกษาวิทยานิพนธ์ร่วม : รศ.น.สพ.ดร.อลงกร อมรศิลป์, 114 หน้า.

ศึกษาในซาลโมเนลลา เอนเทอริกาจำนวน 183 เชื้อ ซึ่งเป็นเชื้อที่แยกจากจากมนุษย์จำนวน 52 เชื้อและจากเนื้อสุกรจำนวน 131 เชื้อ โดยศึกษาความไวต่อยาปฏิชีวนะ การปรากฏของ class 1, 2 และ 3 integrons ยีนดื้อยา และยีนที่ก่อให้เกิดความรุนแรง ทำการศึกษา เชื้อที่ดื้อต่อยา ซิโปรฟลอกซาซินจำนวน 24 เชื้อ โดยตรวจการกลายพันธุ์ของยีน *gyrA gyrB parC* และ *parE* และการแสดงออกของ AcrAB-TolC efflux pump ศึกษาความสัมพันธ์ของเชื้อด้วยวิธี Multilocus sequence typing (MLST) ในเชื้อจำนวน 40 เชื้อ พบว่าเชื้อส่วนใหญ่ดื้อต่อยาเตตร้าซัยคลิน (73%) และแอมพิซิลิน (53%) ไม่มีเชื้อตัวใดดื้อต่อยาเซฟไตรอะโซน การศึกษา integrons พบการปรากฏของยีน *intI1* ร้อยละ 39 ซึ่งมี gene cassette ร้อยละ 15 โดยแบบที่พบคือ *dfrA12-aadA2*, bla_{PSE-1} และ *aadA2* ซึ่งแบบ *dfrA12-aadA2* สามารถถ่ายทอดได้ ไม่ตรวจพบการปรากฏของยีน *intI2* และ *intI3* ตรวจพบ SGI1-G และ SGI1-F ในเชื้อ *S.* Albany และ *S.* Kedougou ตามลำดับ พบยีนดื้อยาจำนวน 18 ชนิด ซึ่งการปรากฏของยีนดื้อยาเป็นไปในทิศทางเดียวกับรูปแ บบการดื้อยา ทุกเชื้อพบการปรากฏของยีน *invA msgA spiA* และ *tolC* ส่วนยีน *spvC* และ *pefA* ซึ่งอยู่บนพลาดมิสนั้นปรากฏในเชื้อจำนวนจำกัด เชื้อส่วนใหญ่เกิดการเปลี่ยนแปลงของ กรดอะมิโน Met-48-Ile ของ GyrA และ Thr-82- Met ของ ParC ระดับการแสดงออกของ ยีน *acrB* คือ 1-430 เท่าโดยการแสดงออกนี้ไม่สัมพันธ์กับค่า MIC ของยาซิโปรฟลอกซาซิน ผลของ MLST แสดงให้เห็นว่า เชื้อจากเนื้อสุกรและมนุษย์ที่ทำการทดสอบส่วนใหญ่ที่มีซีโรวาร์เดียวกันถูกจัดให้อยู่กลุ่มเดียวกัน แสดงให้เห็นว่าเนื้อสุกรเป็นแหล่งกักเก็บเชื้อ ซาลโมเนลลาในมนุษย์

ภาควิชา สัตวแพทยสาธารณสุข ลายมือชื่อนิสิต .....

สาขาวิชา สัตวแพทยสาธารณสุข ลายมือชื่อ อที่ปรึกษาวิทยานิพนธ์หลัก .....

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WECHSIRI WANNAPRASAT: MOLECULAR CHARACTERISTICS OF MULTIDRUG RESISTANT *SALMONELLA ENTERICA* ISOLATED FROM HUMANS AND PORK.

ADVISOR: ASSOC. PROF. RUNGTIP CHUANCHUEN, D.V.M., Ph.D.,

CO-ADVISOR: ASSOC. PROF. ALONGKORN AMONSIN, D.V.M., Ph.D., 114 pp.

Total of 183 *Salmonella enterica* isolates from humans (*n*=52) and pork (*n*=131) were included. All isolates were detected for antimicrobial susceptibilities, the presence of class 1, 2 and 3 integrons, antimicrobial resistance genes and virulence genes. The ciprofloxacin-resistant isolates (n=24) were tested for the presence of mutations in *gyrA*, *gyrB*, *parC* and *parE* and AcrAB-TolC expression. Genetic relatedness of the selected strains (n=40) was examined by multilocus sequence typing (MLST). Most isolates were resistant to tetracycline (73%), followed by ampicillin (53%). None were resistant to ceftriaxone. Thirty-nine percent were positive to *intI1*, of which 15% carried gene cassettes i.e. *dfrA12-aadA2*, bla_{PSE-1} and *aadA2*. Two *Salmonella* isolates carrying class 1 integrons with *dfrA12-aadA2* could horizontally transfer their integrons. None were positive for *int2* and *int3*. SGI1-G and SGI1-F were detected in an Albany and a Kedougou, respectively. Eighteen resistance genes were found in the *Salmonella* isolates with corresponding resistance phenotype. All isolates carried the virulence genes *invA*, *msgA*, *spiA* and *tolC*. The *spvC* and *pefA* gene encoded by *Salmonella* virulence plasmids were present at limited rate. The amino acid substitutions Met-48-Ile and Thr-82-Met were most common in GyrA and ParC, repectively. The *acrB* expression level varied from 1 to 430 folds and was not associated with ciprofloxacin MICs. Based on the MLST results, most of the pork and human isolates with the same serovars were grouped into the same clusters, providing evidence that pork could serve as a reservoir for *Salmonella* in humans.

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

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

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LIST OF ABBREVIATIONS

AMP	ampicillin
bp	base pair(s)
^{o}C	degree(s) Celcius
CEF	ceftriazone
CFU	colony-forming unit
CHP	choramphenicol
CIP	ciprofloxacin
DNA	deoxyribonucleic acid(s)
dNTP	deoxyribonucleoside triphosphate(s)
E.	*Escherichia*
e.g.	exampla gratia, for example
ERY	Erythromycin
GEN	gentamycin
h	hour(s)
i.e.	id est, that is
kb	kilobase(s) or 1000 bp
mg	milligram(s)
MIC	minimal inhibitory concentrations
min	minute(s)
ml	milliliter(s)
mPCR	multiplex polymerase chain reaction
NSS	normal saline solution
PCR	polymerase chain reaction
^{R}	resistance/resistant
rpm	round per minutes

S.	*Salmonella*
sec	seconds
SPC	spectinomycin
STR	streptomycin
SUL	sulfamethoxazole
TET	tetracycline
TRI	trimethoprim
µg	microgram(s)
µl	microliter

CHAPTER I

INTRODUCTION

Food borne diseases are an important public health problem worldwide. The diseases are predominantly associated with ingestion of food contaminated with pathogenic microorganisms. The Centers of Disease Control and Prevention (CDC) estimated that there are up to 48 million cases of food borne diseases each year in the United States, of which 128,000 cases are hospitalized and 3,000 cases are fatal (Foley et al., 2008). In Thailand, the Ministry of Public Health estimated that there are nearly 1 million cases of food borne diseases every year during the period of 2001–2005. In 2006, incidence of food borne diseases was 1,245,022 cases and 9 deaths. The highest incidence occurred in northern and north-eastern region of Thailand including Chiang Mai, Chiang Rai, Khon Kaen and RoiEt provinces (Chaikaew et al., 2009). Of all the foodborne pathogens, bacteria are the major cause of foodborne diseases. The most common bacterial foodborne pathogens that are widespread globally are *Campylobacter* spp., *Escherchia coli*, *Listeria monocytogenes* and *Salmonella enterica* (Scallan et al., 2011).

Salmonella is one of the leading causes of foodborne diseases in many parts of the world, including Thailand. They have been isolated from a variety of food, for example, beef, milk, poultry and pork. Up to 2,500 serovars have been identified so far, of which *S.* Enteritidis is the most common *Salmonella* serotype isolated from human cases in Europe (85%), Asia (38%), and Latin America (31%) followed by S. Typhimurium and S. Newport (Galanis et al., 2006). Most *Salmonella* outbreaks in the United States are associated with

ingestion of contaminated food of animal origins, particularly pork, poultry, beef, eggs and milk (Freitas et al., 2010). European union scientific committee concluded that animal products posing the risk to consumers include beef, poultry, meat, eggs, unpasteurised milk and pork (Forshell and Wierup, 2006). In Thailand, *Salmonella* has been isolated from various food of animal origins e.g. milk, chicken and pork (Padungtod and Kaneene, 2006).

Salmonella serovars are widely distributed in swine production system. They were isolated from tonsils (15%), carcass surface (8%), liver and diaphragm (17%) of swine (Bianchi et al., 2007). As the pathogens may transfer to the food chain and transmit to humans Pork and pork products have been considered a major reservoir. The *Salmonella* strains were isolated from fresh (4.4%), refrigerated (4.4%) and processed pork (4.4%) (Bianchi et al., 2007). It has been shown that *Salmonella* infections in humans are commonly associated with consumption of pork (Thai et al., 2012).

Incidence of salmonellosis outbreaks associated with contamination of pork and pork products has globally reported (Wong et al., 2011). In Vietnam, a high prevalence of *Salmonella* in raw food (78%) was reported, of which up to 64% of pork samples were contaminated with the pathogens. The serovars associated with human salmonellosis in this country included Typhimurium, Paratyphi B biovar java, Anatum, Panama, Rissen and Lexington (Van et al., 2007). In Germany, 20% of salmonellosis in humans were caused by consumption of contaminated pork (Steinbach and Hartung, 1999). In Denmark, pork products were estimated to be responsible for 15% of human salmonellosis cases (Korsak et al., 2003). In Thailand, the *Salmonella* strains are the second most common foodborne pathogens causing diarrhea (Vindigni et al., 2007). Among all the *Salmonella* serovars

identified in the country, *S.* Rissen and *S.* Weltevreden are most commonly found in pork and patients (Padungtod et al., 2008).

Salmonellosis may lead to a significant loss due to its fairly-high morbidity and high medical cost (Rabsch et al., 2001). It was estimated that there are 1.3 billion annual cases of salmonellosis and 3 million deaths worldwide (Galanis et al., 2006). In the United States, there are approximately 1.4 million cases of salmonellosis each year. Among these, 16,000 cases were hospitalized and 600 cases were dead (Zhao et al., 2008). In the European Union, number of salmonellosis has increased to 200,000 cases per year (Olsen et al., 2001).

Symptoms of salmonellosis vary from a self-limiting gastroenteritis to septicemia. People who become ill with salmonellosis get diarrhea, fever, abdominal cramp, headache and vomiting within 12 hours to 3 days after infected Most patients could recover within 4-7 days without antibiotic treatment. However, symptoms could be severe in some group of patients such as elderly, infants, individuals with chronic diseases and immunocompromised patients. In this case, antibiotic treatment may be required.

Since their discovery in the 1940s, antimicrobial agents have been widely used in both veterinary and human medicine (Romani et al., 2008). In food-animal production, antimicrobial agents have been used for three main purposes including disease treatment, infection prophylaxis and growth promotion (McEwen and Fedorka-Cray, 2002). Such extensive use of antimicrobial agents provides selective pressure for antimicrobial resistant

(AMR) bacteria and their resistance determinants, resulting in spread wide distribution of AMR bacteria that could be transmitted to humans the through food chain.

As seen in other food animals, AMR *Salmonella* are commonly found in pigs. The situation has become more complicated since these *Salmonella* are usually resistant to multiple drugs (so called multidrug resistant or MDR). Several studied in different countries have shown that slaughted pigs are the major sources of MDR *Salmonella* strains (Padungtod et al., 2008). A previous study reported high prevalence of tetracycline-resistance in most *Salmonella* serovars (60%) isolated from pigs (Olivera et al., 2002). In the Czech Republic, *S.* Typhimurium, *S.* Derby, *S.* Infantis, *S.* Enteritidis, *S.* Agona, *S.* Schwarzengrund and *S.* Oritamerin that were multidrug resistant were commonly isolated (Sisak et al., 2006). *S.* Agona isolated from patients in Brazil carried class 1 integrons with the gene cassette array *catA1* encoding chloramphenicol resistance and $bla_{\text{TEM-1}}$ for ampicillin resistance (Michael et al., 2005). In Thailand, *S. enterica* isolates from pigs (35%) and healthy adults (42%) were resistant to multiple drugs (Padungtod and Kaneene, 2006). All the isolates from pork and diarrheal patients were resistant to streptomycin, sulfamethoxazole and tetracycline (Angkititrakul et al., 2005). These data may be evident that MDR *Salmonella* from food animals (particularly, pigs) could enter food chain and subsequently infect humans. Infections with MDR *Salmonella* could result in, prolonged hospitalization treatment failure and increased economical losses.

Up to date, several mechanisms of antimicrobial resistance have been characterized in *Salmonella* (Alekshun and Levy, 2007). Among the characterized resistance mechanisms, it is well known that resistance to multiple drugs in *Salmonella* is

mainly attributed to the presence of integrons and overexpression of active efflux systems (Huang et al., 2004). Of all nine integrons types, class 1 integrons is the most common integron type among Gram negative bacteria including *Salmonella* serovars. These genetic elements carry many resistance gene cassettes in their variable regions that could be coselected by only a single antibiotic (Fluit and Schmitz, 2004). Class 1 integrons could be located on conjugative plasmid and play an importance role in horizontal transfer of resistance determinants (Lee et al., 2002). They can integrate into *Salmonella* chromosome, particularly into *Salmonella* genomic island1 (SGI1). Recently, SGI1 variants have been identified in several *S. enterica* and classified as SGI1-A to –G based on the presence of resistance gene clusters (Boyd et al., 2001). As a result, *Salmonella* could maintain their multidrug resistance phenotype even in the absence of antibiotic selective pressure.

In addition to integrons, active effluxes have been shown to be involved in multidrug resistance phenotype of *Salmonella* (Paulsen et al., 2001). Multidrug efflux pumps can extrude drugs of different classes, resulting in the decreased intracellular concentration of substrates that is insufficient to harm the cells and the increased resistance to multiple drugs. Substrates of multidrug efflux pumps are from different classes and not structurally related, therefore; a single antibiotic use may promote cross-resistance to other antibiotics via this mechanism. In *Salmonella,* AcrAB-TolC is the most well known multidrug efflux system. The system has wide-substrate specificity and effectively promotes MDR phenotype. It was previously shown that the mutant *Salmonella* strains overproducing AcrAB-TolC were more resistant to fusidic acid, chloramphenicol, tetracycline, norfloxacin, and penicillin G than their isogenic parents (Giraud et al., 2000).

The pathogenicity of *S. enterica* is associated with various virulence factors that facilitate the organisms in expressing their virulence in the host cells. Some virulence genes are chromosomally encoded, while orthers are associated with transferable plasmids. It is based on the fact that antimicrobial resistance determinants are commonly located on plasmid and cointegration of virulence plasmids and resistance plasmids resulting in newly virulence-resitance plasmids has been previously demonstrated (Colonna et al., 1988). In this case, a single antibiotic use can generate the coselection of both types of determinants leading to the emergence of more resistant and virulent *Salmonella* and widespread of the newly-hybrid virulence-resistance plasmids (Villa and Carattoli, 2005).

Distribution of antimicrobial resistance among bacteria could occur via clonal spread and horizontal transmission (Warnes et al.). Data regarding the presence of resistance determinants and their horizontal transfer may partly explain dynamics of resistance. Therefore, information on the genetic relatedness is required to better describe the relationship of antimicrobial resistant-bacterial pathogens along the food chain. Many typing techniques have been developed and used to trace MDR *Salmonella* strains and investigate the genetic relatedness between *Salmonella* isolated from different sources (Morshed and Peighambari, 2010). Among these, multilocus sequence typing (MLST) has been shown to be a rapid-standardized method for sub-typing isolates of *S. enterica* (Ben-Darif et al., 2010).

Genetic characterization of the MDR *Salmonella* isolates from pork and humans is necessary to explain the distribution and transmission of the pathogens along food chain. Such data will facilitate the development of the strategic plan to prevent and reduce the

spread of the MDR *Salmonella*. While genetic information of multidrug resistance mechanisms, distribution of virulence factors and genetic relatedness in *S. enterica* isolated from pork and humans have been extensively reported in many countries (Galanis et al., 2006) such information in Thailand is still limited. Therefore, the purposes of this study were to characterize mechanisms of multidrug resistance and investigate the genetic association of *S. enterica* isolated from pork and humans in Thailand. In this study, we focused on i) characterization of mechanisms underlying multidrug resistance, particularly class 1 integrons and their transferability, the presence of non-class 1 integrons borne resistance genes and the expression of the AcrAB-TolC efflux system. ii) investigation of the genetic relatedness and iii) examination of dissemination and association of virulence genes and antibiotic resistance genes in *S. enterica* isolated from pork and humans.

CHAPTER II

LITERATURE REVIEW

1. General characteristics of *S. enterica*

Salmonellae are Gram-negative rod with 0.7-1.5 μm width and 2-5 μm length. They are non-spore forming, noncapsulated, facultatively anaerobic bacteria, which classified in the family *Enterobacteriaceae*. The facultative anaerobes can grow under conditions with or without oxygen and survive in various environments. These organisms are able to reduce nitrates to nitrites and produce gas from glucose that can be used for biochemical identification. *Salmonellae* are mesophile and prefer 37°C as the optimum growth temperature. This microorganism is able to survive as long as seven years under freezing conditions (from -23°C to -18°C).

The genus *Salmonella* consists of two species including *S. enterica* and *S. bongori*. Six subspecies are differentiated within *S. enterica* based on their biochemical and genomic characteristics, these six subspecies are as follows: I, *S. enterica* subsp. *enterica*; II, *S. enterica* subsp. *salamae* ; IIIa,*S. enterica* subsp. *arizonae* ; IIIb, *S. enterica* subsp. *diarizonae*; IV, *S. enteric* subsp. *houtenae*, and VI, *S. enterica* subsp. *Indica* (Yamamoto, 2003). Based on host infection, *Salmonella* is classified into 3 groups; the first group includes serotypes that found to infect only humans such as *S.* Typhi. Host-adaped serotypes are classified into second group that found in one host species but can cause diseases in other species. Example of this group is *S.* Pullorum. The third group includes

Salmonella Enteritidis, *Salmonella* Typhimurium and *Salmonella* Heidelberg. These serotypes are associated with infections in humans (Pui et al., 2011).

Six serogroups are classified by the somatic O antigen agglutination including serogroup A, B, C1, C2, D and E. The *Salmonella* serovars are identified by the Kauffman-White classification that is based on the antigenic determinants of flagellar H antigens, somatic O antigens and virulence (Vi) capsular K antigens. Currently, 2,610 serovars have been identified, of which 1,478 serovars within subspecies enterica are major causes of salmonellosis in warm blooded animals and humans. Most cases of human salmonellosis are associated with *S.* Enteritidis infection. They are carried in the intestinal tract of animals and humans. They are generally transmitted to humans through food chain. Humans can get *Salmonellae* via consumption of contaminated food of animal origin such as meat, eggs and milk. Pork is one of important food vehicles of *Salmonella* infection in humans (Freitas et al., 2010).

2. Pathogenesis of *S. enterica*

Salmonella infections are important cause of clinical disease in various animals and major cause of foodborne disease in humans. Many non-typhoidal serotypes have been isolated from various primary hosts such as swine, cattle, poultry, birds and pets. These serotypes also found in humans that are usually final host. In swine, weaners and growers are the most common infection without symptom. The bacteria may be shed in the feces of infected pigs or released during slaughter process. *Salmonella* in contaminated products may enter the food chain and transmit to humans.

Salmonella infection is known as a zoonotic disease that transmits from animal to human. Human infection usually occurs when consuming contaminated foods. Salmonellosis can affect anyone. However, it is more common in children and young adults. The most common symptoms are fever, abdominal pain, diarrhoea, nausea and vomiting. These symptoms usually appear 12–72 hours after infection. *Salmonella* infections can cause severe problems depend on the immune status of the host and virulence factors such as *Salmonella* pathogenicity islands (SPIs) and plasmids (Zou et al., 2010). Most patients could recover within 4-7 days without antibiotic treatment. After infection, this organism multiplies in the intestine and causes enteritis. However, the symtomps will deveop into bacteremia in children, elderly people and the immunocompromised patients. In this case, antimicrobials are essential drugs for treatment (Kim, 2010).

Salmonellosis is one of the most common human foodborne diseases. It is a major public health problem and represents a significant cost in many countries. The WHO has reported that about 17 million annual cases of gastroenteritis due to salmonellosis, of which 3 million people dead. In the United States, *S. enterica* is the most common cause of food-borne gastroenteritis, however in some states Campylobacter is more prevalent than *Salmonella* (Oliver et al., 2005). The CDC has estimated that over 1 million salmonellosis cases occur every year in the United States. Of these cases, about 20,000 and 378 result in hospitalization and death, respectively (Foley et al., 2008). According to Van Pelt et al., 2003, 68% of the foodborne diseases caused by *Salmonella* infection was reported in Europe between 1993 and 1998. In Thailand, *Salmonella* is the second foodborne pathogen causing diarrhea (Vindigni et al., 2007). The most common *Salmonella* serovars causing human salmonellosis in Thailand between 1993 to 2002 was *S.* Weltevreden

(Bangtrakulnonth et al., 2004). These data confirm the importance of *Salmonella* as a source of foodborne disease.

3. Antimicrobial resistance in *S. enterica*

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 the Czech Republic, *S.* Typhimurium, *S.* Derby, *S.* Infantis, *S.* Enteritidis, *S.* Agona, *S.* Schwarzengrund and *S.* Oritamerin isolated from pigs were multidrug resistant (Sisak et al., 2006). Fifty three percent *Salmonella* isolated from food animal products such as chicken, beef and pork in USA showed resistance to at least three antibiotics (White et al., 2001). In Italy, *S.* Typhimurium isolated from raw pork sausages were resistant to ampicillin, streptomycin, sulfonamide and tetracycline (Ranucci et al., 2004). In Thailand, *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%, respectively (Padungtod and Kaneene, 2006). All isolates of *Salmonella* obtained from pork, chicken meat and diarrheal patients were resistant to streptomycin, sulfamethoxazole and tetracycline (Angkititrakul et al., 2005).

Antibiotic resistance in *Salmonella* from humans has been increasing as well. Multidrug-resistant serovar Typhimurium was first reported in the United Kingdom in 1984. Since then, it has been isolated with increasing frequency in other European countries, the United States, Japan and Taiwan (Chiu et al., 2007). In the United States, incidence of multidrug-resistant *S.* Typhimurium has increased from 0.6% in 1979-1980 to 34% in 1996.

In Cuba, several studies showed that *Salmonella* was resistant to several antibiotics such as ampicillin, chloramphenicol, nalidixic acid, trimethoprim and sulfamethoxazole (Cabrera et al., 2006). In Thailand, *S.* Enteritidis isolates from patients were resistant to 6-8 antibiotics and the resistance rates to ceftriaxone, amikacin, and kanamycin in 1994 were significantly higher than those in 1993 (Boonmar et al., 1998). Forty percent of *Salmonella* isolated from children were resistant to trimethoprim and sulfamethoxazole (Hoge et al., 1998). All of non-typhoidal *Salmonlla* isolates from patients in Chulalongkorn hospital were resistant to nalidixic acid (Kulwichit et al., 2007).

4. Genetics of antimicrobial resistance in *S. enterica*

4.1 Integrons in *S. enterica*

Integrons are the mobile genetic element which contain an integrase gene and variable region that contains antimicrobial resistance gene cassettes (Fluit and Schmitz, 2004). They are an important mechanism in the distribution of antimicrobial resistance genes among gram-negative bacteria including *S. enterica*. Nine classes of integrons have been identified based on integrase genes. More than sixty different resiatance gene cassettes have been characterized within integrons (Lee et al., 2002).

Class 1 integrons are the predominant integrons in Gram-negative bacteria and have been reported in *Salmonella*. The structure of class 1 integrons consists of two conserved segments (CS), 5´CS and 3´CS separated by a variable region (Figure 1). The 5´CS region contains the *intI1* gene encoding an integrase enzyme. The *attI* site is responsible for integration of gene cassettes that encode for the resistance phenotypes in

bacteria. The 3´CS contained of *qacEΔ1* encoding resistance to quaternary ammonium compounds, *sul1* encoding resistance to sulphonamides, followed by open reading frame 5 (ORF5) of unknown function. The variable region contains one or more gene cassettes. These gene cassettes have a *attI* that is the target site for cassette integration (Chang et al., 2007). Several different gene cassettes may be present in an integron, which leads to multiresistance. In addition, class 1 integrons can be found on plasmids that transfer resistance gene cassettes more effectively.

Class 1 Integrons were widely found in several *Salmonella* serovars. In Taiwan, *S.* Schwarzengrund (84.49%) isolates from chickens and pigs were positive class 1 integrons with 3 gene cassette patterns including *aadA2, bla*$_{PSE1}$ and *dfraA1* (Wang et al., 2010). *Salmonella* isolates (46%) from swine in Illinois were found class 1 integrons, that associated with resistance to streptomycin, sulfisoxazole, tetracycline, spectinomycin and 91% of these integrons had multidrug resistance patterns (Rao et al., 2008). In Norway, class 1 integrons were found about 22.22% in *S.* Enteritidis isolates and up to 97 % in *S.* Typhimurium isolates from patients (Lindstedt et al., 2003). In China, class 1 integrons (17.39%) were found in *Salmonella* isolates from healthy humans (Zhang et al., 2004).

Class 2 integrons consists of *intI2* gene and gene cassettes in variable region. This interon type is associated with the presence of Tn*7*. Class 2 integrons has been found in limited *Salmonella* serovars such as serovar Panama, Grumpensis, Worthington and Enteritidis (Rodriguez et al., 2006). Most class 2 integrons contain *dfrA1-sat2-aadA1* cassette that common in *Salmonella* Java and *Salmonella* Typhimurium. In addition, previous study reported that class 2 integrons conataining *dfrA1-sat2-aadA1* gene cassette

was found in *E. coli* strains isolated from broilers. The result indicated horizontal gene transfer between *E. coli* and *Salmonella* (van Essen-Zandbergen et al., 2007).

Class 3 integrons have been reported in *Pseudomonas aeruginosa*, *Serratia marcescens*, *Alcaligenes xylosoxidans*, *Pseudomonas putida* and *Klebsiella pneumonia* (Correia et al., 2003). The structure of class 3 integrons is similar to class 2 integrons (Fluit and Schmitz, 2004).

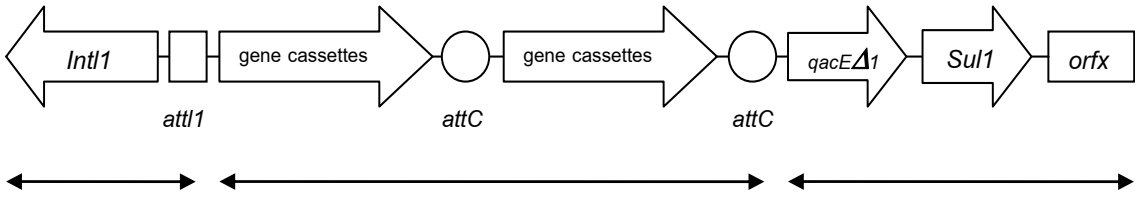

Figure 1: The structure of class 1 integrons

4.2 *Salmonella* Genomic Island 1 (SGI1)

In *Salmonella*, class 1 integrons can integrate into *Salmonella* chromosome that is known as the *Salmonella* genomic island1 (SGI1). SGI 1 has been detected in many *Salmonella* serovars. In S.Typhimirium, the 43 kb SGI1 is located between the *thdf* and *int2* genes that encodes for a thiophene and furan-oxidation protein, respectively. The *int2* gene is not present in other *Salmonella* serovars. Therefor, SGI1 is located between the *thdf* and *yidY* genes in other seovars. (Figure 2) (Boyd et al., 2001). SGI1 consists of two class 1 integrons, each one followed by antimicrobial resistance gene cassettes conferring multidrug resistance phenotype. A cluster of antimicrobial resistance genes is located in the

3' end of SGI1. The typical SGI1 contains first cassette carries the *aadA2* gene, which confers resistance to streptomycin and spectinomycin and a 3'CS with a *sul1* segment. The second cassette contains the bla_{pse-1}, which confers resistance to ampicillin and a 3'CS region with a complete *sul1* gene encoding sulfonamides resistance. FloR, tetR, tetG gene and orfs are present between the two integrons. The *floR* gene confers cross-resistance to chloramphenicol and florfenicol, *tetR* and *tet*G encoding tetracycline resistance (Doublet et al., 2004).

Variations in the structure of SGI1 were generated by chromosomal recombinationation. The variants generated by insertion of new gene cassettes or deletion of one or more gene cassettes. To date, SGI1 variants are classified as SGI1-A to SGI1-O (Levings et al., 2005). The classification depends on a sequence of antimicrobial resistance gene cluster in SGI1. SGI1 and its variants have been found in various serovars with diverse sources. They were found in *Salmonella* isolates from food animal such as *S.* Agona isolated from poultry in Belgium, *S.* Paratyphi B from fish in Singapore and *S.* Albany from fish in Thailand (Doublet et al., 2003). Moreover, they were also found in *S.* Newport isolates from a human in the United States (Boyd et al., 2002).

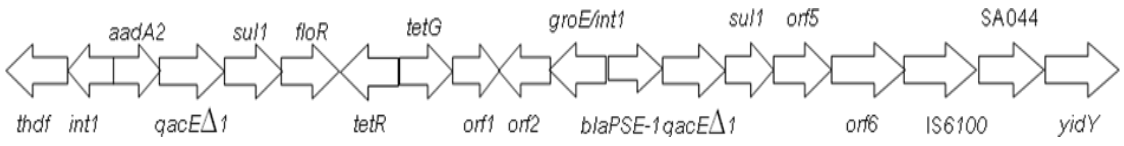

Figure 2: The structure of SGI1

4.3 Drug efflux pump systems in *S. enterica*

Drug efflux pump has been known to play an important role in the antimicrobial resistance in many species of gram-negative bacteria including *Salmonella*. Multidrug efflux systems are currently classified into 5 families including the major facilitator superfamily (MFS), the ATP-binding cassette (ABC) family, the resistance-nodulation-division (RND) family, the small multidrug resistance (SMR) family, and the multidrug and toxic compound extrusion (MATE) family. Overexpression of the multidrug efflux pumps is associated with decreased susceptibility to several classes of antibiotics (Paulsen et al., 2001).

Of the nine drug efflux systems of *Salmonella*, AcrAB-TolC has wide substrate specificity and effective in creating MDR. The AcrAB efflux system is a resistance-nodulation-cell-division (RND) type. This pump is composed of transporter AcrB, a periplasmic accessory protein AcrA and outer membrane protein TolC (Figure 3) (Nikaido et al., 2008). It transports a wide variety of substrates from the cell into the external environment, results in a low concentration of substrates inside the cell. In *Salmonella*, the major mechanism of resistance to quinolones is AcrAB efflux. Previous studie showed that overproduces of AcrAB efflux pump in *S.* Typhimurium is associated with decreased susceptibility to several compounds such as chloramphenicol, tetracycline, norfloxacin, penicillin G and fusidic acid (Giraud et al., 2000).

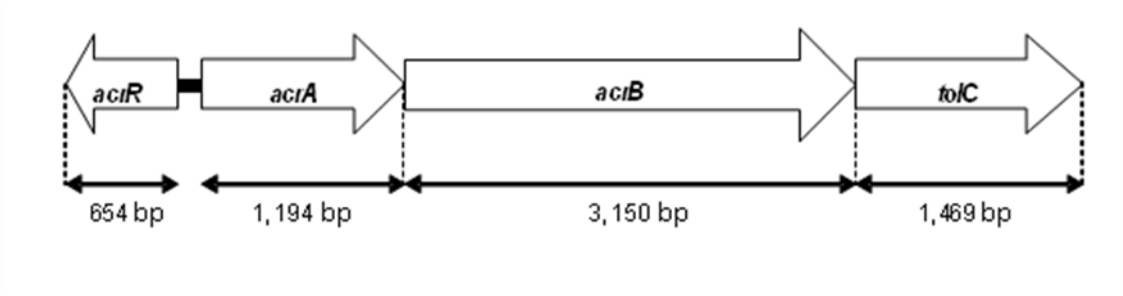

Figure 3: The structure of the AcrAB-TolC multidrug efflux system. The system consists of 3 mcjor components including AcrB, a RND reansporter; AcrA, a periplasmic membrane fusin protein and TolC, an outer membrane protein. AcrB located upstream is a negative regutator.

4.4 Fluoroquinolones resistance in *S. enterica*

Fluoroquinolones are the antimicrobial agents recommended for the treatment of gastroenteritis in humans. *Salmonella* strains with fluoroquinolone-resistance phenotype have rapidly developed and treatment failures have been reported in cases of resistant infection. The emergence of high-level fluoroquinolones resistance has been reported in *S. enterica* serotypes Choleraesuis, Schwarzengrund and Typhimurium (Chiu et al., 2002).

Fluoroquinolones inhibit DNA gyrase and the topoisomerase IV enzyme, which are essential for bacterial DNA synthesis. The DNA gyrase enzyme consists of 2 subunits, GyrA and GyrB encoded by the *gyrA* and *gyrB* genes, respectively. Topoisomerase IV enzyme consists of 2 subunits, which are encoded by the *parC* and *parE* genes, respectively. In *Salmonella*, fluoroquinolone-resistant strains associated with mutation(s) in the quinolone

resistance-determining regions (QRDRs) and overexpression of the AcrAB efflux system (Cloeckaert and Chaslus-Dancla, 2001). Previous studies of fluoroquinolone-resistant strains in *S. enterica* have shown that fluoroquinolone resistance associated with mutation in *gyrA*, *gyrB*, *parC* and *parE* genes. High-level fluoroquinolones resistance in *S.* Schwarzengrund strains were associated with mutations in gyrase and the topoisomerase IV enzyme (Baucheron et al., 2005). In Hong Kong, fluoroquinolone resistance isolates from patients showed mutations in QRDRs regions (Ling et al., 2003). In Taiwan, ciprofloxacin-resistant *S.* Choleraesuis isolates from humans and *S.* Typhimurium isolates from pigs were associated with mutations in the QRDR of the *gyrA* and *parC* genes (Hsueh et al., 2004).

5. Virulence genes in *S. enterica*

The virulence factors are required for colonization, invasion and multiplication of *Salmonella* inside the host (Clements et al., 2001). The pathogenicity of *S. enterica* is associated with the presence of virulence genes. In *Salmonella*, the virulence genes can be located on plasmid and/or on chromosome. *Salmonella* pathogenicity islands (SPI) that harboring several virulence genes have been identified on the chromosome. Several virulence genes have been detected in *Salmonella* for example; *invA, prgH, sifA, sitC, sipB, spaN, spiA* and *tolC* gene. Some genes are known to be involved in host recognition and invasion such as *pef*A, *inv*A, *orgA*, *sopB*, *tolC* and *prgH*. While other genes including *spaN*, *spv*C, *spiA*, *pagC* and *msgA* genes are associated with survival in the host system.

Salmonella plasmid virulence (*spv*) operon has been identified on virulence plasmid. The operon consists of a set of spvABCD. This operon plays a role in multiplication of the

organism inside the reticuloendothelial cells. The *spv* genes have been found on large virulence plasmids in non-typhoid *Salmonella* serovars (Guiney et al., 1995). Plasmid encoding fimbrial (*pef*) gene is one of the most common virulence gene, which located on *Salmonella* virulence plasmid. The presence of *pef* gene plays a role in adhesion and invasion of organism inside the intestine epithelial cells. The *pefA* and *spvC* genes have limited distribution in *Salmonella*. While most *Salmonella* strains appear to contain the *invA* gene, which located on chromosome. All *S.* Typhimurium isolates from beef and human were positive for *invA* gene (Abouzeed et al., 2000). Eighty percent of *Salmonella* isolates from pigs were found *pef* gene (Murugkar et al., 2003). The *spvC* has been found on the *Salmonella* virulence plasmid pSLT (Hauser et al., 2010). The presence of both resistance and virulence genes in bacteria is resulting in more virulent and resistant serovars. In this case, antibiotic use can generate the coselection of both types of determinants leading to the emergence of more antibiotic-resistant and virulence *Salmonella* (Villa and Carattoli, 2005).

CHAPTER III

MATERIALS AND METHODS

The experiment was divided into 3 phases, including Phase 1, Characterization of antimicrobial resistance; Phase 2, Investigation of the genetic relatedness of *S. enterica* strains; and Phase 3, Determination the presence of virulence genes (Figure 4).

Salmonella enterica isolates

A total of 183 *S. enterica* isolates were included in this study. They were isolated from humans (*n*=52) and pork (*n*=131) in northern Thailand during 2005-2007 and stored as stocks in the strain collection of Faculty of Veterinary Medicine, Chiangmai University, Chiangmai. All of the pork isolates were originated from raw pork in retail markets, and all of the human strains were isolated from patients' stools at Suandok hospital of the faculty of Medicine of Chiang Mai University. All the strains were isolated using the standard methods described in International Organization for Standardization (ISO 6579:2002) and tested for their biochemical characteristics (Barrow and Feltham., 1993). The *Salmonella* isolates were sent to the Department of Veterinary Public Health, Faculty of Veterinary Science, Chulalongkorn University. *Salmonella* serovars were identified by using slide agglutination based on the Kauffman-White scheme at the Center of Antimicrobial Resistance Monitoring in Foodborne Pathogen, Faculty of Veterinary Science, Chulalongkorn University. All isolates were stored in 20% glycerol at -80°C.

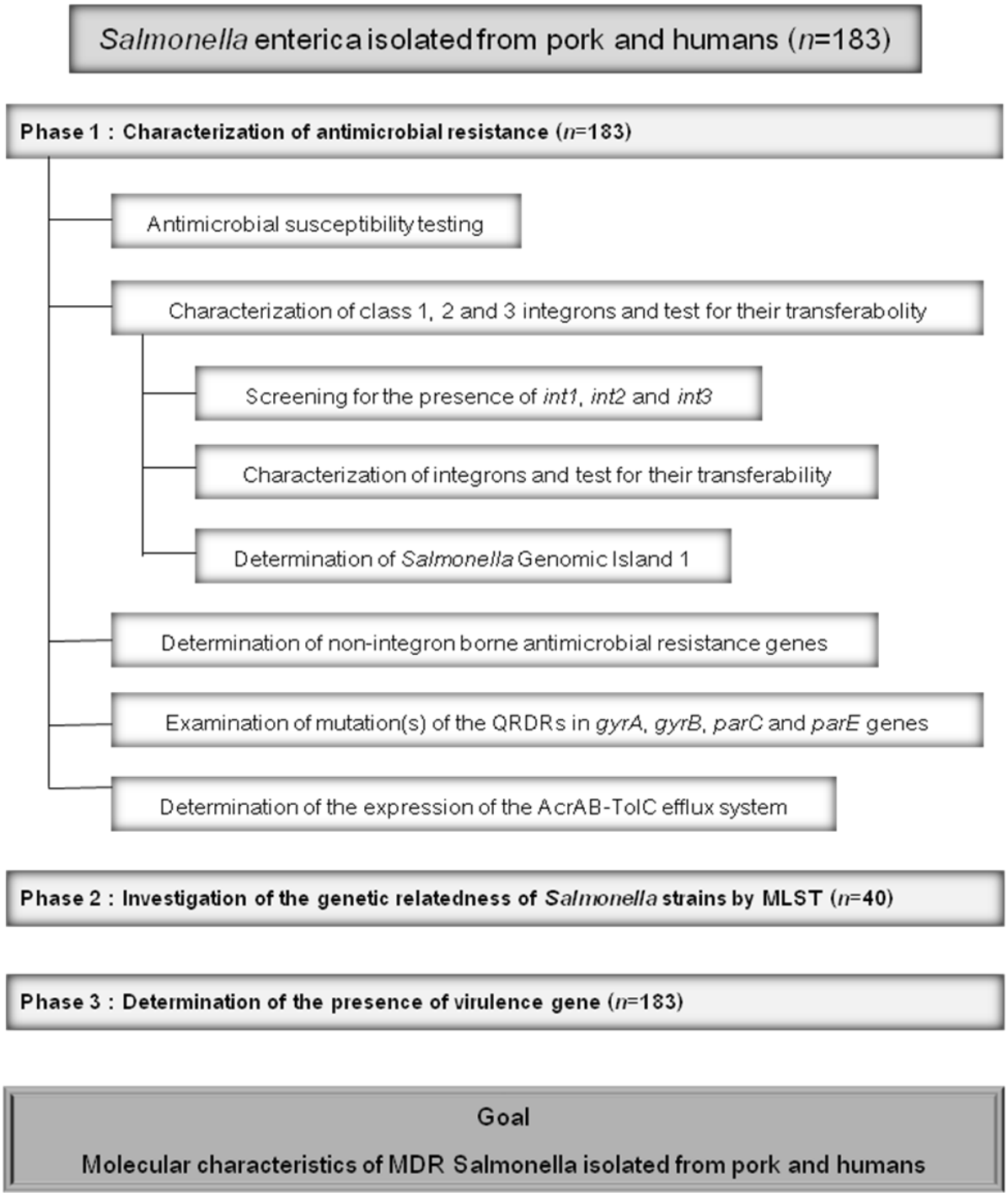

Figure 4: The flow chart of experiments

Phase 1 Characterization of antimicrobial resistance

1.1 Antimicrobial susceptibility test

Antimicrobial susceptibility tests were performed to determine the Minimum Inhibitory Concentrations (MICs) of different classes of antimicrobial agents that are used in food animals and humans including ampicillin (AMP), ceftriaxone (CEF), choramphenicol (CHP), ciprofloxacin (CIP), gentamicin (GEN), spectinomycin (SPC), streptomycin (STR), sulfamethoxazole (SUL), tetracycline (TET) and trimethoprim (TRI). All antimicrobial agents were purchased from Sigma-Aldrich® (Steinheim, Germany). Antimicrobial agents were prepared in suitable solvents at various concentrations (Table 1).

Two-fold agar dilution technique was performed as described by Clinical and Laboratory Standards Institute (CLSI, 2006). The *Salmonella* isolates were grown in Mueller-Hinton agar (MHA, Difco®, MD, USA) at 37°C overnight. Single colonies were picked and transfered to 0.85% NaCl solution (NSS). The turbidity of cell suspension was adjusted to 0.5 McFarland (~10^8 CFU/ml). Then, the suspension was ten-fold diluted to 10^7 CFU/ml in NSS and inoculated onto MHA plates that contained appropriate concentrations of antimicrobial agents using multipoint inoculators (~10^4 CFU/spot). After incubation at 37 °C for 18-24 hr, the MIC value defined as the lowest concentration of an antimicrobial agent that completely inhibits visible growth of bacteria was recorded. The breakpoints used for defining the isolates as susceptible or resistant are according to CLSI (CLSI, 2006). *Pseudomonas aeruginosa* ATCC 27853, *E. coli* ATCC 25922 and *Staphylococcus aureus* ATCC 29212 were used as quality control organisms.

Table 1: Solvents, concentrations and breakpoints of antimicrobial agents used in this study

Antimicrobials	Solvent	Concentrations range (µg/ml)	Breakpoint (µg/ml)
ampicillin	sterile distilled water	0.5-256	32
choramphenicol	95% ethanol	0.5-256	32
ciprofloxacin	0.1M NaOH and sterile distilled water	0.125- 256	4
gentamicin	sterile distilled water	0.125-256	8
spectinomycin	sterile distilled water	0.5-1024	128
streptomycin	sterile distilled water	0.5-1024	32
sulfamethoxazole	0.1M NaOH and sterile distilled water	0.5-1024	512
tetracycline	70% ethanol	0.5-256	16
trimethoprim	dimethyl acetamide	0.5-256	16

1.2 Characterization of class 1, 2 and 3 integrons and test for their transferability

1.2.1 Characterization of class 1, 2 and 3 integrons

For PCR amplification, DNA template was prepared by the whole cell boiled lysate procedure (Levesque et al., 1995). The *Salmonella* strains were grown on Luria-Bertani agar (LB, Difco®, MD, USA) at 37°C overnight. Single colonies was picked and suspended in 50 µl of sterile distilled water and heated in a boiling water bath for 10 min. Then, the cell suspension was centrifuged at 12,000xg for 5 min. The supernatant was removed into a fresh 1.5 ml microcentrifuge tube and stored at -20 °C. All PCR primers in this study are listed in Table2.

All of the *Salmonella* isolates were screened for the presence of the integrase genes, *intI1*, *int2* and *int3* using PCR with specific primer pairs Int1F and In1R, Int2F and In2R and Int3F and In3R, respectively as previously described (Chuanchuen et al., 2007). The PCR reaction consisted of 12.5 µl of 2X Reddymix™ PCR MasterMix (0.625 units Taq polymerse, 1.5 nM $MgCl_2$ and 0.2 mM each of dNTP/reaction), 1 µl of each primer (10 µM), 5 µl of DNA template and 5.5 µl of sterile-distilled water. PCR thermocycling conditions were an initial denaturation at 94°C for 5 min, and 30 cycles of denaturation for 45 sec at 94°C, primer annealing for 45 sec at 50°C and extension for 1 min at 72°C and one cycle of final extension at 72°C for 5 min. PCR amplification were conducted on a PCR Tpersonal combi model (Biometra®, Gottingen, Germany). PCR amplicons were separated on 1% agarose gel electrophoresis (Bioexpress, Kaysville, UT, Spain) in 1xTris-acetate/EDTA (TAE) buffer.

The gels were strained in ethidium bromide solution (Sigma-Aldrich®) and visualized using the Bio-Rad Gel-Documentation System (Bio-Rad Laboratories, Ventura, Ca, USA).

All the strains containing the *int*1 gene were detected for the presence of gene cassettes in variable regions using PCR with a specific primer pair 5'CS and 3'CS (Levesque et al., 1995). The PCR reaction consisted of 12.5 µl of 2X Reddymix™ PCR MasterMix, 1 µl of each primer (10 µM), 5 µl of DNA template and 5.5 µl of sterile-distilled water. PCR thermal cycling conditions consisted of an initial denaturation at 95°C for 5 min, and 30 cycles of denaturation for 45 sec at 95°C, primer annealing for 54 sec at 54°C and extension for 3 min at 72°C and a final extension at 72°C for 5 min. The PCR product were purified using Nucleospin Gel Extension kit (Nucleospin®, Gutenberg, France) and submitted for DNA sequencing at 1st base Ptl Ltd, Selangor, Malaysia). DNA sequences were compared with the published sequence using NCBI blast search available at the National Center for Biotechnology Information website (www.ncbi.nlm.nih.gov). The PCR products with the same size were digested with a restriction enzyme including *Eco*RI, *Alu*I and *Taq*I. The PCR amplicons with the same restriction patterns were considered identical.

1.2.2 Localization of integrons and test for their transferability

Test for integron transfer was performed using biparental mating technique as previously described (Chen et al., 2004). All the *Salmonella* strains carrying class 1 integrons with the resistance gene cassettes were used as donors and *E. coli* MG1655 rifampicin-resistant derivatives (MG1655 rif^R) was used as recipients (Chuanchuen et al., 2008). *E. coli* MG1655 rif^R is susceptible to all antimicrobials tested and does not contain

class 1 integrons and plasmid. The donors and recipients were inoculated into 4 ml of LB broth and 4 ml of LB broth containing rifampicin (32 µg/ml), respectively at 37ºC overnight. Eighty-µl of each overnight culture of donors and recipients were transferred into 4 ml of fresh LB broth and grown to log phase at 37ºC for 4 hours in shaking incubator. Then, 700 µl of each donor and recipient were mixed in 1.5 ml microcentrifuge tube and centrifuged at 8,000 rpm for 1 min. The supernatant was removed and the pellet was resuspended in 30 µl of LB broth. The cell suspension was dropped onto a 0.45 µm membrane filter (Millipore, Massachusetts, USA) placed on LB agar plate without antibiotic. After incubated at 37ºC overnight, the filter was transferred into 1 ml of NSS and the attached cells were removed by vortexing. The suspension was centrifuged at 12,000xg for 1 min and the supernatant was discarded. A hundred-µl of LB broth was added into the cell pellets. The cell mixture was spread on LB agar with rifampicin (32 µg/ml) and one of the following antibiotics: ampicillin (100 µg/ml), streptomycin (50 µg/ml), trimethoprim (10 µg/ml), tetracycline (16 µg/ml), chloramphenicol (32 µg/ml) and incubated at 37ºC overnight. The single colonies of transconjugants were streaked on Eosin Methylene Blue agar (EMB, Difco®, MD, USA) with corresponding antibiotics and incubated at 37ºC overnight. The *E. coli* transconjugants showed metallic sheen appearance on EMB agar. Plasmid DNA was extracted from transconjugants using alkaline lysis method (Liou et al., 1999) and investigated for the presence of corresponding integrons with the corresponding-resistance gene cassettes as described above.

For determination of SGI-1and its variants, only the class 1 integrons positive strains (*n*=18) were screened for the presence of SGI1 and its variants using PCR with specific primer pairs as previously describe(Khemtong and Chuanchuen, 2008). The isolates were

detected for the presence of left and right junction of SGI1 by PCR with primer set thdF1 and yidY. Then, the order of the antibiotic resistance gene clusters was characterized based on antimicrobial resistance pattern and integron profile using PCR with specific primer pairs. The PCR reaction consisted of 12.5 µl of 2X Reddymix™ PCR MasterMix, 1 µl of each primer (10 µM), 5 µl of DNA template and 5.5 µl of sterile-distilled water. PCR thermocycling conditions were an initial denaturation at 94°C for 5 min, and 30 cycles of denaturation for 45 sec at 94°C, primer annealing for 45 sec at 55°C and extension for 1 minute at 72°C and one cycle of final extension at 72°C for 5 min. The PCR amplicons were visualized on 1.2% agarose gel, purified and submitted for DNA sequencing.

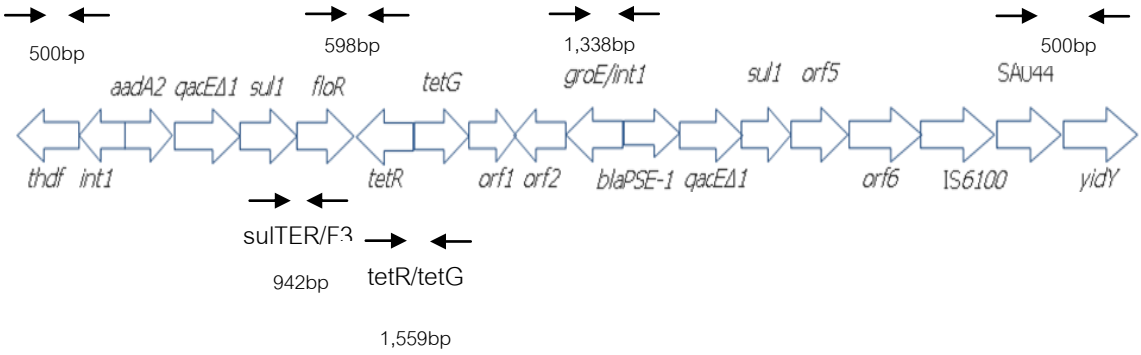

Figure 5: Localization of primers used for characterization of SGI1 and its variants. The arrows indicate the direction of primers. The number (bp) below indicate the size of PCR amplifications. The map is not drawn to scale.

1.3 Determination of non-integron borne antimicrobial resistance genes

All the *Salmonella* isolates were detected for the presence of 18 antimicrobial resistance genes corresponding to their resistance phenotypes (Table 2) by multiplex

Polymerase Chain Reaction (mPCR) with specific primers as previously described (Chuanchuen et al., 2008). The PCR reaction consisted of 12.5 µl of 2X Reddymix™ PCR MasterMix, 1 µl of each primer (10 µM), 5 µl of DNA template and 5.5 µl of sterile-distilled water. PCR thermocycling conditions were an initial denaturation at 94°C for 5 min, and 30 cycles of denaturation for 45 sec at 94°C, primer annealing for 45 sec at 54°C and extension for 1 min at 72°C and one cycle of final extension at 72°C for 5 min. PCR products were visualized on 1.2% agarose gel. The PCR products were purified and submitted for sequencing. DNA sequences were compared with the published sequence using NCBI blast search available at the NCBI website.

Table 2: Antimicrobial resistance phenotypes and their corresponding resistance encoding genes tested

Resistance phenotype	Resistance genes
Ampicillin	bla_{PSE1}, bla_{TEM}
Chloramphenicol	*catA*, *catB*, *cmlA*
Gentamicin	*aadA*1, *aadA*2, *aadB*
Streptomycin	*strA*, *strB*
Sulphamethoxazole	*sul1*, *sul2*, *sul3*
Tetracycline	*tetA*, *tetB*
Trimethoprim	*dfrA1*, *dfrA10*, *dfrA12*

1.4 Examination of mutation(s) of the QRDRs in *gyrA, gyrB, parC* and *parE* genes

The *Salmonella* isolates resistant to ciprofloxacin (*n*= 18) were tested for the presence of mutation(s) in Quinolone Resistance Determining Regions (QRDRs) of *gyrA*, *gyrB*, *parC* and *parE* using PCR with specific primers as previously described (Chuanchuen et al., 2010). Amplification of *gyrA* was performed using a primer set gyrAsalF and gryAsalR. The *gyrB* gene was amplified using primer pair gyrBF and gyrBR. For *parC* and *parE* genes, the QRDRs were amplified using primer pairs parCF/parCR and parEF/parER, respectively. The PCR reaction consisted of 12.5 µl of 2X Reddymix™ PCR MasterMix, 1 µl of each primer (10 µM), 5 µl of DNA template and 5.5 µl of sterile-distilled water. PCR thermocycling conditions were an initial denaturation at 94°C for 5 min, and 30 cycles of denaturation for 45 sec at 94°C, primer annealing for 45 sec at 55°C and extension for 1 min at 72°C and one cycle of final extension at 72°C for 5 min. PCR products were visualized on 1.2% agarose gel. All PCR products were purified and submitted for DNA sequencing. Both stands of DNA sequence were compared with the published DNA sequence in GenBank (GenBank accession numbers AE008801, AE008878 and AE008846 for *gyrA*, gyrB, *parC* and *parE*, respectively) and analysed by Edit seq and Seqman (DNA-STAR) program.

1.5 Determination of the expression of the AcrAB-TolC efflux system

The *Salmonella* strains with different ciprofloxacin MICs (*n*=24) were randomly selected for determination the expression of the AcrAB-TolC efflux system. Total RNA was extracted using QIAGEN RNeasy mini kit (Qiagen® Hilden, Germany) as described by the manufacturer. The isolates were grown in LB broth and incubated at 37°C overnight. RNA

was treated with DNaseI (Fermentas®, mainz, Germany) according to the manufacturer's protocol.

For RT-PCR reaction, 1 µg of DNaseI treated RNA sample was used to prepare cDNA by ImProm-II™ Reverse Transcriptase (Promega, Madison, USA) as described by the manufacturer. The cDNA synthesis reactions were performed in a final volume 20 µl containing 1 µl of 10 pmol/µl acrBR primer (Nishino et al., 2006) and 0.5 µg of RNA. The mixture was incubated at 70°C for 5 min and on ice for 5 min. Then, 4 µl of ImProm-II™ 5X Buffer, 2 µl of 25mM $MgCl_2$, 1 µl of 10 mM dNTP mix, 7 µl of DEPC-treated water and 1 µl of of ImProm-II™ Reverse Transcriptase were added. The cDNA synthesis thermocycles were as follows: 25°C for 5 min, 45°C for 45 min and inactivation of reaction at 25°C for 15 min. The cDNA was stored at -20 °C and used as cDNA template for real-time qRT-PCR.

Transcription level of *acrB* gene was determined by quantitative realtime PCR using acrBF and acrBR primers (Nishino et al., 2006). The housekeeping gene, *rrs* was used as internal control. The transcription level of *rrs* was determined by quantitative realtime PCR using rrsF and rrsR primers. The real-time qRT-PCR was performed using Biotools QuantiMix EASY SYG Kit (Biotools B&M Labs S.A., Madrid, Spain) according to the manufacture's instructions. The cDNA templates were 1:100 diluted in sterile-distilled water. The reactions were performed in a final volume of 25 µl consisting of 10 µl of QUANTISYG 2X, 8 µl of sterile-distilled water, 1 µl of each primer and 5 µl of cDNA template. The PCR amplification was carried out in triplicate in a Rotor-Gene™ 3000 Real Time Thermal Cycler (Corbett Research, Sydney, Australia). The PCR thermocycles were an initial denaturation at 95°C for 5 min, followed by 30 cycles of 95°C for 45 sec, 50°C for 45 sec and 72°C for 1

min. A final extension at 72°C for 10 min. The Ct value was used to calculate the average *acrB* copy number. The copy number of each sample was normalized using the *rrs* copy number. The *acrB*-expression level of these strains tested was compared with that of *S.* Typhimurium strain 13311.

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Phase 2 Investigation of the genetic relatedness of the *Salmonella* strains by Multilocus sequence typing (MLST)

Salmonella serovars Typhimurium, Weltrevreden and Kedougou (*n*=40) with different antimicrobial resistance patterns were selected for determination of their genetic relationship by MLST based on *manB, mdh* and *fimA* genes with specific primer pairs manBF/ manBR, mdhF/ mdhR and fimAF/ fimAR as previously described (Kotetishvili et al., 2002). The PCR reactions were performed in a final volume of 25 µl consisting of 12.5 µl of 2X Reddymix™ PCR MasterMix, 1 µl of each primer, 5 µl of DNA template and 5.5 µl of sterile-distilled water. PCR thermocycling conditions for *manB* were an initial denaturation at 95°C for 9.5 min, and 40 cycles of denaturation for 45 sec at 95°C, primer annealing for 45 sec at 55°C and extension for 1 min at 72°C and one cycle of final extension at 72°C for 7 min. PCR amplification for *mdh* and *fimA* were performed using the following cycle: an initial denaturation at 95°C for 9.5 min, and 40 cycles of denaturation for 45 sec at 95°C, primer annealing for 45 sec at 58°C and extension for 1 min at 72°C and one cycle of final extension at 72°C for 7 min. The PCR amplicons were visualized on 1.2% agarose gel. All PCR products were purified and submitted for sequencing. The nucleotide sequences were assembled using Segman and aligned by the Clustal W algorithm in MEGA 4.0 (Tamura et al., 2007).

Phase 3 Determination of the presence of virulence genes

All *S. enterica* isolastes were detected for the presence of virulence genes including those associated with recognition and invasion i.e. *pefA*, *invA*, *orgA*, *sopB*, *tolC*, *prgH* and those involved in survival in the host system i.e. *spaN*, *spv*C, *spiA*, *pagC* and *msgA* genes. The virulence genes were detected using PCR and multiplex PCR with specific primers (Table 3) as previously described (Chiu and Ou, 1996; Skyberg et al., 2006). Each PCR reactions were performed in a final volume of 25 µl consisting of 12.5 µl of 2X Reddymix™ PCR MasterMix, 1 µl of each primer, 5 µl of DNA template and 5.5 µl of sterile-distilled water. The amplification cycles were as follow: initial denaturation at 95°C for 5 min, and 25 cycles of denaturation for 30 sec at 94°C, primer annealing for 30 sec at 66.5°C, and DNA extension for 2 min at 72°C and a final extention at 72°C for 10 min (Skyberg et al., 2006). For *spvC* gene, the PCR cycles were an initial denaturation at 94°C for 1 min, and 30 cycles of denaturation for 30 sec at 94°C, primer annealing for 30 sec at 56°C, and DNA extension for 2 min at 72°C and a final extention at 72°C for 10 min (Chiu and Ou, 1996). The PCR amplicons were visualized on 1.2% agarose gel, purified and submitted for sequencing. DNA sequences were compared with the published sequence using NCBI blast search available at the NCBI website.

Table 3: Primers used in this study for determination integrons, antimicrobial resistance genes and virulence genes

Primers	Sequence (5'-3')	PCR type	Size (bp)	Reference
Class 1 integrons				
Int1F	CCTGCACGGTTCGAATG	*int1*	497	Chuanchuen et al., 2007
Int1R	TCGTTTGTTCGCCCAGC	*int1*	497	Chuanchuen et al., 2007
Int2F	GGCAGACAGTTGCAAGACAA	*int2*	247	Chuanchuen et al., 2008
Int2R	AAGCGATTTTCTGCGTGTTT	*int2*	247	Chuanchuen et al., 2008
Int3F	CCGGTTCAGTCTTTCCTCAA	*int3*	155	Chuanchuen et al., 2008
Int3R	GAGGCGTGTACTTGCCTCAT	*int3*	155	Chuanchuen et al., 2008
3'CS	AAGCAGACTTGACCTGA	Variable	Variable	Levesque et al., 1995
5'CS	GGCATCCAAGCAGCAAG	regions	Variable	Levesque et al., 1995
SGI1				
thdf1	ACACCTTGAGCAGGGCAAG	*thdf*	500	Khemtong and Chuanchuen, 2008
thdf2	AGTTCTAAAGGTTCGTAGTCG	*thdf*	500	Khemtong and Chuanchuen, 2008
SO44-1	TGACGAGCTGAAGCGAATTG	*SO44-yidY*	500	Khemtong and Chuanchuen, 2008
SO44-2	AGCAAGTGTGCGTAATTTGG	*SO44-yidY*	500	Khemtong and Chuanchuen, 2008
sulTER	AAGGATTTCCTGACCCTG	*sul1-floR*	942	Khemtong and Chuanchuen, 2008
F3	AAAGGAGCCATCAGCAGCAG	*sul1-floR*	942	Khemtong and Chuanchuen, 2008
F4	TTCCTCACCTTCATCCTACC	*floR-tetR*	942	Khemtong and Chuanchuen, 2008
F6	TTGGAACAGACGGCATGG	*floR-tetR*	942	Khemtong and Chuanchuen, 2008
tetR	GCCGTCCCGATAAGAGAGCA	*tetR*-tetG	1,559	Khemtong and Chuanchuen, 2008
tetG	GAAGTTGCGAATGGTCTGCG	*tetR*-tetG	1,559	Khemtong and Chuanchuen, 2008
groEL	TTCTGGTCTTCGTTGATGCC	*groEL- pse1*	1,338	Khemtong and Chuanchuen, 2008
pse-1	CATCATTTCGCTCTGCCATT	*groEL- pse1*	1,338	Khemtong and Chuanchuen, 2008
Resistance genes				
aadA1F	CTCCGCAGTGGATGGCGG	*aadA1*	631	Chuanchuen et al., 2008
aadA1R	GATCTGCGCGCGAGGCCA	*aadA1*	631	Chuanchuen et al., 2008
aadA2F	CATTGAGCGCCATCTGGAAT	*aadA2*	500	Chuanchuen et al., 2008
aadA2R	ACATTTCGCTCATCGCCGGC	*aadA2*	500	Chuanchuen et al., 2008
aadBF	CTAGCTGCGGCAGATGAGC	*aadB*	300	Chuanchuen et al., 2008
aadBR	CTCAGCCGCCTCTGGGCA	*aadB*	300	Chuanchuen et al., 2008
bla_{PSE}F	GCAAGTAGGGCAGGCAATCA	bla_{PSE}	422	Chuanchuen et al., 2008
bla_{PSE}R	GAGCTAGATAGATGCTCACAA	bla_{PSE}	422	Chuanchuen et al., 2008
bla_{TEM}F	ATCAGTTGGGTGCACGAGTG	bla_{TEM}	608	Chuanchuen et al., 2008
bla_{TEM}R	ACGCTCACCGGCTCCAGA	bla_{TEM}	608	Chuanchuen et al., 2008
catAF	CCAGACCGTTCAGCTGGATA	*catA*	452	Chuanchuen et al., 2008
catAR	CATCAGCACCTTGTCGCCT	*catA*	452	Chuanchuen et al., 2008
catBF	CGGATTCAGCCTGACCACC	*catB*	416	Chuanchuen et al., 2008
catBR	ATACGCGGTCACCTTCCTG	*catB*	416	Chuanchuen et al., 2008

Table 3 (continued):

Primers	Sequence (5'-3')	PCR type	Size (bp)	Reference
cmlAF	TGGACCGCTATCGGACCG	*cmlA*	641	Chuanchuen et al., 2008
cmlAR	CGCAAGACACTTGGGCTGC	*cmlA*	641	Chuanchuen et al., 2008
dfrA1F	CAATGGCTGTTGGTTGGAC	*dfrA1*	254	Chuanchuen et al., 2008
dfrA1R	CCGGCTCGATGTCTATTGT	*dfrA1*	254	Chuanchuen et al., 2008
dfrA10F	TCAAGGCAAATTACCTTGGC	*dfrA10*	432	Chuanchuen et al., 2008
dfrA10R	ATCTATTGGATCACCTACCC	*dfrA10*	432	Chuanchuen et al., 2008
dfrA12F	TTCGCAGACTCACTGAGGG	*dfrA12*	330	Chuanchuen et al., 2008
dfrA12R	CGGTTGAGACAAGCTCGAAT	*dfrA12*	330	Chuanchuen et al., 2008
strAF	TGGCAGGAGGAACAGGAGG	*strA*	405	Chuanchuen et al., 2008
strAR	AGGTCGATCAGACCCGTGC	*strA*	405	Chuanchuen et al., 2008
strBF	GCGGACACCTTTTCCAGCCT	*strB*	621	Chuanchuen et al., 2008
strBR	TCCGCCATCTGTGCAATGCG	*strB*	621	Chuanchuen et al., 2008
sul1F	CGGACGCGAGGCCTGTATC	*sul1*	591	Chuanchuen et al., 2008
sul1R	GGGTGCGGACGTAGTCAGC	*sul1*	591	Chuanchuen et al., 2008
sul2F	GCGCAGGCGCGTAAGCTGAT	*sul2*	514	Chuanchuen et al., 2008
sul2R	CGAAGCGCAGCCGCAATTC	*sul2*	514	Chuanchuen et al., 2008
sul3F	GGGAGCCGCTTCCAGTAAT	*sul3*	500	Chuanchuen et al., 2008
sul3R	GGGTGCGGACGTAGTCAGC	*sul3*	500	Chuanchuen et al., 2008
tetAF	GCTGTCGGATCGTTTCGG	*tetA*	658	Chuanchuen et al., 2008
tetAR	CATTCCGAGCATGAGTGCC	*tetA*	658	Chuanchuen et al., 2008
tetBF	CTGTCGCGGCATCGGTCAT	*tetB*	615	Chuanchuen et al., 2008
tetBR	CAGGTAAAGCGATCCCACC	*tetB*	615	Chuanchuen et al., 2008
AcrAB-TolC				
acrBF	TGAAAAAAATGGACCCGTTCTTC	*acrB*	420	Nishino et al., 2006
acrBR	CGAACGGCGTGGTGTCA	*acrB*	420	Nishino et al., 2006
rrsF	CCAGCAGCCGCGGTAAT	*rrs*	215	Nishino et al., 2006
rrsR	TTTACGCCCAGTAATTCCGATT	*rrs*	215	Nishino et al., 2006
MLST				
manBF	CATAAYCCGATGGACTACAACG	*manB*	800	Kotetishvili et al., 2002
manBR	ACCAGCAGCCACGGGATCAT	*manB*	800	Kotetishvili et al., 2002
mdhF	GATGAAAGTCGCAGTCCTCG	*mdh*	900	Kotetishvili et al., 2002
mdhR	TATCCAGCATAGCGTCCAGC	*mdh*	900	Kotetishvili et al., 2002
fimAF	TCAGGGGAGAAACAGAAAACTAAT	*fimA*	900	Kotetishvili et al., 2002
fimAR	TCCCCGATAGCCTCTTCC	*fimA*	900	Kotetishvili et al., 2002

Table 3 (continued):

Primers	Sequence (5'-3')	PCR type	Size (bp)	Reference
Virulence genes				
invA-1	ACAGTGCTCGTTTACGACCTGAAT	*invA*	243	Chiu and Ou., 1996
invA-2	AGACGACTGGTACTGATCGATAAT	*invA*	243	Chiu and Ou., 1996
spvC-1	ACTCCTTGCACAACCAAATGCGGA	*spvC*	571	Chiu and Ou., 1996
spvC-2	TGTCTTCTGCATTTCGCCACCATCA	*spvC*	571	Chiu and Ou., 1996
pefAF	GCGCCGCTCAGCCGAACCAG	*pefA*	157	Skyberg et al., 2005
pefAR	GCAGCAGAAGCCCAGGAAACAGTG	*pefA*	157	Skyberg et al., 2005
spiAF	CCAGGGGTCGTTAGTGTATTGCGTGAGATG	*spiA*	550	Skyberg et al., 2005
spiAR	CGCGTAACAAAGAACCCGTAGTGATGGATT	*spiA*	550	Skyberg et al., 2005
pagCF	CGCCTTTTCCGTGGGGTATGC	*pagC*	454	Skyberg et al., 2005
pagCR	GAAGCCGTTTATTTTTGTAGAGGAGATGTT	*pagC*	454	Skyberg et al., 2005
msgAF	GCCAGGCGCACGCGAAATCATCC	*msgA*	189	Skyberg et al., 2005
msgAR	GCGACCAGCCACATATCAGCCTCTTCAAAC	*msgA*	189	Skyberg et al., 2005
sipBF	GGACGCCGCCCGGGAAAAACTCTC	*sipB*	875	Skyberg et al., 2005
sipBR	ACACTCCCGTCGCCGCCTTCACAA	*sipB*	875	Skyberg et al., 2005
prgHF	GCCCGAGCAGCCTGAGGAGTTAGAAA	*prgH*	756	Skyberg et al., 2005
prgHR	TGAAATGAGCGCCCCTTGAGCCAGTC	*prgH*	756	Skyberg et al., 2005
spaNF	AAAAGCCGTGGAATCCGTTAGTGAAGT	*span*	504	Skyberg et al., 2005
spaNR	CAGCGCTGGGGATTACCGTTTTG	*span*	504	Skyberg et al., 2005
orgAF	TTTTTGGCAATGCATCAGGGAACA	orgA	255	Skyberg et al., 2005
orgAR	GGCGAAAGCGGGGACGGTATT	orgA	255	Skyberg et al., 2005
tolCF	TACCCAGGCGCAAAAAGAGGCTATC	*tolC*	161	Skyberg et al., 2005
tolCR	CCGCGTTATCCAGGTTGTTGC	*tolC*	161	Skyberg et al., 2005
iroNF	ACTGGCACGGCTCGCTGTCGCTCTAT	*iroN*	1205	Skyberg et al., 2005
iroNR	CGCTTTACCGCCGTTCTGCCACTGC	*iroN*	1205	Skyberg et al., 2005
sitCF	CAGTATATGCTCAACGCGATGTGGGTCTCC	*sitC*	768	Skyberg et al., 2005
sitCR	CGGGGCGAAAATAAAGGCTGTGATGAAC	*sitC*	768	Skyberg et al., 2005
sopBF	CGGACCGGCCAGCAACAAAACAAGAAGAAG	*sopB*	220	Skyberg et al., 2005
sopBR	TAGTGATGCCCGTTATGCGTGAGTGTATT	*sopB*	220	Skyberg et al., 2005
lpfCF	GCCCCGCCTGAAGCCTGTGTTGC	*lpfC*	641	Skyberg et al., 2005
lpfCR	AGGTCGCCGCTGTTTGAGGTTGGATA	*lpfC*	641	Skyberg et al., 2005

CHAPTER IV

RESULTS

1. *Salmonella enterica* isolates

Serovars were examined in all isolates by the Kauffman-White based on slide agglutination. Serovar Anatum was most frequently found (26.2%) followed by serovars Kedougou (14.7%), Stanley (14.2%) and Rissen (13.6%). Serovar Weltevreden, Typhimurium, Enteritidis, Corvallis and Give were identified in 6.5%, 4.3%, 3.8% 2.7% and 2.7% of the isolates, respectively. Two percent of Newport and 1.6% of Panama were found. Two isolates of each were serovars Albany and Bovismorbificans. Serovars Agona, Baiboukoum, Bradford, Coeln, Langensalza, Paratyphi B and Virchow were identified only one isolate of each. The *Salmonella* serovars isolated from human and pork shown in table 4.

Table 4: *Salmonella enterica* serovars isolated from human and pork (*n*=183)

Salmonella serovars	No. of isolates		% of isolates
	Human (*n*=52)	Pork (*n*=131)	
Agona	0	1	0.5
Albany	0	2	1.0
Anatum	2	46	26.2
Baiboukoum	0	1	0.5
Bovismorbificans	0	2	1.0
Bradford	0	1	0.5
Coeln	0	1	0.5
Corvallis	5	0	2.7
Enteritidis	7	0	3.8
Give	0	5	2.7
Kedougou	13	14	14.7
Langensalza	0	1	0.5
Newport	4	0	2.1
Panama	2	1	1.6
Paratyphi B	0	1	0.5
Rissen	0	25	13.6
Singapore	0	2	1.0
Stanley	11	15	14.2
Typhimurium	5	3	4.3
Virchow	0	1	0.5
Weltevreden	3	9	6.5
Total	52	131	

2. Characterization of antimicrobial resistance

2.1 Antimicrobial susceptibility

Eighty-three percent of the isolates were resistant to at least one antimicrobial agent, while 11% of the isolates were susceptible to all antimicrobial agents tested. Most isolates were resistant to tetracycline (73%), followed by ampicillin (53%), spectinomycin (53%), sulfamethoxazole (51%), choramphenicol (48%), trimethoprim (39%), streptomycin (37%), ciprofloxacin (12%) and gentamicin (9%). None of the isolates were resistant to ceftriaxone.

Eighty-six percent of the isolates were resistant to at least 3 different antimicrobial classes and were defined as multidrug-resistant (MDR). Distribution of antimicrobial resistance is shown in Figure 6. Among the *Salmonella* isolates from pork, the highest frequencies of resistance to tetracycline (67%). Resistance rates to sulfamethoxazole, ampicillin, chloramphenicol, spectinomycin, streptomycin and trimethoprim were 55%, 35%, 27%, 35%, 18% and 30%, respectively. All of the human *Salmonella* isolates were resistant to ampicillin, chloramphenicol and spectinomycin. These isolates also exhibited resistance to tetracycline (90%), streptomycin (84%), trimethoprim (63%), sulfamethoxazole (44%), ciprofloxacin (30%), nalidixic acid (27%) and gentamicin (21%) (Figure 7).

Antimicrobial resistance phenotypes of all *Salmonella* isolates from humans were classified into 19 patterns (Table 5). The most common resistance pattern was AMP-CHP-SPC-STR-SUL-TET (15.4%). For the pork isolates, 46 antimicrobial resistance patterns were observed, of which the most common pattern was TET (Table 6).

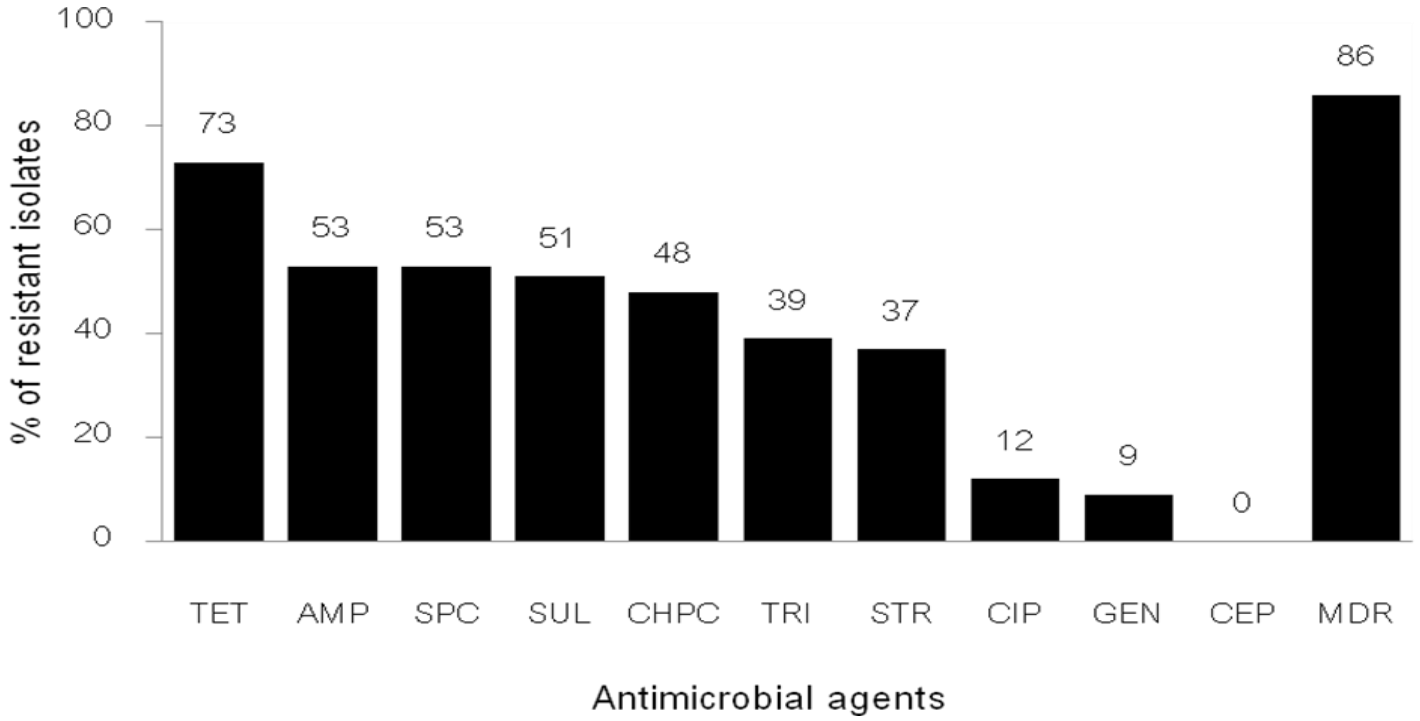

Figure 6: Distribution of antimicrobial resistance among *S. enterica* (*n*=183). AMP, ampicillin; CHPC, chloramphenicol; CIP, ciprofloxacin, GEN, gentamicin; SPC, spectinomycin; STR, streptomycin; SUL, sulfamethoxazole; TET, tetracycline; TRI, trimethoprim; CEF, ceftriazole; MDR, multidrug resistance.

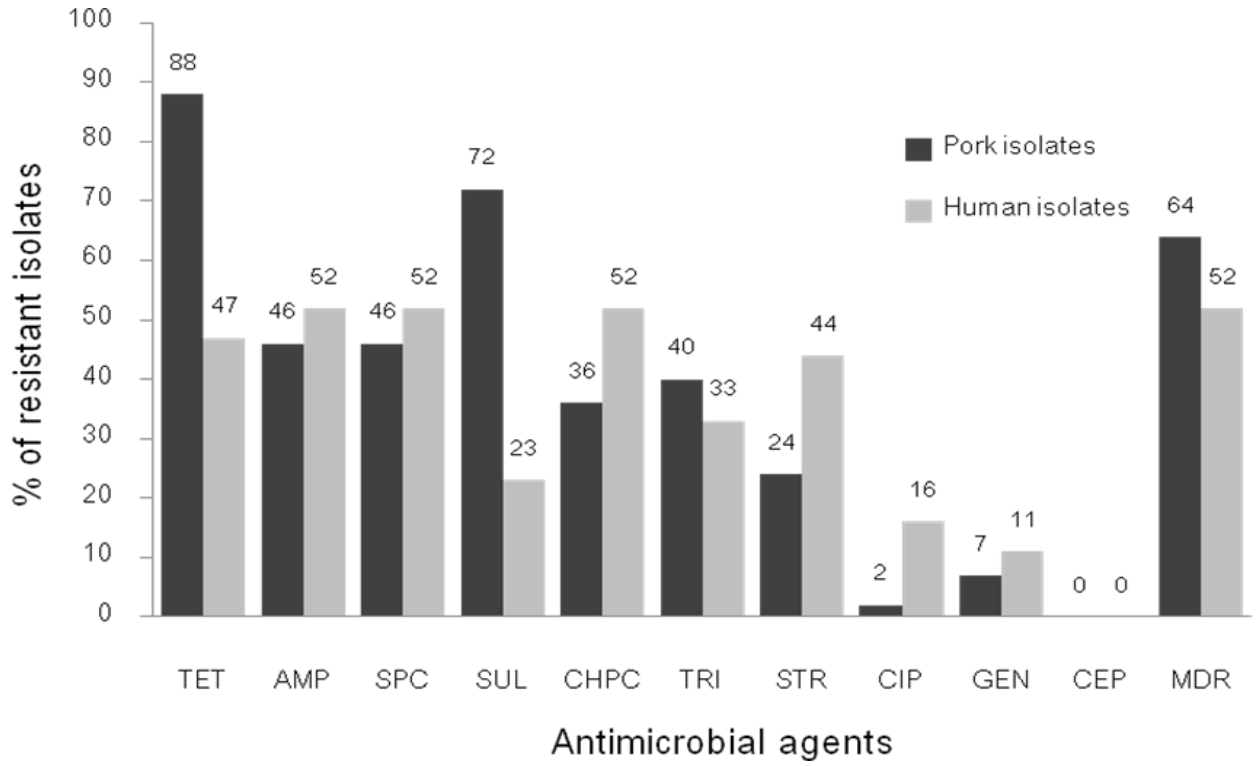

Figure 7: Distribution of antimicrobial resistance among *Salmonella* isolates from pork (*n*=52) and humans (*n*=131).

Table 5: Antimicrobial resistance patterns of *Salmonella* resistant isolates from humans (*n*=52)

Antimicrobial resistance patterns	No. of isolates (%)
AMP-CHP-SPC	1(1.9)
AMP-CHP-CIP-SPC	1(1.9)
AMP-CHP-SPC-SUL	1(1.9)
AMP-CHP-CIP-SPC-TMP	1(1.9)
AMP-CHP-SPC-STR-SUL	1(1.9)
AMP-CHP-SPC-STR-TET	7(13.5)
AMP-CHP-GEN-SPC-TET-TMP	1(1.9)
AMP-CHP- SPC-STR-SUL-TET	8(15.4)
AMP-CHP- SPC-STR-TET-TMP	7(13.5)
AMP-CHP- SPC- SUL-TET-TMP	1(1.9)
AMP-CHP- CIP-SPC-STR- TET-TMP	4(7.6)
AMP-CHP- CIP-SPC- SUL-TET-TMP	1(1.9)
AMP-CHP-GEN- SPC-STR- TET-TMP	4(7.6)
AMP-CHP- SPC-STR- SUL-TET-TMP	4(7.6)
AMP-CHP- CIP- GEN-SPC-STR-TET-TMP	2(3.8)
AMP-CHP- CIP- GEN- SPC- SUL-TET-TMP	1(1.9)
AMP-CHP- CIP- SPC-STR- SUL-TET-TMP	4(7.6)
AMP-CHP- GEN-SPC -STR- SUL-TET-TMP	1(1.9)
AMP-CHP- CIP- GEN-SPC -STR- SUL-TET-TMP	2(3.8)

Table 6: Antimicrobial resistance patterns of *Salmonella* resistant isolates from pork (*n*=110)

Antimicrobial resistance patterns	No. of isolates (%)
SPC	3(2.7)
STR	1(0.9)
SUL	9(8.2)
TET	19(17.3)
TMP	1(0.9)
AMP-SUL	1(0.9)
AMP-TET	1(0.9)
CHP-TMP	1(0.9)
SPC-TET	1(0.9)
STR-TET	3(2.7)
SUL-TET	2(1.8)
SUL-TRI	3(2.7)
TET-TMP	1(0.9)
AMP- SPC- TET	2(1.8)
AMP -STR-TET	4(3.6)
AMP - SUL-TET	2(1.8)
AMP-TET-TMP	1(0.9)
CHP-TET-TMP	1(0.9)
GEN- SUL-TET	1(0.9)
SPC - SUL-TET	1(0.9)
STR - SUL-TET	1(0.9)
AMP-CHP-SUL-TET	2(1.8)
AMP-CHP-SUL-TMP	1(0.9)
AMP-SPC-SUL-TET	1(0.9)

Table 6 (continued):

Antimicrobial resistance patterns	No. of isolates (%)
AMP-SPC-SUL-TMP	1(0.9)
AMP-STR-SUL-TET	3(2.7)
CHP-SPC-SUL-TET	4(3.6)
CHP-SPC-SUL-TMP	1(0.9)
CHP-STR-SUL-TET	2(1.8)
SPC-STR-SUL-TET	1(0.9)
SPC-SUL-TET-TMP	1(0.9)
AMP-CHP-SPC-SUL-TET	2(1.8)
AMP-CHP-STR-SUL-TET	1(0.9)
AMP-CHP- SUL-TET-TMP	1(0.9)
AMP-SPC-SUL-TET-TMP	6(5.4)
AMP-STR-SUL-TET-TMP	1(0.9)
CHP-SPC-SUL-TET-TMP	3(2.7)
CIP- SPC-SUL-TET-TMP	1(0.9)
AMP-CHP-GEN-SPC-SUL-TET	2(1.8)
AMP-CHP- SPC-SUL-TET-TMP	7(6.3)
AMP- SPC-STR-SUL-TET-TMP	1(0.9)
CHP- CIP- SPC-SUL-TET-TMP	1(0.9)
CHP- SPC-STR-SUL-TET-TMP	1(0.9)
AMP-CHP-GEN-SPC-SUL-TET-TMP	1(0.9)
AMP-CHP-SPC-STR-SUL-TET-TMP	3(2.7)
AMP-CHP-GEN-SPC-STR-SUL-TET-TMP	3(2.7)

2.2 Characterization of class 1, 2 and 3 integrons

Of all isolates, 39.8 % of the *Salmonella* isolates were positive to *intI1* (Twenty-nine percent and eighty-six percent of the isolates from pork and human, respectively). The PCR amplicons of *intI1* are shown in Figure 8. The *int2* and *int3* genes were not detected in the *Salmonella* isolates in this study.

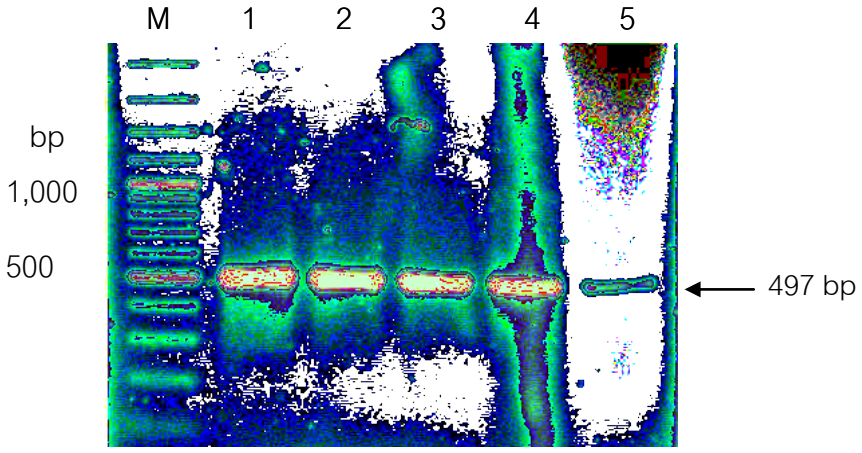

Figure 8: PCR amplicons of *intI1* gene. DNA template was PCR amplified using primer int1F and int1R that generated the amplicon size of 497 bp. Lane M, 100 bp marker; Lane 1, positive control; Lane 2-4 the *intI1*-positive *Salmonella* strains.

2.3 Gene cassettes of class 1 integrons

All the *int1*-positive isolates (*n*= 73) were detected for the presence of gene cassettes in variable regions. Up to 62 isolates are found to carry empty integrons. Eleven isolates from pork carried class 1 integrons carrying gene cassettes with size ranging from

1.0 to 1.9 kb. Among these, 8 isolates carried gene cassettes of 1.9 kb in size that were confirmed to be the *dfrA12-aadA2* gene cassette arrays. One isolate generated 1.2 kb PCR amplification of variable region that was identified to be bla_{PSE-1}. Two isolates contained two gene cassettes of 1.0 and 1.2 kb and were confirmed to be the bla_{PSE-1} and *aadA2* gene cassette array, respectively. (Figure 9). None of the int1-positive isolates from human carried gene cassettes. The 200 bp amplicon of *purG* gene encoding phosphoribosylformylglycinamide synthetase was found as an artefact PCR amplicon in thirteen isolates from humans. Three integron profiles (IP1-3) were defined number of integrons and size of variable regions. All class 1 integrons carried gene cassettes isolates were resistant to at least 4 antimicrobial agents.

All sulfametoxazole-resistant isolates were screened for the presence of *sul1*, *sul2* and *sul3*, a serovar Albany with class 1 integrons (i.e. SA710) was positive to only *sul2*. Four isolates positive to class 1 integrons (i.e. SA564, SA717, SA715, and SA583) carried only *sul3* and the genetic structure of their unusal 3'CS was confirmed to be linked with *qacH-sul3* domain by sequencing analysis. A class 1 integrons-positive Weltevreden (i.e. SA592) was sensitive to sulfamethoxazole and did not carry *sul1*, *sul2* or *sul3.*

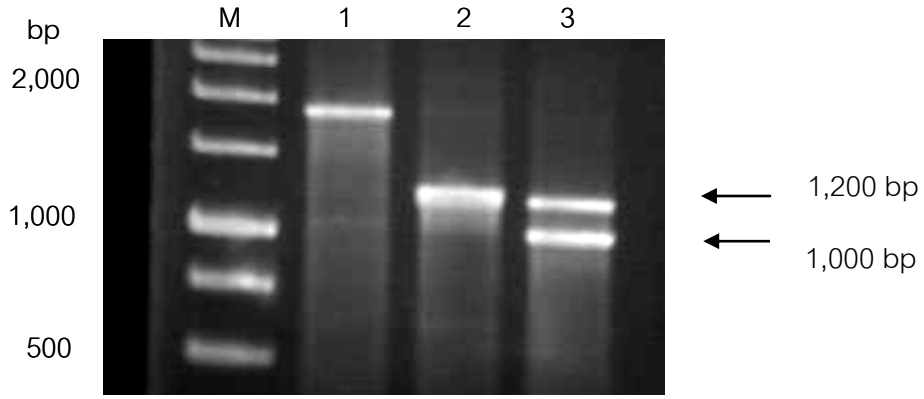

Figure 9: PCR Amplification of variable regions in the *intI1*-positive strains. Three integrons profile (IP1-3) were defined by the number and size of PCR amplicons. Lane M, 100 bp marker; Lane 1,IP1: *dfrA1-aadA2*; Lane 2, IP2: bla_{PSE-1} ; Lane 3, IP3: bla_{PSE-1},*aadA2.*

Table 7: Integron profile and antimicrobial resistance of the *Salmonella* isolates (*n*= 11)

IP	Approximate size of amplicons (kb)	Insert gene cassette	Antimicrobial resistance pattern	Number of isolates	Serotype
I	1.9	*dfrA12-aadA2*	AMP-SPC-SUL-TET-TRI	2	Rissen
			AMP-SPC-SUL-TET-TRI	2	Anatum
			CHPC-SPC-SUL-TET-TRI	1	Anatum
			AMP-CHPC-SPC-STR-SUL-TET-TRI	1	Anatum
			AMP-CHPC-SPC-STR-SUL-TET-TRI	1	Baiboukoum
			AMP-CHPC-SPC-STR-SUL-TET-TRI	1	Rissen
II	1.2	bla_{PSE-1}	AMP-CHPC-SUL-TRI	1	Albany
III	1.2,1.0	bla_{PSE-1}, *aadA2*	CHPC-STR-SUL-TET	1	Anatum
			AMP-CHPC-SPC-STR-SUL-TET-TRI	1	Anatum

2.4 Localization of class 1 integrons and their transferability

Eleven pork isolates carrying class 1 integrons with different gene cassettes i.e. *dfrA12-aadA2*, bla_{PSE1} and bla_{PSE1}-*aadA2* were tested for integrons transferability by biparental mating. The conjugation experiments showed that two *S.* Rissen isolates and one *S.* Baiboukoum isolate carrying class 1 integrons with *dfrA12-aadA2* gene cassette array could horizontally transfer their integrons to the *E. coli* recipient.

All the *int1*-positive isolates were examined for the presence of SGI1. Two *Salmonella* isolates from pork were found to contain for SGI1 variants. *S.* Albany carrying class 1 integrons with bla_{PSE1} gene cassette array carried SGI1-G. The SGI1-F was identified in *S.* Kedougou that carried empty integrons. These strains were positive to genetic components of SGI1-G and SGI1-F, respectively (Figure 10).

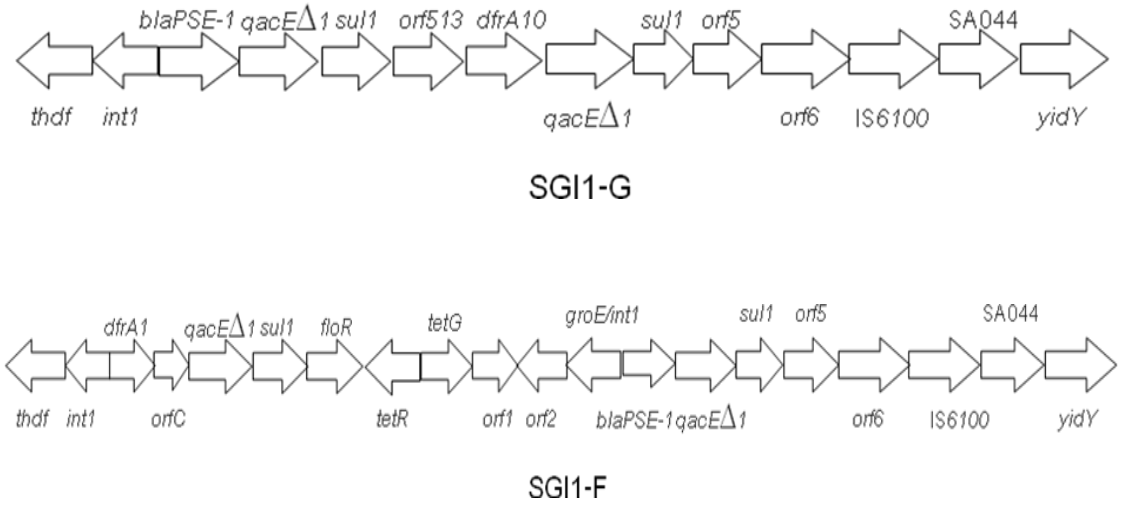

Figure 10: The genetic components of SGI1-G and SGI1-F.

2.5 Determination of non-integron borne antimicrobial resistance genes

Eighteen antimicrobial resistance genes including $bla_{PSE1,}$ $bla_{TEM,}$ *aadA1, aadA2, aadB, strA, strB, tetA, tetB, cmlA, catA, catB, sul1, sul2, sul3, dfrA1, dfrA10* and *dfrA12* were detected in the *Salmonella* isolates with corresponding resistance phenotype (Table 8). The PCR amplicons of these genes are shown in Figure 11-12. Fifty-two percent of ampicillin-resistant strains were positive to bla_{TEM} and followed by bla_{PSE1} (39%). The *cmlA, sul3* and *dfrA12* were detected in chloramphenicol- (50%), sulfamethoxazole- (34.7%) and trimethoprim-resistant strains (24.6%), respectively. The *aadA1* gene was the most commonly found in gentamicin-resistant strains (83.3%). The *aadB* gene was identified in only one gentamicin-resistant isolate. Four kinds of the streptomycin-resistance encoding genes *aadA1, aadA2, aadB, strA* and/or *strB* were detected in the streptomycin-resistant *Salmonella* strains (55%). Fifty-seven and fifty-four percent of spectinomycin-resistant strains were positive to *aadA1* and *aadA2* gene, respectively. The *tetA* and/or *tetB* genes were detected in the tetracycline-resistant strains, of which the *tetB* gene was most commonly found among the resistant genes tested in this study (55%). Seven percent of the antimicrobial-resistant *Salmonella* strains did not carry any resistance-encoding genes tested. Distribution of antimicrobial resistance genes in *Salmonella* resistant isolates from pork and humans was varied. All gentamicin-resistant isolates from pork carried *aadA1* gene. The high percentage of streptomycin- (79%) and tetracycline-resistant (63%) isolates from pork carried *strA* and *tetB*, respectively. The sulfamethoxazole-resistant isolates from humans has a higher percentage of the presence of *sul2* and *sul3* genes (Figure 13).

Table 8: Antimicrobial resistance genes in *Salmonella* isolates (*n*=183)

Resistant isolate (n)	Resistance genes	Number (%)
Ampicillin (98)	bla_{PSE1}	39 (39.7%)
	bla_{TEM}	52 (53%)
Chloramphenicol (88)	*catA*	26 (29.5%)
	catB	19 (21.5%)
	cmlA	44 (50%)
Gentamicin (18)	*aadA1*	15 (83.3%)
	aadA2	5 (27.7%)
	aadB	1 (5.5%)
Streptomycin (68)	*aadA1*	29 (42.6%)
	aadA2	26 (38.2%)
	strA	38 (55.8%)
	strB	10 (14.7%)
Spectinomycin (98)	*aadA1*	56 (57.1%)
	aadA2	53 (54%)
Sulfamethoxazole (95)	*sul1*	29 (30.5%)
	sul2	30 (31.5%)
	sul3	33 (34.7%)
Tetracycline (134)	*tetA*	9 (6.7%)
	tetB	75 (55.9%)
Trimethoprim (73)	*dfrA1*	17 (23.2%)
	dfrA10	11 (15%)
	dfrA12	18 (24.6%

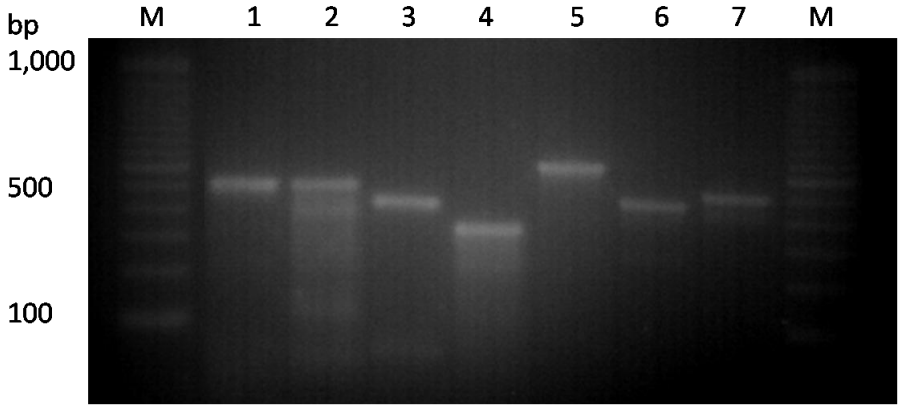

Figure 11: PCR amplicons of resistance genes. Lane M, 100 bp marker; Lane 1-7, the result of the PCR reaction amplifying *sul2, sul3, dfrA10, dfrA12, catA* and *aadA2*

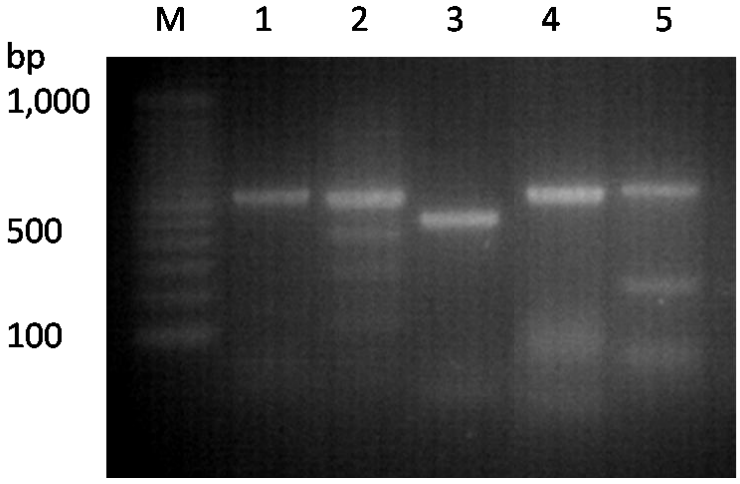

Figure 12: PCR amplicons of resistance genes. Lane M, 100 bp marker; Lane 1-5, the result of the PCR reaction amplifying bla_{TEM}, *cmlA, dfrA10, tetB* and *aadA1*

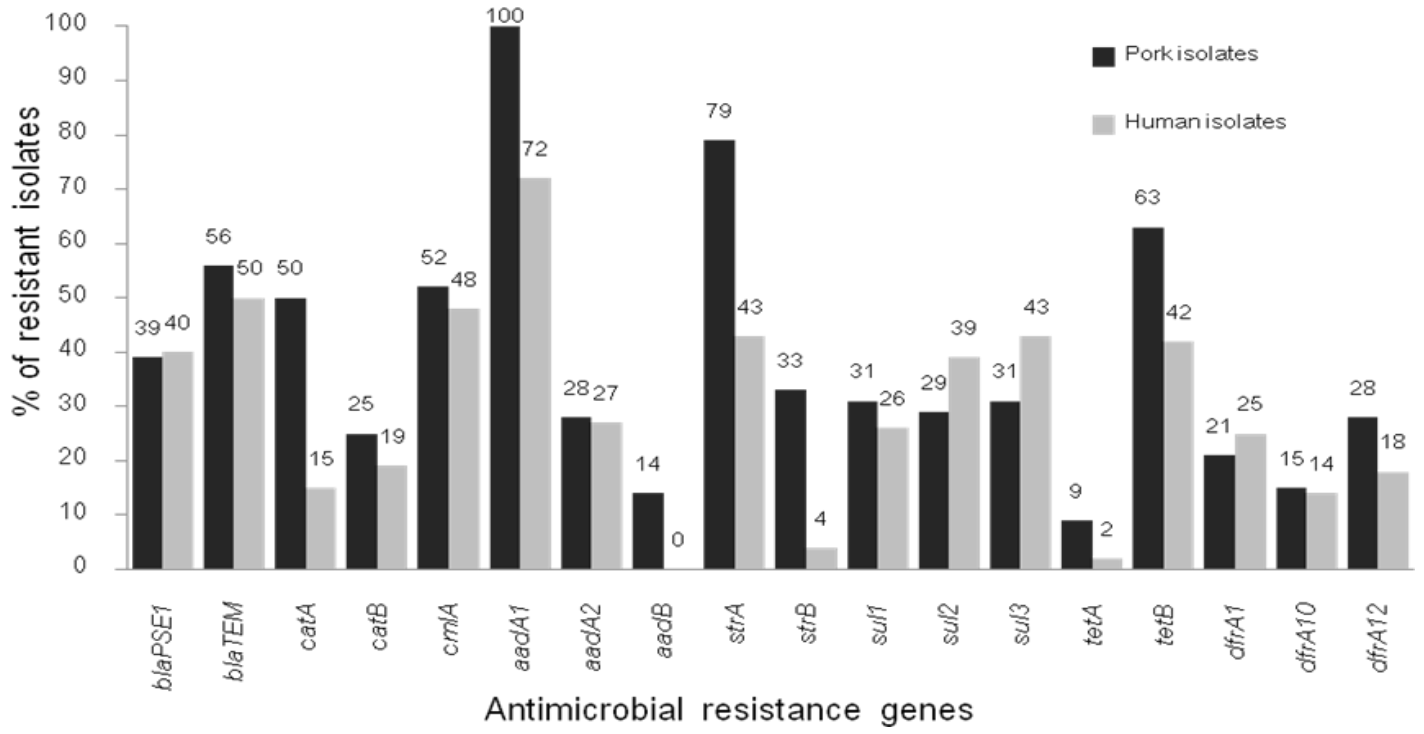

Figure 13: Distribution of antimicrobial resistance genes in *Salmonella* resistant isolates from pork and humans.

2.6 Mutations in QRDRs of *gyrA*, *gyrB*, *parC* and *parE* genes

Eighteen *Salmonella* isolates resistant to ciprofloxacin were examined for the presence of mutations in the QRDRs of *gyrA*, *gyrB*, *parC* and *parE* genes (Table 9). Six isolates were found to contain two point mutations in *gyrA* that were G-144-T leading to a Met-48-Ile substitution in GyrA (28%) and C-133-T leading to a Pro-45-Ser substitution in GyrA (6%). Replacement of T at position of 188 with C in *gyrB* leading to a Val-63- Ala substitution in GyrB was found in six isolates (33%). Eight silent mutations were found in *gyrB* gene. There were 13 point mutations identified in *parC* gene. Replacement of C at position of 245 with T leading to a Thr-82- Met substitution in ParC was the most common mutations (78%) followed by G-31-C (67%), C-62-T (67%), T-86-C (67%) and A-212-G (67%) leading to Ala-11-Pro, Ser-21- Leu, Ile-29- Thr and Gln-71- Arg, respectively. Twelve silent mutations were found in *parE* gene.

The *Salmonella* strains with combination of *gyrA*, *gyrB* or *parC* mutations were divided into ten groups (gruop I-X, Table 10). There were 2 isolates had a mutations in *gyrA*, *gyrB* and *parC* (group I and VIII). One pork isolate (SA463) had 1 point mutation in *gyrA*, *gyrB* and 8 point mutations in *parC.* One human isolate (SA614) had 1 point mutation in *gyrA*, *gyrB* and 6 point mutations in *parC* gene. Four *Salmonella* isolates with point mutation in *gyrB* additionally carried mutation in *parC* (group II and V). Eight ciprofloxacin-resistant strains carried mutations only in *parC* (group III, IV, VII and X).

Table 9: Mutations observed in the *gyrA*, *gyrB*, *parC* and *pare* genes from the ciprofloxacin-resistant isolates (*n*=18)

Gene	Mutation		No.(%)
	Nucleotide substitution	Amino acid substitution	
gyrA	G-144-T	Met-48-Ile	5 (27.7)
	C-133-T	Pro-45-Ser	1 (5.5)
gyrB	T-188-C	Val-63- Ala	6 (33.3)
parC	T-2-C	Met-1- Thr	6 (33.3)
	A-13-C	Ser-5- Arg	1 (5.5)
	G-31-C	Ala-11-Pro	12 (66.6)
	C-62-T	Ser-21- Leu	12 (66.6)
	T-86-C	Ile-29- Thr	12 (66.6)
	C-92-T	Thr-31- Met	7 (38.8)
	C-152-T	Thr-51- Ile	6 (33.3)
	G-173-A	Gly-58- Glu	1 (5.5)
	C-182-G	Ala-61- Gly	6 (33.3)
	A-212-G	Gln-71- Arg	12 (66.6)
	T-230-C	Leu-77- Pro	7 (38.8)
	T-230-G	Leu-77- Arg	4 (22.2)
	C-245-T	Thr-82- Met	14 (77.7)

2.7 The expression of the AcrAB-TolC efflux system

Twenty-four *S. enterica* isolates with different ciprofloxacin MICs (MIC ranged 0.125-8 µg/ml) were selected for determination of the expression level of *acrB* by real-time qRT-PCR. The *acrB* expression level was 1 up to 430 fold higher than *S.* Typhimurium 13311 strain and not associated with ciprofloxacin MICs. Two isolates with ciprofloxacin MIC 0.125 µg/ml had high-level expression of *acrB* (101 and 221 fold). The highest expression level (430 fold) was detected in *S.* Rissen (SA 671) isolate from pork. This isolate was resistant to 5 antimicrobial agents i.e., ciprofloxacin (MIC= 8 µg/ml), spectinomycin, sulfamethoxazole, tetracycline and trimethoprim. Mutations in *gyrB* and *parC* were found in SA 671.

The lowest expression level (1 fold) was detected in five isolates. These isolates were resistant to at least 4 antimicrobial tested and include ciprofloxacin (MIC= 4 µg/ml). The mutations of *gyrA*, *gyrB* and/or *parC* were detected in these isolates. Two hundred-twenty one fold expression of *acrB* was detected in *S.* Anatum that susceptible to all antimicrobial tested. The expression level of *acrB*, the antimicrobial resistance patterns and mutations in QRDRs of the selected *S. enterica* isolates are shown in table 10.

Table 10: The expression level of *acrB*, the antimicrobial resistance patterns and mutations in QRDRs of the selected *Salmonella* isolates.

Group	Strain	Mutation			CIP MIC	Resistance pattern	Expression level of *acrB*
		gyrA	*gyrB*	*parC*			
I	SA463	pro-45-ser	val-63-ala	ser-5- arg, ala-11-pro, ser-21- leu, ile-29- thr, thr-31- met, gln-71- arg, leu-77-pro, thr-82- met	8	CHPC-CIP-SPC-SUL-TET-TRI	131
II	SA613	-	val-63-ala	thr-82- met	8	AMP-CHPC-CIP-STR-SPC-SUL-TET-TRI	49
	SA622	-	val-63-ala	thr-82- met	8	AMP-CHPC-CIP-STR-SPC-TET-TRI	63
	SA671	-	val-63-ala	thr-82- met	4	CIP-SPC-SUL-TET-TRI	430
III	SA578	-	-	thr-82- met	4	AMP-CHPC-CIP-SPC	1
	SA579	-	-	thr-82- met	4	AMP-CHPC-CIP-SPC-TRI	19
IV	SA601	-	-	ala-11-pro, ser-21- leu, ile-29- thr, gln-71- arg, leu-77- pro, thr-82- met, met-1- thr, thr-51- ile, ala-61- gly	4	AMP-GEN-CHPC-CIP-STR-SPC-SUL-TET-TRI	2
	SA602	-	-	ala-11-pro, ser-21- leu, ile-29- thr, gln-71- arg, leu-77- pro, thr-82- met, met-1- thr, thr-51- ile, ala-61- gly	4	AMP-GEN-CHPC-CIP-STP-SPC-TET-TRI	3

Table 10 (continued):

Group	Strain	Mutation			CIP MIC	Resistance pattern	Expression level of *acrB*
		gyrA	*gyrB*	*parC*			
	SA624	-	-	ala-11-pro, ser-21- leu, ile-29- thr, gln-71- arg, leu-77- pro, thr-82- met, met-1- thr, thr-51- ile, ala-61- gly	8	AMP-GEN-CHPC-CIP-STR-SPC-TET-TRI	26
V	SA603	-	val-63-ala	thr-82- met, gly-58- glu	4	AMP-CHPC-CIP-STR-SPC-TET-TRI	55
VI	SA608	met-48-ile	-	ala-11-pro, ser-21- leu, ile-29- thr, thr-31- met, gln-71- arg, leu-77- pro	4	AMP-GEN-CHPC-CIP-SPC-TET-TRI	273
	SA609	met-48-ile	-	ala-11-pro, ser-21- leu, ile-29- thr, thr-31- met, gln-71- arg, leu-77- pro	4	AMP-CHPC-CIP-SPC-SUL-TET-TRI	1
VII	SA610	-	-	ala-11-pro, ser-21- leu, ile-29- thr, thr-31- met, gln-71- arg, leu-77- pro	4	AMP- CHPC-CIP-SPC-STR-SUL-TET-TRI	1
	SA611	-	-	ala-11-pro, ser-21- leu, ile-29- thr, thr-31- met, gln-71- arg, leu-77- pro	4	AMP-CHPC-CIP-SPC-TET-TRI	1
	SA612	-	-	ala-11-pro, ser-21- leu, ile-29- thr, thr-31- met, gln-71- arg, leu-77- pro	8	AMP-CHPC-CIP-STR-SPC-SUL-TET-TRI	24
VIII	SA614	met-48-ile	val-63-ala	ala-11-pro, ser-21- leu, ile-29- thr, thr-31- met, gln-71- arg, leu-77- pro	8	AMP-GEN-CHPC-CIP-STR-SPC-SUL-TET-TRI	58

Table 10 (continued):

Group	Strain	Mutation			CIP MIC	Resistance pattern	Expression level of *acrB*
		gyrA	*gyrB*	*parC*			
IX	SA615	met-48-ile	-	ala-11-pro, ser-21- leu, ile-29- thr, thr-31- met, gln-71- arg, leu-77- pro, thr-31- met, gln-71- arg, leu-77- pro, thr-82- met	8	AMP-CHPC-CIP-STR-SPC-TET-TRI	58
X	SA616	-	-	ala-11-pro, ser-21- leu, ile-29- thr	8	AMP-CHPC-CIP-STR-SPC-SUL-TET-TRI	106
XI	SA545	-	-	-	0.125	AMP-TET-SUL	101
	SA666	-	-	-	0.125	AMP-CHPC-SPC-SUL-TET-TRI	18
	SA717	-	-	-	0.125	AMP-SPC-SUL-TET-TRI	1
	SA721	-	-	-	0.125	TET	18
	SA734	-	-	-	0.125	AMP-GEN-CHPC-CIP-STR-SPC-TET	6
	SA736	-	-	-	0.125	Susceptible to all antimicrobial tested	221

3. Investigation of the genetic relatedness of the *Salmonella* strains by Multilocus sequence typing (MLST)

DNA sequence alignments of *manB, mdh* and *fimA* were used for construction of phylogenetic trees (Figure 14). Forty *Salmonella* isolates of 3 different serotypes including Typhimurium, Kedougou and Weltrevreden from pork and humans were divided into 3 clusters (cluster A, B and C). Strains of the same serovar generally cluster into distinct groups; cluster A contained 20 *S.* Kedougou strains of 10 pork- and 10 human isolates, cluster B contained 8 *S.* Typhimurium strains of 3 pork- and 5 human isolates and cluster C contained 10 *S.* Weltevreden strains of 7 pork- and 3 human isolates. MLST could not differentiate 2 strains in this study i.e. the *S.* Weltevreden SA 652 and *S.* Typhimurium SA 576 were mixed with *S.* Typhimurium and *S.* Weltevreden, respectively. Most of pork and human isolates showed a highly similar in each cluster.

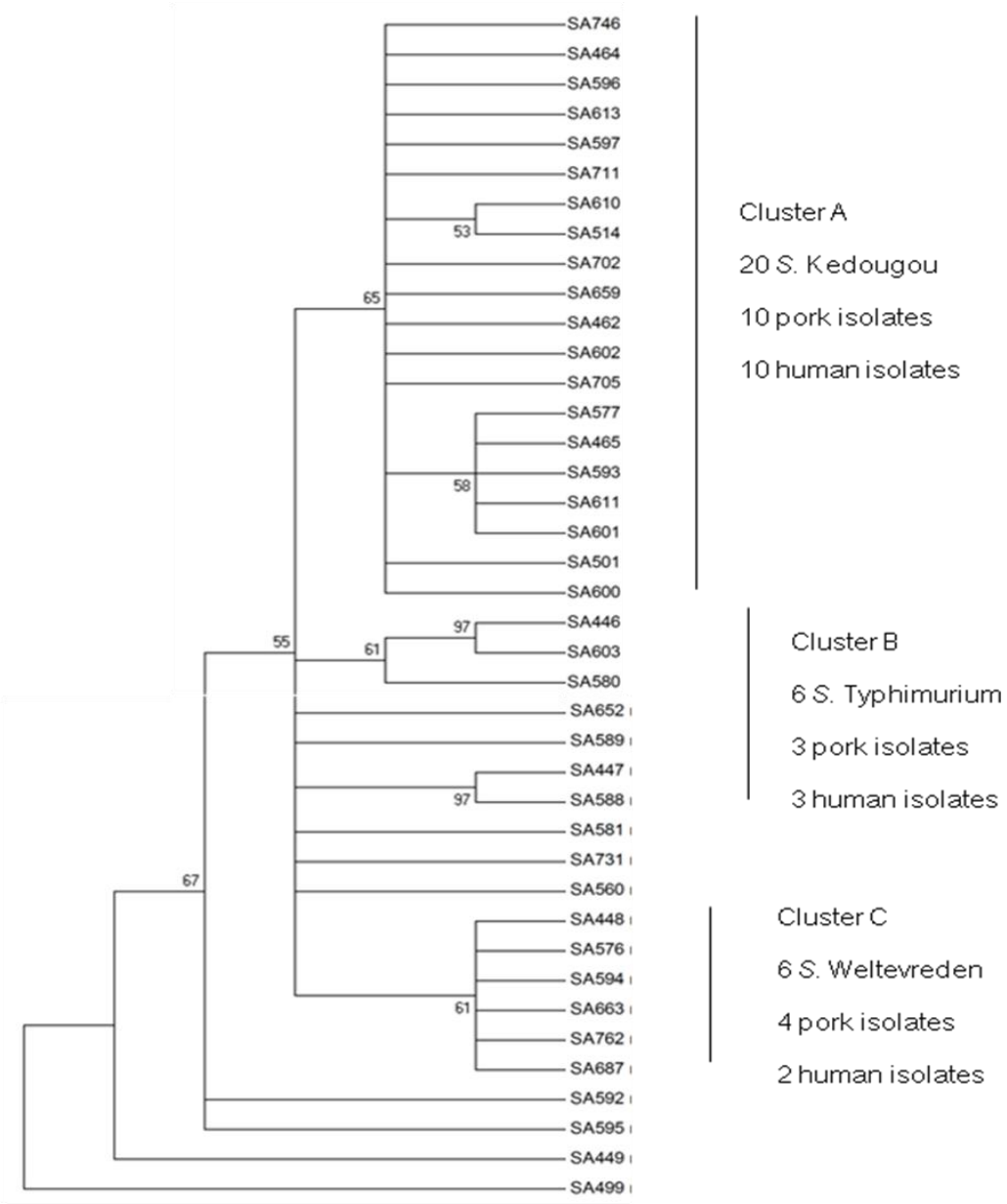

Figure 14: Phylogenetic relationships of *Salmonella* strains based on the sequence of *manB, mdh* and *fimA* genes, constructed by MEGA (*n*=40).

4. Occurrence of virulence genes

All isolates were detected for the presence of sixteen virulence genes by multiplex PCR. The PCR amplicons of these genes are shown in Figure 15. All the *Salmonella* isolates from pork carried the *invA*, *msgA*, *spiA* and *tolC* gene, whereas all the isolates from humans harbored the *invA*, msgA, *prgH*, *sifA, sipB, sitC, spaN*, *spiA* and *tolC* genes. One isolate from pork (0.7%) and 7 human isolates (13.4%) were positive to *spvC* gene. The *pefA* gene was detected in a pork isolate (0.7%) and 3 human isolates (5.7%). The *iroN, lpfC, prgH, sifA, sitC, sipB, sopB* and *spaN* genes were found in more than 50% of the pork isolates. Fifty-seven and eleven percent of pork and human isolates, respectively were positive to *orgA*. The *lpfC* (82%) and *sopB* (80%) genes were detected at high rate in the human isolates. Distribution of virulence genes in pork and human isolates are shown in Figure 16.

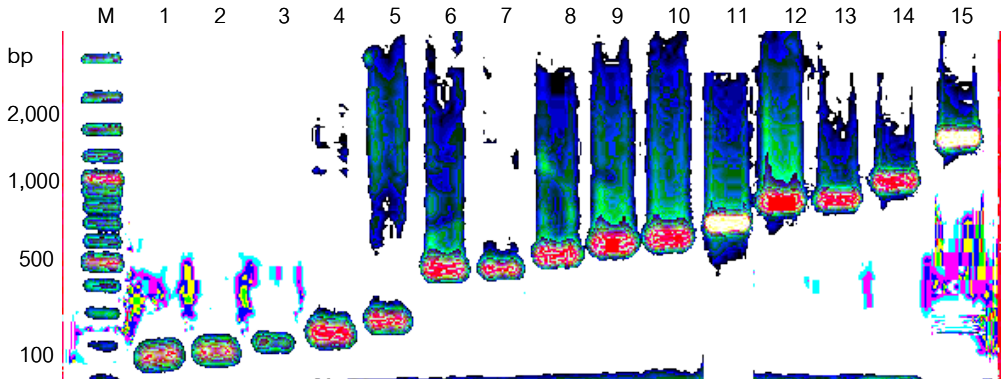

Figure 15: PCR amplicons of virulence genes. Lane M, 100 bp marker; Lane 1-15, the result of the PCR reaction amplifying *pefA*, *tolC*, *msgA*, *sopB*, *orgA*, *sifA*, *pagC*, *spaN*, *spiA*, *spvC*, *lpfC*, *prgH*, *sitC*, *sipB* and *iron*

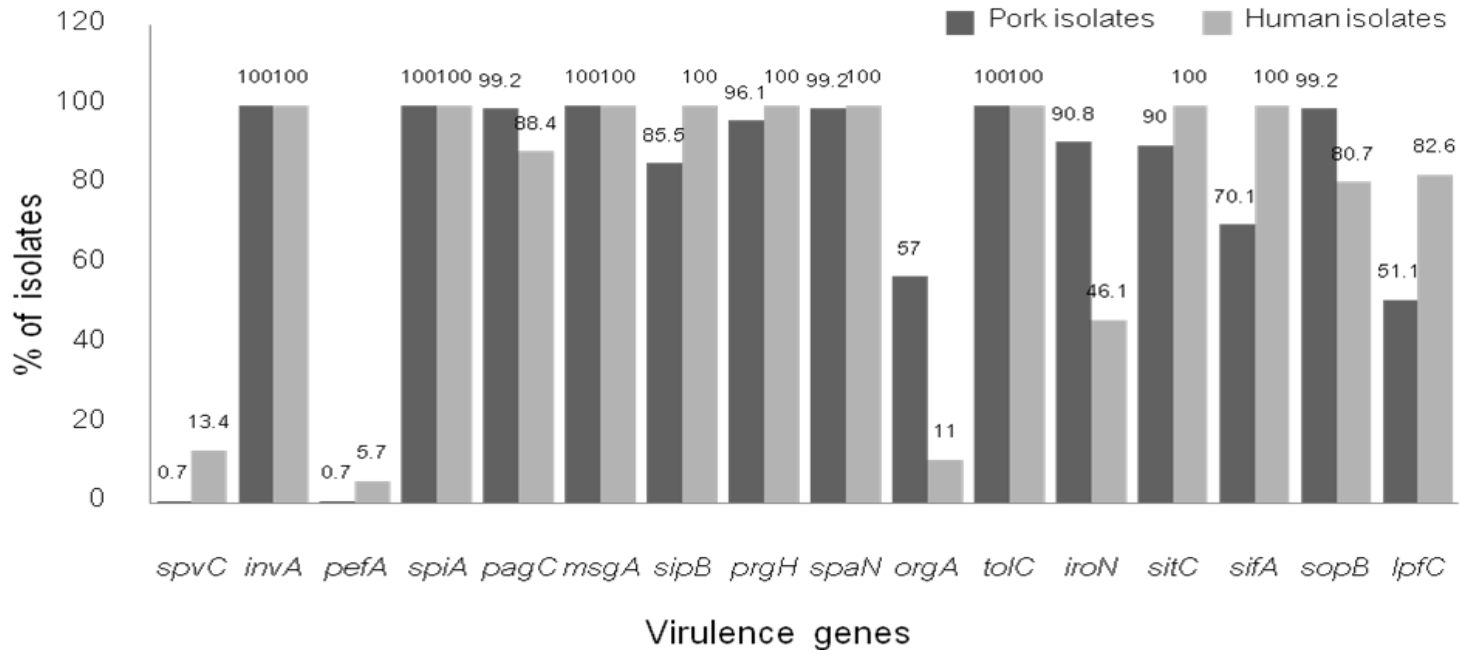

Figure 16: Occurrence of virulence genes among *Salmonella* isolates from pork and humans (*n*=183).

CHAPTER V

DISCUSSIONS

Antimicrobial agents have been widely used in pig production, mainly for growth promotion, disease prophylaxis and infection therapeutics (McEwen and Fedorka-Cray, 2002) It is well accepted that the overuse of these antimicrobials is a major cause of the increasing of MDR bacteria including *Salmonella*. Pigs have been considered one of the major sources of MDR *Salmonella* (Padungtod et al., 2008). It has been shown that that MDR *Salmonella* pigs could enter the food chain and subsequently infect humans (White et al., 2003). Infection with MDR *Salmonella* could result in prolonged treatment and increased economical losses and finally treatment failure. Genetic characterization of the MDR *Salmonella* isolates from pork and humans is necessary to explain the distribution and transmission of the pathogens along food chain. The results of the present study may explain link between human and pork, which consist of

The results of the present study are discussed based on the publications and falls into three as follows:

1. Class 1 integrons and virulence genes in *Salmonella* enterica isolates from pork and humans.
2. Mutations in Topoisomerase genes and expression of the AcrAB-TolC efflux system in *Salmonella enterica* from pork and humans.
3. Genetic relatedness among *Salmonella enterica* from pork and humans.

Part 1 Class 1 integrons and virulence genes in *Salmonella* enterica isolates from pork and humans.

In this study, most of the *Salmonella* isolates were MDR. Up to 64% of pork isolates were MDR. This is in agreement with the earlier work in United States (Chen et al., 2004), Mexico (Miranda et al., 2009), Vietnam (Thai et al., 2012), and Germany (Schwaiger et al., 2012). These data confirmed the widespread of MDR *Salmonella* among pork and indicated that pigs may serve as reservoir of MDR strains that may be transmitted to humans via food chain. Therefore, the high prevalence of MDR *Salmonella* strains in pork could increase the potential risk for human health. Most of humans isolates (52%) were MDR, consistent with previous studies in Portugal (Antunes et al., 2006), Vietnam (Vo et al., 2008), Oman (Al-Bahry et al., 2007), Morocco (Ammari et al., 2009) and Iran (Ranjbar et al., 2011). Taken together, all the data confirm the wide distribution of MDR *Salmonella* in food of animal origin and human.

Up to eighty-eight percent of *Salmonella* isolates from pork were resistant to tetracycline, which is consistent with the results of former studies in Belgium (100%) (Doublet et al., 2004) and France (48%) (Cailhol et al., 2006). In Thailand, it was previously reported that the pork isolates from Khon Kaen (88.5%) and Sa Kaew (68.9%) province were resistant to tetracycline at high rate (Pulsrikarn et al., 2012). This is likely to be due to the extensive use of tetracycline in the farmed pig (Ha et al., 2003). The human isolates exhibited resistance to tetracycline (47%) but less than those previously reported in other countries e.g. Finland (100%) (Lailler et al., 2002) and China (60%) (Zhang et al., 2004)

High resistance rate to ampicillin (53%), spectinomycin (53%), sulfamethoxazole (51%) and chloramphenicol (48%) were observed in this study. The *Salmonella* strains resistant to these drugs has been reported in many studies. It is not surprising, because these drugs have been used in both animals and humans for a quite long time (Su et al., 2005). The occurrence of resistance to tetracycline, ampicillin, spectinomycin, sulfamethoxazole and chloramphenicol were higher among the food-borne isolates than among those of human isolates, consistent with a previous study in *Salmonella* isolated from pork and human clinical isolates (Antunes et al., 2006)

The occurrence of Ceftriaxone-resistant *Salmonella* strains is a new public health concern because this third generation cephalosporin is a drug of choice for treatment of severe *Salmonella* infections, especially in children. There are increasing reports of ceftriaxone resistant *Salmonella* in Taiwan and United States (Chen et al., 2004), while these resistant isolates are still rare in Singapore (Su et al., 2005). Winokur et al. (2000) reported that pig is one of the important food animal reservoirs of ceftriaxone-resistant *Salmonella*. However, none of isolates tested in this study were resistant to ceftriaxone.

In the present study, 40% of the *Salmonella* isolates were positive to *intI1* gene. This result was similar to those previously reported among *Salmonella* in Portugal (Antunes et al., 2006), United States (Zhao et al., 2008) Vietnam (Van et al., 2007) Japan (Ahmed et al., 2009) and China (Yang et al., 2010). It was suggested that distribution of class 1 integrons among *Salmonella* may be related to its location on plasmid that could be horizontally transferred to the same or different bacteria species (Meng et al., 2011).

Fifteen percent of the *intI1* positive isolates contained resistance gene cassettes in variable region. In contrast, much higher rate of class 1 integrons with gene cassette was reported in *Salmonella* strains in previous studies, e.g. 46% in *Salmonella* isolates from pork (Antunes et al., 2006) and 78% in *Salmonella* isolates from pig (Wang et al., 2010). In addition, all of the class 1 integrons-positive isolates in this study were MDR, confirming that MDR of *Salmonella* is usually related to the presence of class 1 integrons (Vo et al., 2008).

Three integron patterns were identified in this study, including *dfrA12-aadA2*, bla_{PSE-1} and bla_{PSE-1}, *aadA2*. All of these gene cassettes have been reported previously in *Salmonella* (Antunes et al., 2006). The gene cassette array *dfrA12-aadA2* was commonly found in the pork isolates. This gene cassette was previously reported in *Salmonella* from different sources and different geographical areas, for example, from food products and humans in Portugal (Antunes et al., 2006), from poultry and swine in Thailand (Khemtong and Chuanchuen, 2008), from patients in Hong Kong (Jin and Ling, 2009) and from broiler chicken and pigs in Taiwan (Wang et al., 2010). This data provides more evidence that foods of animal origins could serve as vehicles of transmission of antimicrobial resistance.

In conjugation experiment, only 2 *Salmonella* isolates could horizontally transfer class 1 integrons with *dfrA12-aadA2* to *E.coli* recipient. This is similar to our previous study in *Salmonella* from pork and humans in Portugal (Antunes et al., 2006), from poultry and swine (Khemtong and Chuanchuen, 2008) and from patients in Hong Kong (Jin and Ling, 2009). This information suggests a worldwide dissemination of the *dfrA12-aadA2* gene and confirms the important role in widespread of resistance to trimethroprim and aminoglycosides among *Salmonella* (Wang et al., 2010).

It is interesting to find that class 1 integrons containing bla_{PSE1} with a nonsense point mutation was observed. This antibiotic-resistance gene cassette could not yield any benefits to the *Salmonella* host strain. It is unclear how this mutated gene was embedded in the integron cassette. Since the host cells do not gain any profit from catching a non-functional gene, it is likely that the mutational event occurred after the site-specific recombination event.

High prevalence of class 1 integrons without genes cassettes (86%) or empty integrons was detected in this study. Up to 33% of empty integrons were found in pork isolates. This was consistent with previous study in China showing that 50% of *intI1* positive-*Salmonella* from pork were empty integrons (Meng et al., 2011). The origin of these empty integrons is still unclear. It has been suggested that the empty integrons may lost their gene cassettes in the absence of antimicrobial selective pressure or have exchanged the gene cassettes with other integrons (Partridge et al., 2002). Regardless, these empty variable regions are available to receive new gene cassettes and contribute to resistance dissemination among the pathogens (Bissonnette and Roy, 1992).

While a typical 3'-CS region of class 1 integrons is usually a *sul1*-type consisting of *qacEΔ1-sul1* fused structure (Fluit and Schmitz, 2004), integrons lacking this region have been previously found (Lindstedt et al., 2003). In this study, four class 1 integrons lacking the 3'-CS were associated with an atypical 3'-CS region linked to the *qacH-sul3* domain. High frequency of such non-classic class 1 integrons was previously observed in different

Salmonella serovars (Antunes et al., 2006), suggesting that the presence of *qacEΔ1-sul1* is not a perfect marker for prediction of the presence of class 1 integrons.

None of the isolates tested were positive for class 2 and class 3 integrons. This finding was similar to previous study among *Salmonella* from patients in Hong Kong (Jin and Ling, 2009) and Japan (Ahmed et al., 2009). In contrast, the previous studies reported the *Salmonella* strains in Spain (Rodriguez et al., 2006) and Chile (San Martin et al., 2008) were carried class 2 integrons with *dfrA1-aadA1* array. However, the prevalence of class 2 and 3 integrons in *Salmonella* is less common than class 1 integrons from poultry and swine in Thailand (Naghoni et al., 2010).

The variant SGI1-G and SGI1-F were found in two pork isolates i.e. Albany and Kedougou, respectively. The SGI1-G type has been previously detected in a serovar Agona from poultry (Doublet et al., 2003). To our knowledge, this was the first documented report of SGI1-G in a serovar Albany. The SGI1-F variant has been identified in other serovars, including Albany (Doublet et al., 2003), Emek (Khemtong and Chuanchuen, 2008), Cerro and Dusseldrof (Levings et al., 2005). Identification of an SGI1 cluster in the chromosome of different *Salmonella* serovars suggesting potential for horizontal transfer of SGI1 (Ebner et al., 2004). SGI1 and its variants are acquired by several serovars and contribute to antimicrobial resistance in the pathogens. However, it is unclear how the SGI1-like variants originate and what the origin of SGI-1 antimicrobial gene clusters is (Boyd et al., 2002).

In this study, most tetracycline-resistant isolates was contained *tet*B (57%), while a few isolates contained *tet*A (6%). In contrast, Aarestrup et al, (2003) reported that

Salmonella from animals and humans in many countries of South-East Asia had a higher prevalence of *tetA* (65%) than *tetA* (20%). However, both *tetA* and *tetB* have been found widespread among *Salmonella* (Frech and Schwarz, 2000).

Most chloramphenicol-resistant isolates carried *cmlA* followed by *catA* and *catB* genes. Similar finding was observed in *Salmonella* from humans in Hungary (Nogrady et al., 2005). The *catB* has been reported to be located on class 1 integrons in *Salmonella* (Stokes and Hall, 1989). However, this gene not associated with class 1 integrons in this study.

In addition, the streptomycin-resistant isolates in our collection carried *strA* and *strB* genes. This result was similar to a previous study in *Salmonella* isolates from humans in Brazil (Peirano et al., 2006) and Denmark (Madsen et al., 2000). Both genes can confer streptomycin resistance and are widely distributed among gram-negative bacteria, including *Salmonella* (Kikuvi et al., 2010).

In most cases, the resistance genes were detected where the corresponding individual resistance phenotypes were observed, suggesting the expression of the genes present. Some of class 1 integrons-carrying strains additionally contained the relevant-resistance genes located outside the integron structure. Some *Salmonella* isolates harbored multiple genes for the identical resistance phenotype e.g. *sul1*, *sul2* and *sul3* and *cmlA*, *catA* and *catB*. The presence of multiple genes mediating the similar resistance phenotypes have been frequently reported (Randall et al., 2004). In this case, one gene may be chromosomally encoded and the others may be associated with integrons or located on a plasmid. Further studies are required to elucidate the alternative possibilities.

By screening of 17 virulence genes, fourteen were found at high frequency among *Salmonella* in this study. High prevalence of the virulence genes have been demonstrated in *Salmonella* isolates from bison carcasses and sick and healthy birds, suggesting the wide distribution of these virulence factors among *Salmonella* (Skyberg et al., 2006). In contrast, *spvC* (4%) and *pefA* (2%) were present at limited rate. The possible explanation for this difference is that all 14 genes tested are located in the *Salmonella* genome, while *spvC* and *pefA* are encoded by *Salmonella* virulence plasmids that can be serovar specific (Skyberg et al., 2006). However, the absence of *pefA* from plasmid preparation of a *Salmonella* isolate suggested that the *Salmonella* strains harbored multiple plasmids and the virulence plasmid had integrated into the chromosome (Abouzeed et al., 2000). All isolates containing virulence genes were resistant to antimicrobial agent. Coexistence of virulence genes and resistance determinants on the same plasmid has been previously reported (Chu and Chiu, 2006) and become a particular threat to human health.

Part 2 Mutations in Topoisomerase genes and expression of the AcrAB-TolC efflux system in *Salmonella enterica* from pork and humans.

In *Salmonella,* the more common amino acid substitution in GyrA previously demonstrated to be associated with fluoroquinolone resistance were the replacement of Ser-85 with Tyr, Phe, or Ala and Asn-87 with Tyr, Gly, or Asn (Eaves et al., 2004). The most common mutation in ParC was Thr-57-Ser substitution (Eaves et al., 2004). In contrast, none of these amino acid changes were observed in our *Salmonella* collection. The isolates in this study carried novel mutations i.e. C-133-T and G-144-T in *gyrA* and A-13-G, C-92-T and T-230-G in *parC* that caused only slight decreases in the ciprofloxacin susceptibilities. Since these mutations have never been previously reported in GyrA or ParC in *Salmonella*, their

contribution to ciprofloxacin resistance need to be further determined in a single genetic background. It has been demonstrated that mutation in GyrA conferred low ciprofloxacin-resistance level and those with a ParC mutation in addition to a GyrA mutation exhibited more resistance. In contrast, the ciprofloxacin MICs of the strains carrying only a mutation in GyrA or ParC in this study were not different from those of the isolates with mutations in both genes.

In comparison to other pathogenic Enterobacteriaceae, *Salmonella* infrequently exhibit high fluoroquinolone resistance level and it was suggested that such limitation was associated with a prohibitive fitness cost (Giraud et al., 2006; Giraud et al., 2003). This was in agreement in this study where the ciprofloxacin-resistant isolates showed the MIC value of 4-8 μg/ml. Resistance to fluoroquinolones in *S. enterica* initially arises from mutation (s) in one or more topoisomerase genes (Piddock, 2002). While the mutations in the quinolone resistance-determining region (QRDR) of *gyrA* have mainly contributed to reduced susceptibility to fluoroquinolones in the clinical *Salmonella* human and animal isolates (Eaves et al., 2004), those in *gyrB*, *parC* and *parE* have been rarely reported. It was suggested that combination of mutations in the same or different topoisomerase gene resulted in increased fluoroquinolone-resistance level. For this instance, the *Salmonella* isolates with reduced susceptibility contained a single mutation in *gyrA* and the resistant isolates carried at least two mutations in *gyrA* and/or *gyrB* and/or *parC* and/or *parE* (Eaves et al., 2004).

Fluroquinolone resistance in *Salmonella* is commonly associated with a single point mutation between nucleotides 67 to 122 in the QRDR of *gyrA* (Giraud et al., 1999), leading

to the more common GyrA amino acid changes at position Gly81, Ser83 or Asp87 (Marimon et al., 2004). In contrast, the strains in this study lacked these prominent mutations. However, our strains carried mutations in *gyrA*, *gyrB* and/or *parC.* Based on our knowledge, all the mutations identified are novel in ciprofloxacin-resistant *S. enterica*. Since these mutations have never been found, their actual contribution may not be stated. Further experiment such as site specific mutagenesis or study in a larger population may worth it.

In Gram-negative bacteria, a single *gyrA* mutation confers low ciprofloxacin resistance level and the additional mutations in *gyrA* or within the other topoisomerase genes results in the increased-resistance level (Dimitrov et al., 2009; Liebana et al., 2002). However, none of the isolates in the present study harbored only *gyrA* mutations. Therefore, it may not be precisely assess the contribution of mutations in *gyrA* and other two genes.

No nucleotide changes in *parE* were identified, consistent with previous studies (Marimon et al., 2004). This is not beyond expectation and supports that *parE* mutation is much less common than mutations in the others.

Mutations in *parC* are seldom seen in the ciprofloxacin-resistant *S. enterica* (Eaves et al., 2004) and role of these mutations is still not apparent. In contrast, *parC* mutations (s) were identified in all the resistant strains in this study. While up to 13 different point mutations were observed in *parC*, some single strains simultaneously carried 9 *parC* mutations. Still, it cannot ascertain that all these mutations contributed to ciprofloxacin resistance of the bacterial hosts. However, the susceptible isolates did not carried the same mutations in any of three topoisomerase genes, supporting that nucleotide changes

observed were not likely associated with strain variation and indicating the significance of mutations in topoisomerase genes in ciprofloxacin resistance. Taken together, there are no associations between the number and type of mutations and ciprofloxacin resistance level among the ciprofloxacin-resistant *Salmonella* isolates in this collection.

In addition to topoisomerase mutations, overexpression of the AcrAB-TolC efflux pump has been shown to mediate reduced-susceptibility to fluoroquinolones in *Salmonellae* (Chen et al., 2007). As the AcrAB-TolC efflux pump was originally found in *Escherichia coli*, there are considerable structural and functional similarities between the pump in *E. coli* and *Salmonella* (Pomposiello and Demple, 2000).

Transcription of *acrB* was detected in all 24 *Salmonella* isolates, supporting the constitutive expression of the AcrAB-TolC efflux pump in *Salmonella*. The AcrAB-TolC pump was originally isolated in *Escherichia coli* and identified as a major mechanism in fluoroquinolone resistance (Piddock et al., 2000). It was shown that inactivation of the pump resulted in loss of fluoroquinolone resistance in the strains with *gyrA* mutations (Oethinger et al., 2000). This may not be always the case since some ciprofloxacin resistant isolates in this study (i.e. SA578, SA609, SA610 and SA611) did not overproduce AcrB when compared to the reference strain. Concurrently, the ciprofloxacin susceptible isolates in Gr.5 (MIC=0.125 μg/ml) produced AcrB from 6 to 221 folds. The contribution of the AcrAB-TolC pump in these strains should be minimal (if any) and may not be accurately determined due to high susceptibility to ciprofloxacin of the strains. In addition to fluoroquinolone resistance, the AcrAB-TolC efflux pump plays a major role in multiple antibiotic resistance in *Salmonella*. Deletion of the *acrAB* operon resulted in increased

susceptibility to multiple drugs e.g. chloramphenicol, ceftiofur, tetracycline, cephalothin, trimethoprim-sulfamethoxazole. Despite these previous reports, the same way may not be true in our strains. The best evidences were overproduction of AcrB in SA736 that was susceptible to all antimicrobials (221 folds) and SA721 that was resistant to tetracycline only (18 folds).

Particular attention was paid to SA736 that was susceptible to ciprofloxacin and all other antimicrobials tested while lacked mutation in topoisoamease genes and overproduced AcrB up to 221 folds. Its genetic and phenotypic property illustrated the inconsistent role of the AcrAB-TolC efflux pump, while substantiated role of mutations in the target genes in fluoroquinolone resistance in the *Salmonella* clinical isolates. Therefore, further studies are warranted to elucidate the actual involvement of these mechanisms in the clinical isolates.

Part 3 Genetic relatedness among *Salmonella enterica* from pork and humans.

MLST is a sequencing-based subtyping method that generally examines the DNA sequences of full or partial housekeeping genes and has been used to infer genetic relatedness between various bacterial isolates including *Salmonella* (Kotetishvili et al., 2002). MLST was one of the best molecular typing methods due to its discriminatory ability, the nonambiquous resulting DNA sequences, easy comparison through the web-based databases and providing phylogenetic relationships among isolates (Noda et al., 2011). Therefore, MLST has been increasingly applied as an epidemiological tool for the evolutionary analyses among clinical isolates.

MLST for *Salmonella* was previously described and the sequences of 450-600 nucleotide fragments of seven-housekeeping genes were originally used (Kotetishvili et al., 2002). It was recently suggested that MLST using several house-keeping genes may not be appropriate for discrimination the closely-related Salmonella strains within a given serovar due to high sequence homology and slow accumulation of variations in the house-keeping genes. Therefore, the MLST based on seven house-keeping genes may be satisfactorily used for distinguishing among diverse *Salmonella* serovars.

The MLST schemes based on sequencing of three virulence genes including *manB, mdh* and *fimA* was later developed for *Salmonella*. A previous study suggested that the MLST scheme using *fimA* virulence gene failed to discriminate between some outbreak strains (Stepan et al., 2011). However, the MLST schemes using the sequencing combination of *manB, mdh* and *fimA*) was designed for epidemiological investigation of *Salmonella* outbreaks (Alcaine et al., 2005; Sukhnanand et al., 2005). The three-gene MLST were shown to provide discriminatory power similar to that of a seven genes-based MLST (Alcaine et al., 2006), more discriminative than PFGE (Kotetishvili et al., 2002) and more economical. The *manB, mdh* and *fimA* genes have been used to study the genetic relatedness among *Salmonella* (Alcaine et al., 2005; Sukhnanand et al., 2005). However, the results may not be appropriate to compare with those available online-databases. This is because the web-databases are usually derived from seven-housekeeping gene MLS (Alcaine et al., 2006).

In this study, 40 *Salmonella* isolates of 3 different serotypes including Typhimurium, Kedougou and Weltrevreden from pork and humans were analyzed using the scheme

based on sequencing of *manB, mdh* and *fimA*. These serovars were most common pork- and human-associated serotypes among *Salmonella* in this collection. A previous study reported that *S.* Weltevreden was commonly found in pork and patients in Thailand (Padungtod et al., 2008) and it was also suggested that the human cases caused by these three serotypes may be linked to the consumption of contaminated pork. In the present study, the *Salmonella* isolates were grouped into 3 different clusters and most *Salmonella* of the same serovars were in the same cluster. All the Kedogou serovars (10 pork isolates and 10 human isolates) were sequenced typed into the same cluster A, indicating the overlapping populations of the *Salmonella* subtypes from these two different sources. Similarly, all the Typhimurium serovars but one were included in cluster B and all the Weltrevreden serovars but one were in cluster C. This was consistent with previous studies demonstrating that overall *Salmonella* isolates in the same cluster were in the same serotypes (Alcaine et al., 2005). The data from phylogenetic tree indicated that the *Salmonella* in all three clusters are presumed to have the common ancestor. Besides, most of pork and human isolates in each cluster showed a high similarity, indicating the closed relationship among the isolates. Taken together, the results provided evidence that pork is a reservoir for the *Salmonella* serotypes in humans. The similar results were previously observed among the isolates in other countries e.g. *Salmonella* from pork and humans in Mexico (Zaidi et al., 2006) and Germany (Hauser et al., 2010). In addition, different serovars were classified into a single cluster. For example, a serovar Weltrevreden was in cluster B and a serovar Typhimurium was also in cluster B. Such multiple serotypes within the same cluster were observed in former studies (Alcaine et al., 2006) and supported the suggestion made in the latter that MLST provided a slight-more sensitivity than serotyping.

Conclusion and suggestions

From the findings of this study, we conclude that there is the widespread of class 1 integrons among *Salmonella* isolated from pork and humans. Class 1 integrons carrying resistance-encoding gene cassettes play an important role in multidrug-resistance phenotype and dissemination of antibiotic resistance among the pathogens. The gene cassette array *dfrA12-aadA2* was the most prevalent among our isolates and previously reported in *Salmonella* from different countries. These data confirm that horizontal gene transfer play a major role in worldwide dissemination of the *dfrA12-aadA2* gene cassette array and of course, class 1 integrons.

Based on the previous studies in our laboratory, the *dfrA12-aadA2* was idenfified in in *Salmonella* from different sources including poultry, swine (Khemtong and Chuanchuen, 2008; Padungtod et al., 2011) and dairy cows (Chuanchuen et al., 2010). In addition, this cassette was also found in other bacterial pathogens, including *Aeromonas hydrophila* from Nile Tilapia (Lukkana et al., 2011) and *E. coli* from swine (Lay et al., 2012). When these data are combined, they provide more evidence that food producing animals and their products could serve as vehicles of transmission of antimicrobial resistance determinants. The gene cassette has been identified in bacteria of different geographical regions. Taken together, it confirms that horizontal transfer of resistance determinants plays an important role in the dissemination of multi-drug resistance among bacterial pathogens (Figure 17).

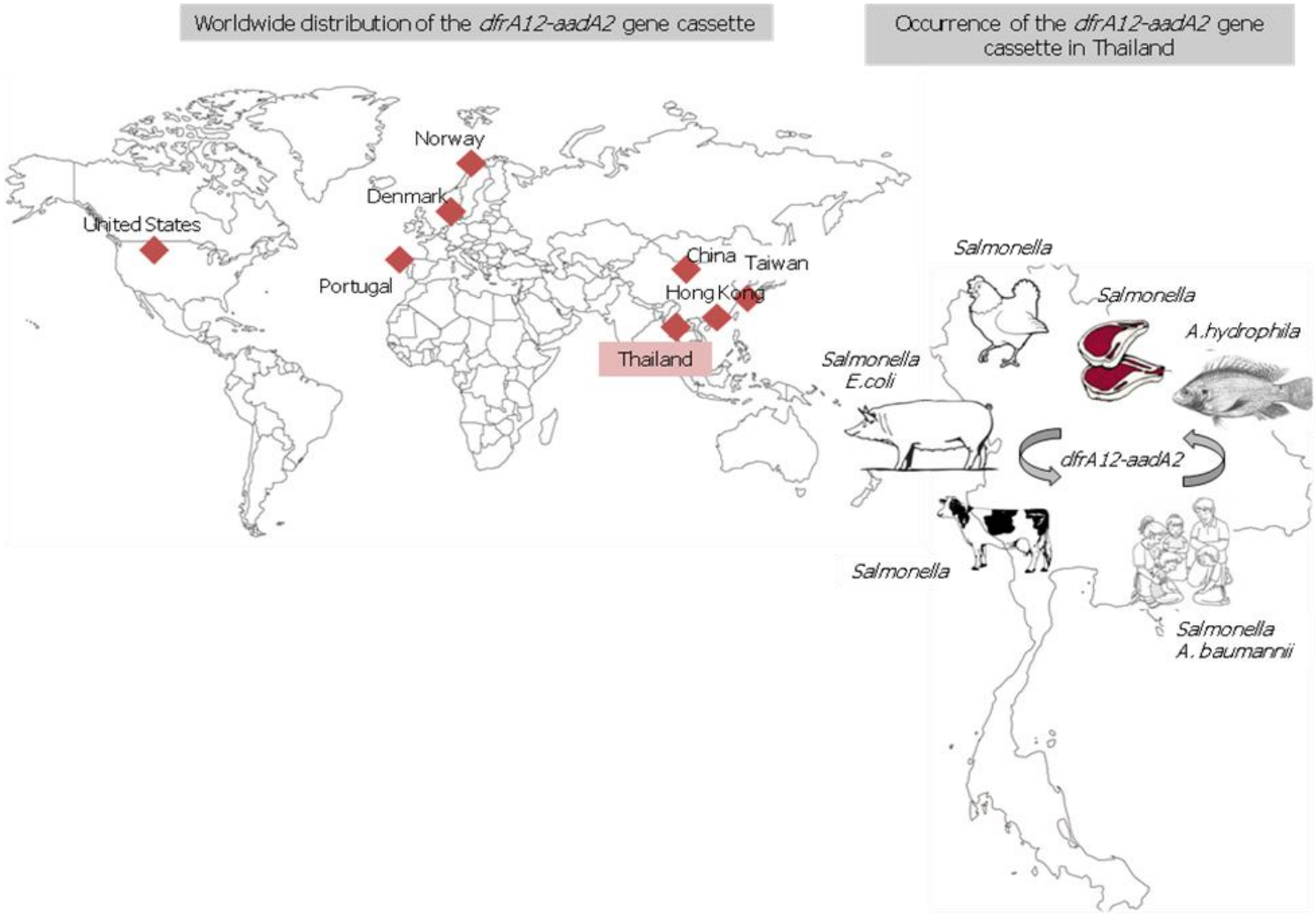

Figure 17: Dissemination of class 1 integrons containing *dfra12-aadA2* gene cassette array in Thailand and other countries

Our data demonstrated clonal relationship of *Salmonella* MDR strains from pork and humans. Therefore, both horizontal transfer and clonal dissemination of MDR *Salmonella* were confirmed and the possible route of dissemination through the food chain could be drawn (Figure 18).

High prevalence of reistance and virulence genes observed confirms that *Salmonella* contribute to the pool of these two determinants. The presence of class 1 integrons and virulence genes on the same plasmid supports that a single antimicrobial could coselect for both determinants, resulting in the more resistant and more virulent *Salmonella* strains.

The results from this study confirmed the need of prudent and responslbe use of antimicrobials. Many antimicrobials are used in both animals and humans. Therefore, these antimicrobials must be used carefully. Up to date, there are a number of antimicrobial regulations for *Salmonella* authorized by relevent authorities in various countries and such regulations vary from one country to another. In general, the common-important principles include that antimicrobials should be used only when it is necessary. The prescription of antimicrobials should be made after the laboratory results i.e. causative bacteria and antimicrobial susceptibility is obtained. Role of veterinarian in control of antimicrobial use, particularly in food-producing animals, needs to be strengthened and educating the public about the issue of antimicrobial resistance needs to be provoked.

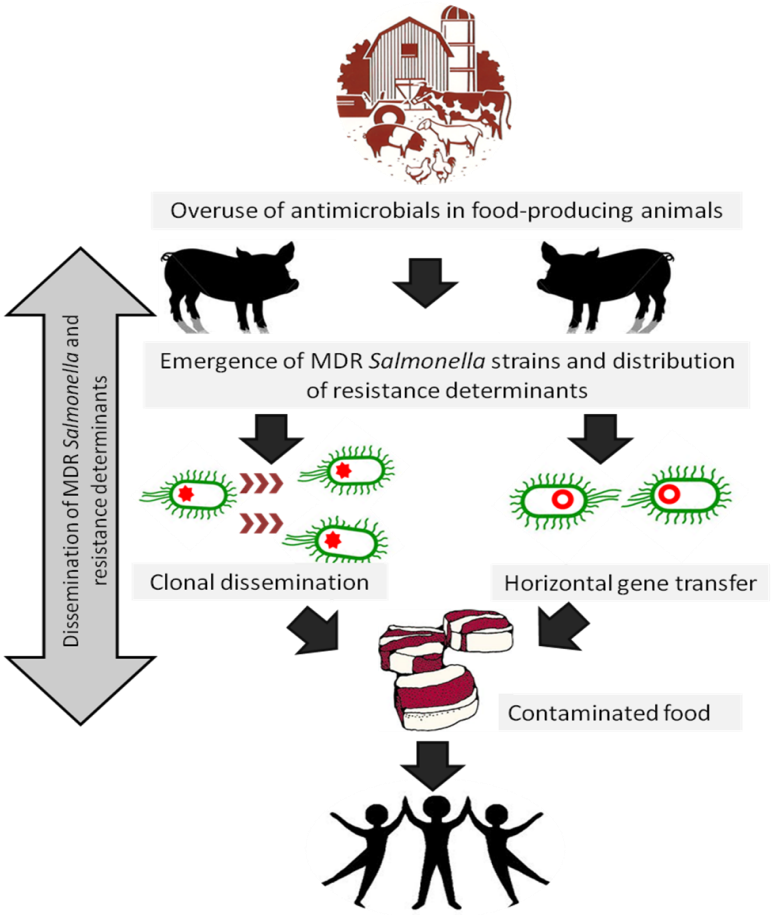

Figure 18: Dissemination of MDR *Salmonella* strains from farm to fork.

Additional suggestions and further studies are as follows:

- The data obtained could be applied in development of antimicrobial guidelines to reduce the use of antimicrobial in food animals.

- National database of antimicrobial resistance should be established. The main purpose is to monitor antimicrobial usage in food animals, identify problems of

antimicrobial resistance in food animals and humans and provide necessary data for risk assessment.

- Further studies include:

    1. Study of non-antimicrobial alternatives to compensate the requirement of antimicrrbial use in food-producing animals.

    2. Characterization of antimicrobial resistance in other animals and along the food chain.

    3. Characterization of antimicrobial resistance in commensal bacteria. This is because commensal bacteria serve as a reservoir of resistance determinants for both pathogenic and noptahogenic bacterial strains.

    4. Risk assessment of antimicrobial resistance in bacteria.

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APPENDICES

APPENDIX A

Mutation in *gyrA*, *gyrB*, *parC* and *parE* genes of the ciprofloxacin resistant *Salmonella* isolates

Gene	Mutation		No.(%)
	Nucleotide substitution	Amino acid substitution	
gyrA	G-144-T	Met-48-Ile	5 (27.7)
	C-133-T	Pro-45-Ser	1 (5.5)
gyrB	C-144-T	Silent mutation	13 (72.2)
	G-147-A	Silent mutation	14 (77.7)
	A-171-G	Silent mutation	2 (11.1)
	T-183-C	Silent mutation	12 (66.6)
	T-188-C	Val-63- Ala	6 (33.3)
	T-216-C	Silent mutation	17 (94.4)
	C-222-T	Silent mutation	17 (94.4)
	G-237-T	Silent mutation	16 (88.8)
	C-243-T	Silent mutation	1 (5.5)
parC	T-2-C	Met-1- Thr	6 (33.3)
	A-13-C	Ser-5- Arg	1 (5.5)
	G-31-C	Ala-11-Pro	12 (66.6)
	C-62-T	Ser-21- Leu	12 (66.6)
	T-86-C	Ile-29- Thr	12 (66.6)
	C-92-T	Thr-31- Met	7 (38.8)
	C-152-T	Thr-51- Ile	6 (33.3)
	G-173-A	Gly-58- Glu	1 (5.5)
	C-182-G	Ala-61- Gly	6 (33.3)
	A-212-G	Gln-71- Arg	12 (66.6)
	T-230-C	Leu-77- Pro	7 (38.8)
	T-230-G	Leu-77- Arg	4 (22.2)
	C-245-T	Thr-82- Met	14 (77.7)

Mutation in *gyrA*, *gyrB*, *parC* and *parE* genes of the ciprofloxacin resistant *Salmonella* isolates

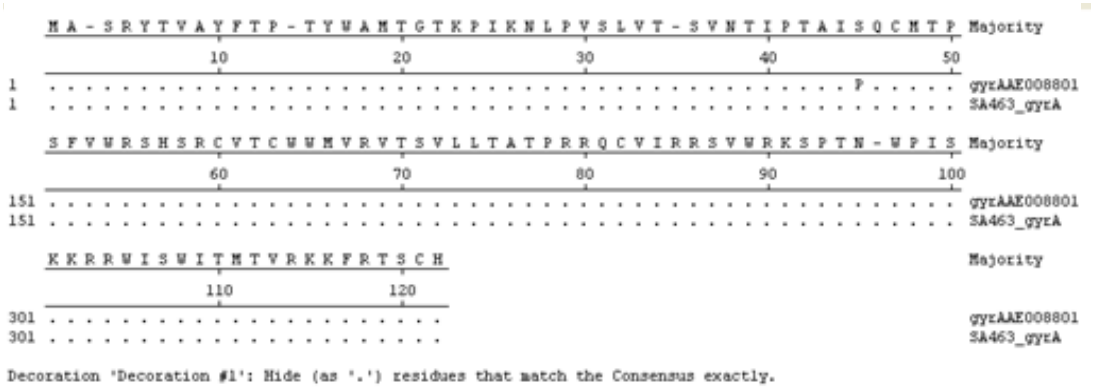
Gene	Mutation		No.(%)
	Nucleotide substitution	Amino acid substitution	
parE	C-231-T	Silent mutation	1 (5.5)
	C-255-T	Silent mutation	1 (5.5)
	G-258-T	Silent mutation	1 (5.5)
	A-270-G	Silent mutation	18 (100)
	G-273-A	Silent mutation	11 (61.1)
	C-285-T	Silent mutation	2 (11.1)
	T-390-G	Silent mutation	4 (22.2)
	C-403-T	Silent mutation	2 (11.1)
	C-417-T	Silent mutation	18 (100)
	C-450-T	Silent mutation	6 (33.3)
	A-459-G	Silent mutation	9 (50)
	A-468-G	Silent mutation	4 (22.2)

APPENDIX B

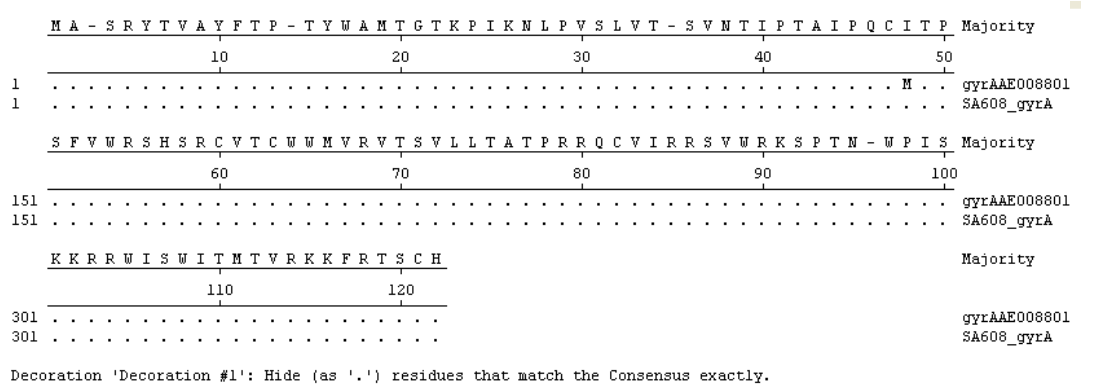
Nucleotide sequences

Mutation in *gyrA* gene

SA463

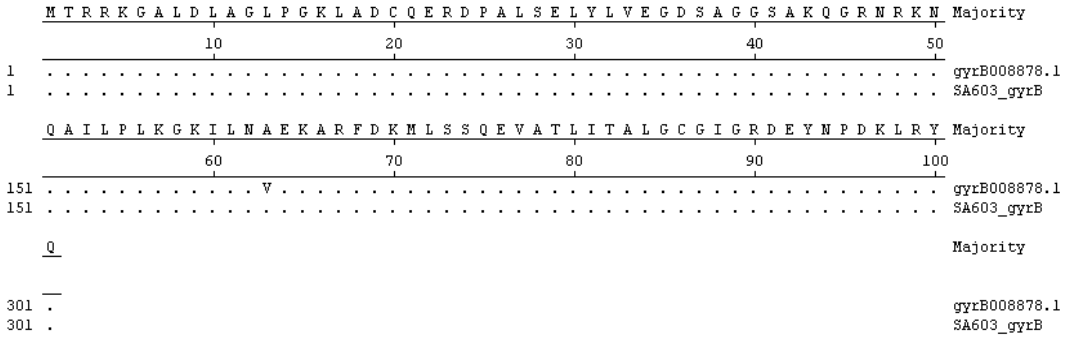


SA608



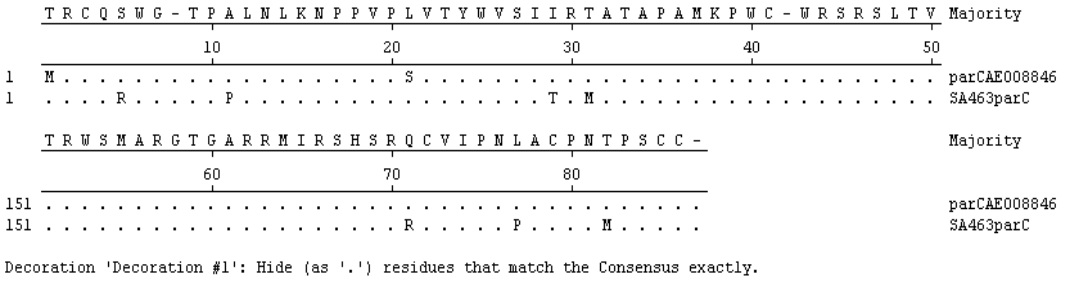
Mutation in *gyrB* gene

SA671

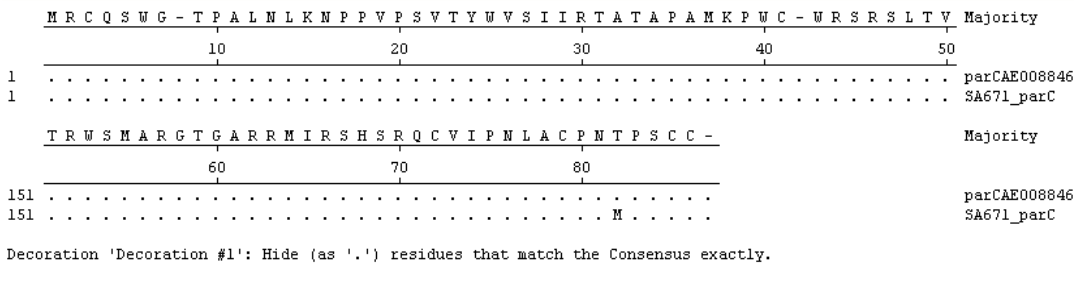


Mutation in *parC* gene

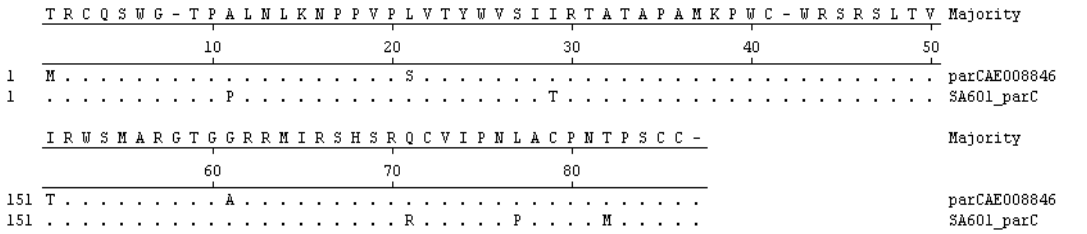
SA463



SA671

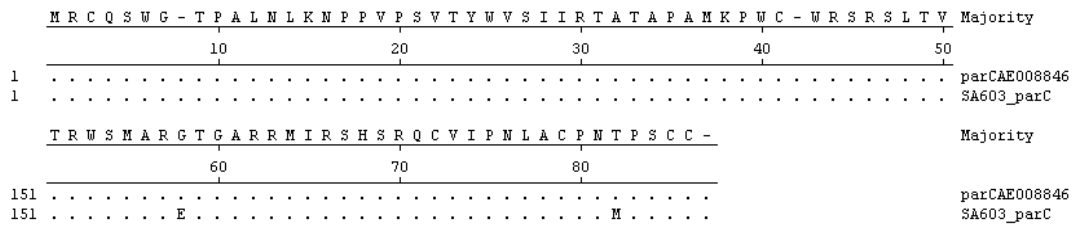


SA601



Decoration 'Decoration #1': Hide (as '.') residues that match the Consensus exactly.

SA603



Decoration 'Decoration #1': Hide (as '.') residues that match the Consensus exactly.

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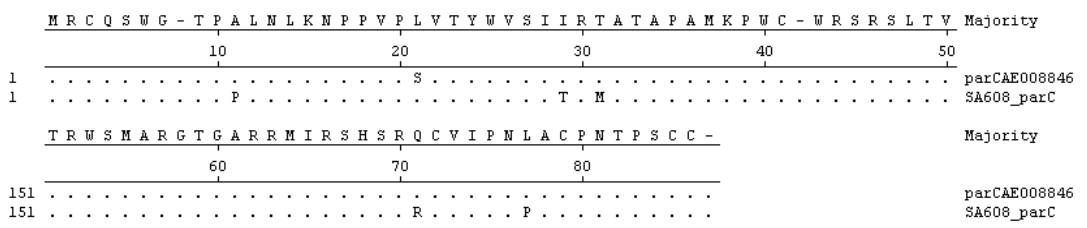
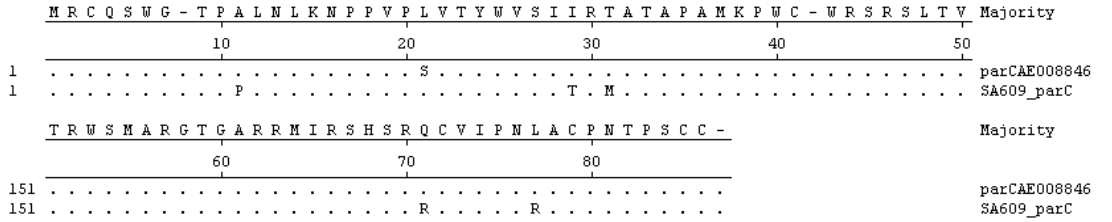
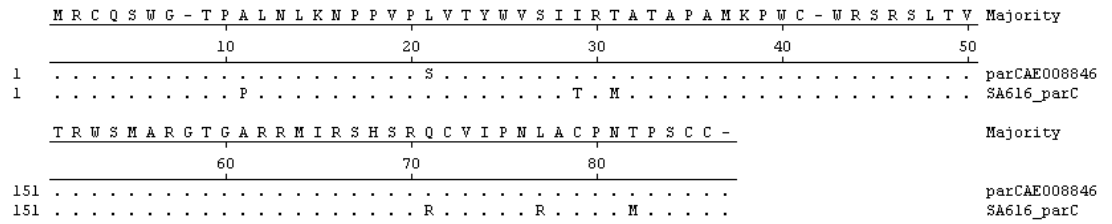


Decoration 'Decoration #1': Hide (as '.') residues that match the Consensus exactly.

SA609



SA616



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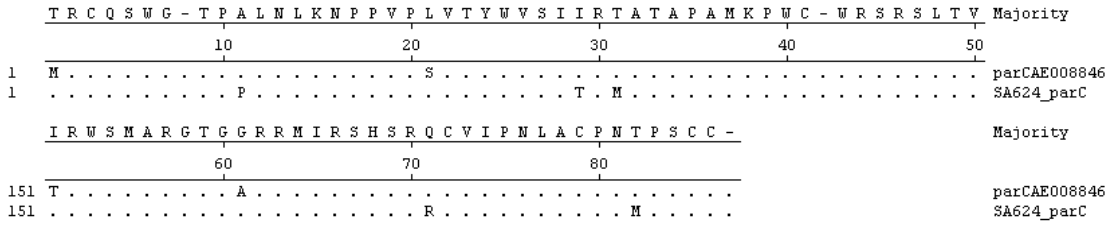
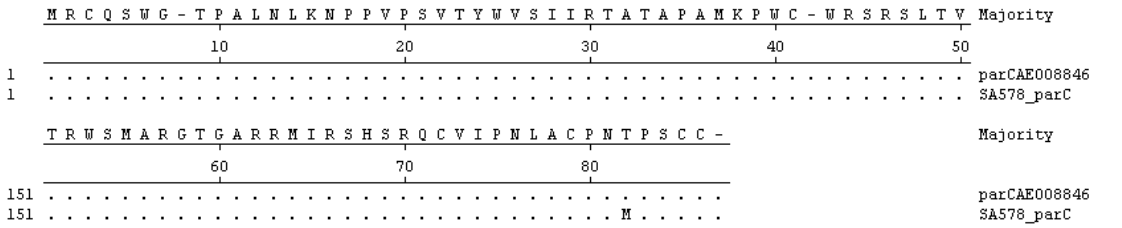
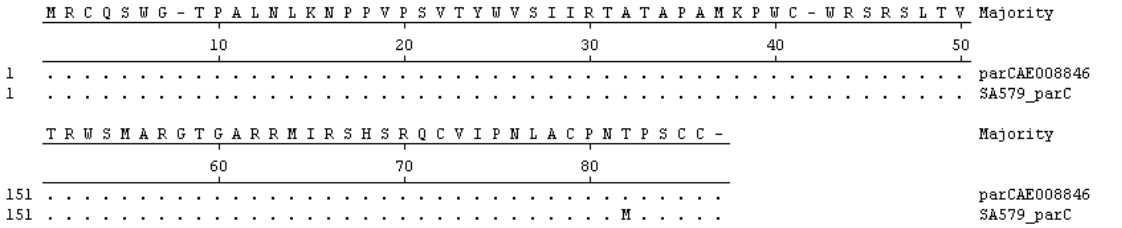


SA578



Decoration 'Decoration #1': Hide (as '.') residues that match the Consensus exactly.

SA579



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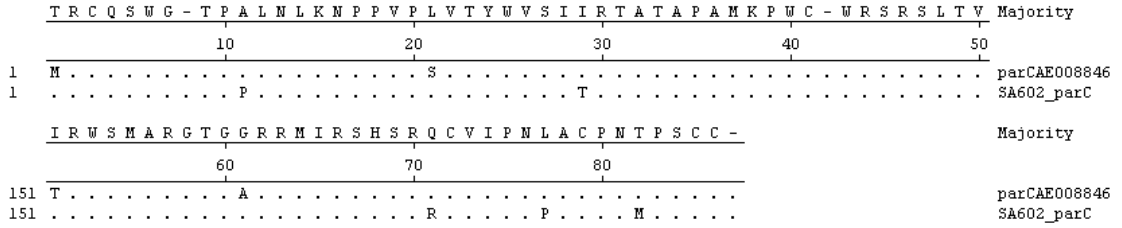
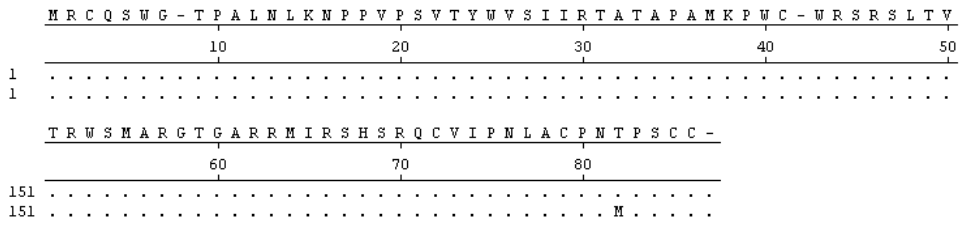
SA602



Decoration 'Decoration #1': Hide (as '.') residues that match the Consensus exactly.

SA613



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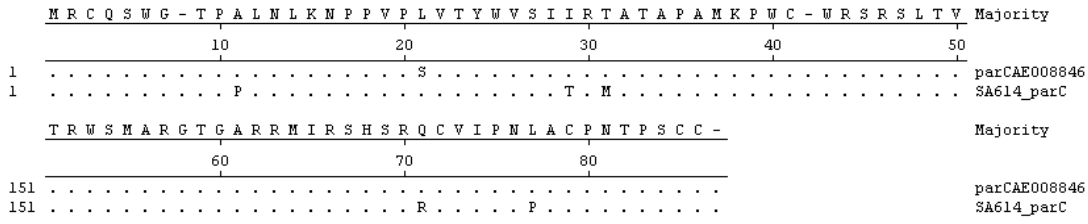
SA614



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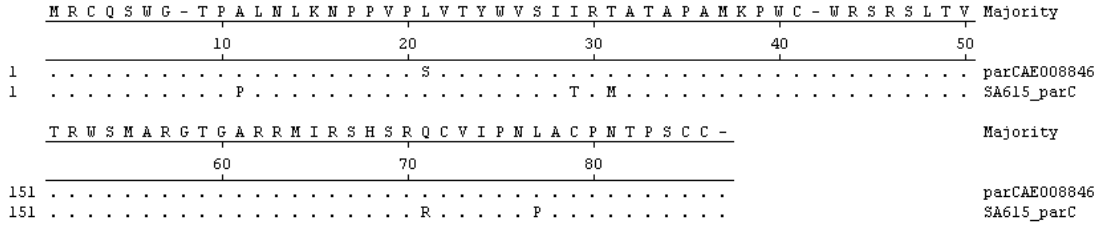

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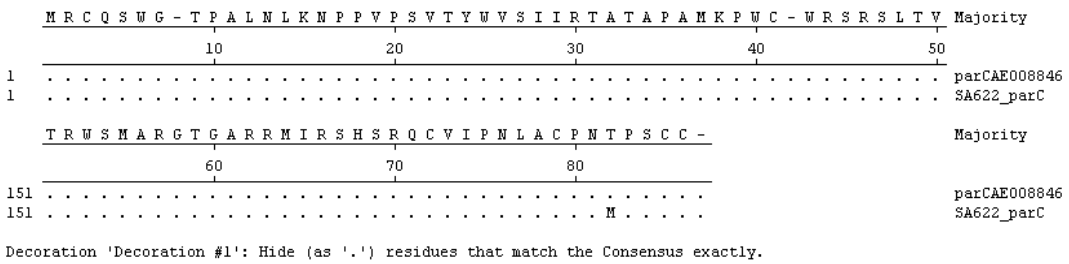
SA622



QRDRs region of *gryA* gene, 367 nucleotides; GenBank: AE008801

gagatggcctgaagccggtacaccgtcgcgtactttacgccatgaacgtattgggcaatgactggaacaaagcctataaa
aaatctgcccgtgtcgttggtgacgtaatcggtaaataccatccccacggcgattccgcagtgtatgacaccatcgttcgtat
ggcgcagccattctcgctgcgttacatgctggtggatggtcagggtaacttcggttctattgacggcgactccgcggcggc
aatgcgttatacggagatccgtctggcgaaaatcgcccacgaactgatggccgatctcgaaaaagagacggtggatttc
gtggataactatgacggtacggaaaaaattccggacgtcatgcc

QRDRs region of *gryB* gene, 302 nucleotides; GenBank: AE008878.1

atgacccgtcgtaaaggcgcgctcgatttagccggtctgccgggcaaactggcggactgtcaggaacgcgacccggc
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gcgattctgccgctgaaaggtaaaatccttaacgtcgagaaagcgcgcttcgacaagatgctttcctcccaggaagtggc
gacgctgatcaccgcgctgggctgcggtatcggtcgcgacgagtacaacccggacaagctgcgctatca

QRDRs region of *parC* gene, 262 nucleotides; GenBank: AE008846

ctatgcgatgtcagagctggggctgaacgccagcgctaaatttaaaaaatccgcccgtaccgtcggtgacgtactgggta
agtatcatccgcacggcgacagcgcctgctatgaagccatggtgctgatggcgcagccgttctcttaccgttacccgctg
gtcgatggccaggggaactggggcgcgccggatgatccgaagtcattcgcggcaatgcgttataccgaatctcgcctgt
ccaaatacgccgagctgctgtta

QRDRs region of *parE* gene, 543 nucleotides; GenBank: AE008846

atggcgattgccagcgcgcagcggcgactgcgcgccgcaaaaaaagtggtgcgcaaaaagctcaccagcggcccg
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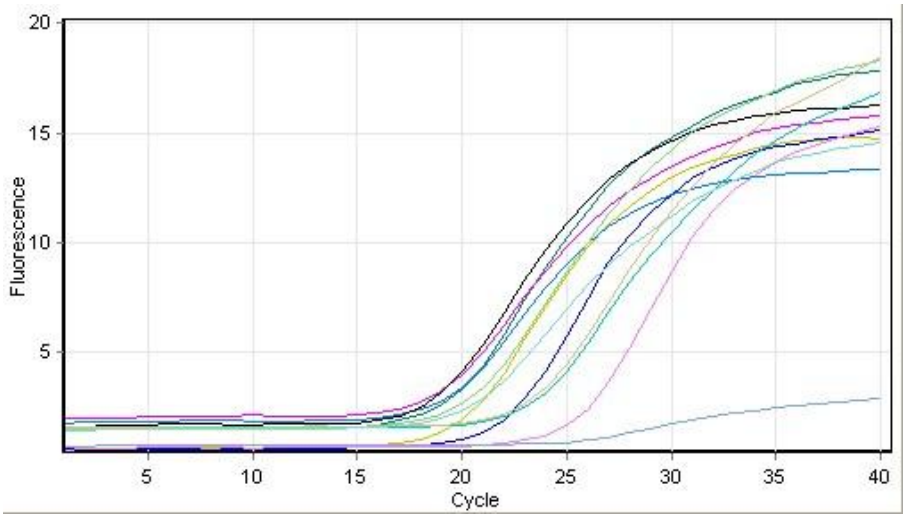
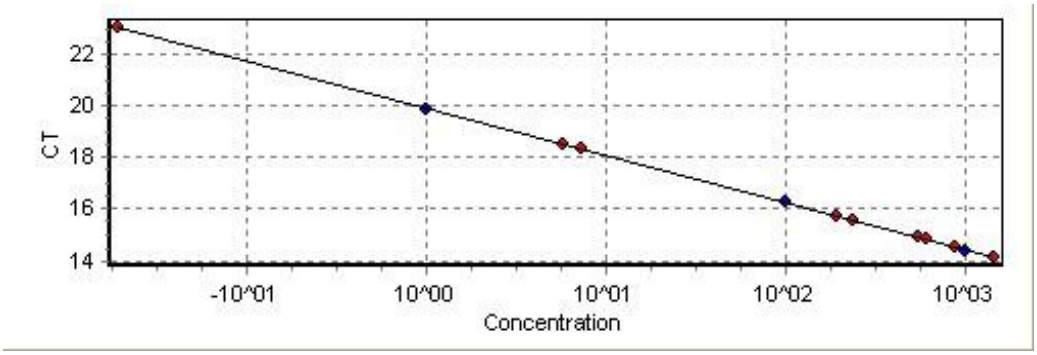
APPENDIX C

Quantitative realtime PCR

Raw Data For Cycling A.FAM



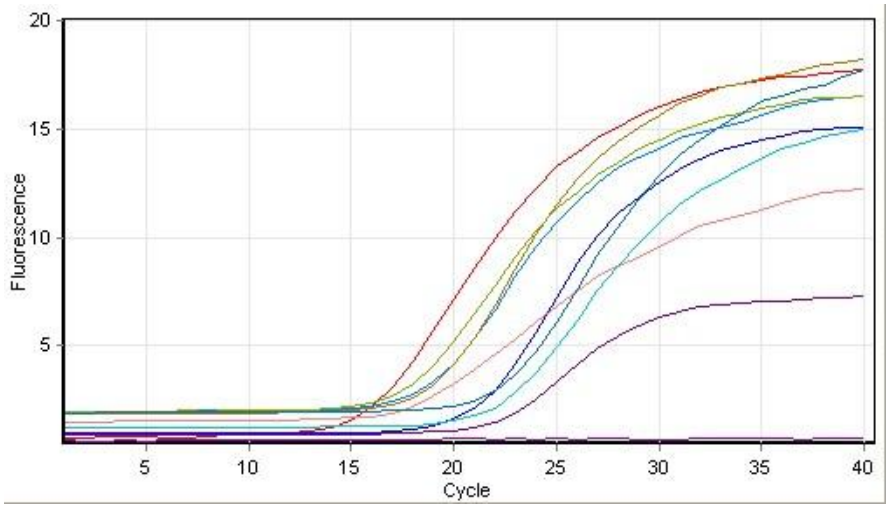
Standard Curve



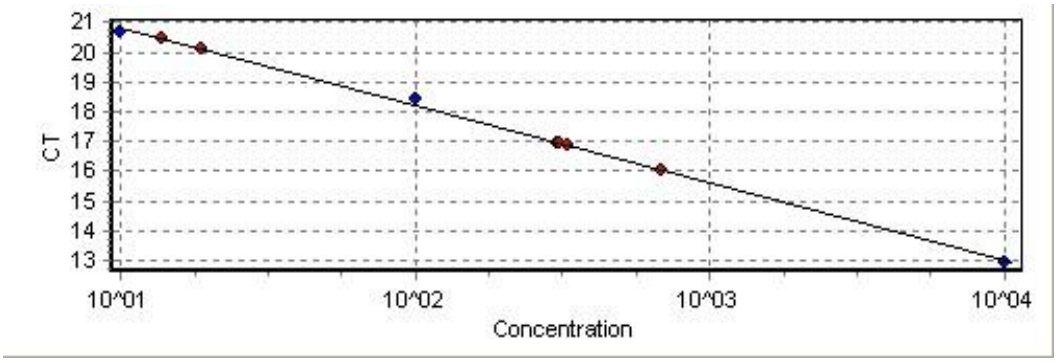
Quantitative information

No.	Colour	Name	Type	Ct	Given Conc (Copies)	Calc Conc (Copies)
2		SAWt	Standard	14.37	1,000	1,054
3		SAWt	Standard	16.30	100	92
5		SAWt	Standard	19.86	1	1
6		SA612	Unknown	14.89		547
7		SA612	Unknown	14.82		598
10		SA624	Unknown	14.52		874
11		SA624	Unknown	14.13		1,431
12		SA615	Unknown	18.32		7
13		SA615	Unknown	18.50		6
14		SA622	Unknown	15.55		237
15		SA622	Unknown	15.72		191
16		neg	Negative Control	23.01		

Raw Data For Cycling A.FAM



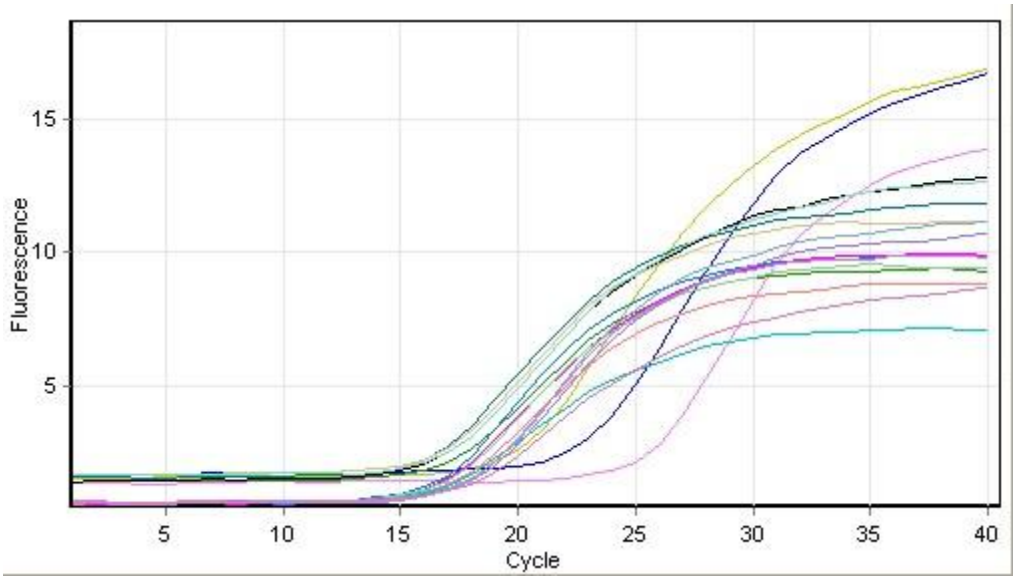
Standard Curve



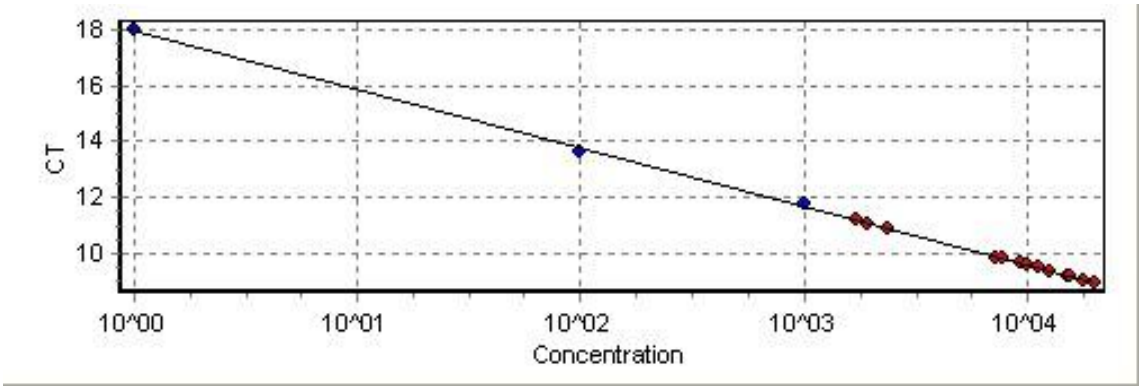
Quantitative information

No.	Colour	Name	Type	Ct	Given Conc (Copies)	Calc Conc (Copies)
1		SAWt	Standard	12.94	10,000	10,719
3		SAWt1:100	Standard	18.45	100	81
4		SAWt1:1000	Standard	20.66	10	11
6		SA721	Unknown	16.94		309
8		SA721	Unknown	16.96		305
12		SA734	Unknown	20.10		19
21		SA666	Unknown	16.87		330
22		SA666	Unknown	16.05		684
24		SA734	Unknown	20.46		14
26		neg acr	Negative Control			

Raw Data For Cycling A.FAM



Standard Curve



Quantitative information

No.	Colour	Name	Type	Ct	Given Conc (Copies)	Calc Conc (Copies)
2		SAWt	Standard	11.77	1,000	921
3		SAWt	Standard	13.66	100	113
5		SAWt	Standard	17.97	1	1
6		SA671	Unknown	9.85		7,714
7		SA671	Unknown	9.67		9,387
8		SA463	Unknown	9.20		15,775
9		SA463	Unknown	10.91		2,379
10		SA734	Unknown	9.61		10,000
11		SA734	Unknown	8.98		20,156
12		SA608	Unknown	9.51		11,259
13		SA608	Unknown	9.23		15,284
14		SA603	Unknown	9.09		17,902
15		SA603	Unknown	9.90		7,308
16		SA602	Unknown	9.39		12,777
17		SA602	Unknown	9.62		9,974
18		SA579	Unknown	11.11		1,916
19		SA579	Unknown	11.20		1,734

BIOGRAPY

Miss Wechsiri Wannaprasat was born on July 16, 1981 in Saraburi, Thailand. She obtained Doctor of Veterinary Medicine degree and Master of Science degree from Chulalongkorn University, Thailand in 2005 and 2007, respectively. She enrolled in the degree of Doctor of Philosophy Program in Veterinary Public Health, Department of in Veterinary Public Health, Faculty of Veterinary Science, Chulalongkorn University since academic year 2009. During study in PhD program, she received the scholarship from the Royal Golden Jubilee Ph.D. Program.

This dissertation results in 3 publications as follows: 1. **Wannaprasat W.**, Padungtod P. and Chuanchuen R. 2011. Class 1 integrons and virulence genes in *Salmonella enterica* isolates from pork and humans. Int. J. Antimicrob. Agents. 37(5):457-61. 2. Mutations in Topoisomerase genes and expression of the AcrAB-TolC efflux system in *Salmonella enterica* from pork and humans. Thai J. of Vet. Med. Submitted. 3. Genetic relatedness among *Salmonella enterica* from pork and humans. (Manuscript in preparation).

Previous publications:

1. Chuanchuen, R., **Wannaprasat W.**, Ajariyakhajorna K. and Schweizer H.P. 2007. Role of the MexXY multidrug efflux pump inaminoglycoside resistance in *Pseudomonas aeruginosa* isolates from *Pseudomonas* mastitis. Microbiol. Immunol. 52(8):392-398.

2. Chuanchuen, R., **Wannaprasat W.**, Ajariyakhajorna K. and Schweizer H.P. 2007. Functional characterization of MexXY and OpmG in aminoglycoside efflux in *Pseudomonas aeruginosa*. Southeast Asian J Trop Med Public Health. 39(1):115-122.

3. Chuanchuen, R., Pathanasophon P., Khemtong S., **Wannaprasat W.** and Padungtod P. 2007. Susceptibility to and cross-resistance between antibiotics and disinfectants in *Salmonella enterica* isolates from poultry and swine. J. Vet. Med. Sci. 70(6):595-601.

4. **Wannaprasat W.**, Koowatananukul C., Ekkapobyotin C. and Chuanchuen R. 2009. Numbers, species and antimicrobial resistance of *Lactobacillus* and *Bacillus* in commercial probiotic products for food animals. J. Vet. Med. Sci. 40(5):1103-12.

5. Chuanchuen R., Ajariyakhajorn K., Koowatananukul C, **Wannaprasat W**, Khemtong S and Samngamnim S. 2010. Antimicrobial resistance and virulence genes in *Salmonella enterica* isolates from dairy cows. Foodborne Pathog. Dis. 7(1):63-9.