STUDIES ON MOLECULAR EPIDEMIOLOGY OF ANTIMICROBIAL RESISTANCE GENES IN SALMONELLA ISOLATED FROM PIG FARMS

INTRODUCTION

Salmonella are gram-negative bacterial pathogens causing food-borne diseases and resulting in public health concern worldwide. These pathogens are infected by consumption of contaminated raw food or direct contact with carrier animals (Pang *et al.*, 1995). Many evidences have been reported that human illnesses are attributed to food-borne pathogens from animal origin, especially the most favorite food including pork (Swartz, 2002). Although, these illnesses are generally considered as self-limiting diarrhea, in some cases, antimicrobial therapy is still required, particularly in children, elder and immunocompromised patients (Lee *et al.*, 1994). However, an improper using of antimicrobials in both medication and agriculture probably also results in an emergence of resistant strains.

The development of antimicrobial resistance among *Salmonella* is becoming a serious problem, especially the emergence of multi-drug resistant (MDR) *Salmonella* strains (Duijkeren *et al.*, 2003). According to the report in 1960, *Salmonella* showed resistance to multiple antimicrobial agents such as ampicillin, chloramphenicol, and sulfamethoxazole/trimethoprim (Smith *et al.*, 1984). Moreover, *Salmonella* Typhimurium DT104 (*S.* Typhimurium DT104) known as penta-resistant phenotype has been widespread and brought about food-borne diseases in both developed and developing countries (Glynn *et al.*, 1998; Duijkeren *et al.*, 2003). These MDR strains have led to the increasing therapeutic concern throughout the world.

Many studies have revealed that *Salmonella* can carry their antimicrobial resistance genes on either chromosome or plasmids. Especially, chromosomally-encoded resistance genes can be expressed stably. However, the major mechanism in dissemination of antimicrobial resistance genes is related to mobile genetic elements including transposons and conjugative plasmids. The conjugative plasmids are

implicated potentially in dissemination of antimicrobial resistance among gram-negative bacteria that could transfer antimicrobial resistance genes from one to another strain by conjugation (Lawley *et al.*, 2004). Such MDR *S.* Typhi has been reported to be plasmid mediated (Mirza *et al.*, 2000). For the last decade, a new genetic element has been reported which was described as integrons. The integrons contain resistance gene cassettes that can be moved to other integrons (Fluit and Schmitz, 1999). The integrons have been classified into three classes, of which mostly the class 1 integrons disseminated among gram-negative bacteria (Tosini *et al.*, 1998; Dalsgaard *et al.*, 2000; Soto *et al.*, 2003).

According to Portillo (2000) conclusion, the genus *Salmonella* comprising more than 2,600 serotypes have been identified by using serological test (Popoff, 2001) and phage typing methods (Rubin and Weinstein, 1997), however, only some *Salmonella* serotypes are associated with animal-producing food which lead to human diseases (Swartz, 2002). Therefore, serotype identification of *Salmonella* is very important in epidemiologic studies. Although, serotyping is considered as a conventional scheme to characterize *Salmonella* strains, but it is laborious and time consuming. Other genotyping methods, such as pulsed field gel electrophoresis (PFGE) and random amplified polymorphic DNA (RAPD) have been successfully applied for typing. Furthermore, restriction fragment length polymorphism (RFLP) and polymerase chain reaction (PCR) based on *fliC* (phase-1 flagellin) and *fljB* (phase-2 flagellin) genes have also been used to determine serotypes of *Salmonella* strains (Dauga *et al.*, 1998; Hong *et al.*, 2003). This method has been proven to be an alternative approach to serotype *Salmonella* strain.

Majority of human food-borne illnesses is occurred by consumption of contaminated food from animal origin. Therefore, the aims of this study are to characterize *Salmonella* isolated from pig farms including their antimicrobial resistance phenotypes, their class 1 integrons and resistance genes cassettes, and examine transferring capability of antimicrobial resistance genes. Moreover, the study on molecular epidemiology of these isolates was also performed in order to

determine the diversity among these isolates. The information would be useful to control and prevent the dissemination of these MDR strains.

Objectives

1. To identify class 1 integrons and antimicrobial resistance gene cassettes in *Salmonella* isolated from pigs.

2. To examine transferring of antimicrobial resistance genes by plasmid conjugation in particular MDR *Salmonella* isolates from pigs.

3. To determine the diversity among these isolates by plasmid profiles and RFLP-PCR analysis.

LITERATURE REVIEW

1. The genus *Salmonella*

The genus *Salmonella* is a member of the family Enterobacteriaceae which comprises a large group of gram-negative bacilli. *Salmonella* are zoonotic and can be pathogenic in humans and animals (Gray and Fedorka-Cray, 2002). These pathogens are one of the most prevalent agents causing food-borne diseases in both developed and developing countries (Glynn *et al.*, 1998; Duijkeren *et al.*, 2003). *Salmonella* are ubiquitous and recovered from some insects and almost vertebrates, especially humans, livestock, pig and chicken. They can grow in both aerobic and anaerobic conditions and have ability to utilize a variety of substrates (Gray and Fedorka-Cray, 2002).

1.1 Characteristics of *Salmonella*

Salmonella are gram-negative, rod-shaped and facultative anaerobic bacteria. They are non-lactose fermenting, non-spore forming and most are motile. *Salmonella* are not fastidious organisms, they can grow under various environmental conditions outside of living hosts. The optimum condition of growth is at temperature 37^{o}C and pH 6.5-7.5. However, the organisms can not growth at the temperature below 7^{o}C and pH < 3.8. These organisms can be eliminated by cooking and pasteurization of milk (71.7^{o}C, 15 seconds) and fruit juices (70-74^{o}C, ≤20 seconds) (Gray and Fedorka-Cray, 2002).

According to Portillo (2000) conclusion, the genus *Salmonella* comprises more than 2,600 serotypes on the basis of serological and phage typing methods. The former method is based on the reaction of O antigen (lipopolysaccharide) and H antigen (flagella protein) with specific antisera (Popoff, 2001). The latter typing method is further classified using bacteriophages. Definitive types are based on the patterns of resistance or susceptibility to a set of bacteriophages (Rubin and Weinstein, 1997). However, only a limit set of *Salmonella* serotypes is associated

with food animals which lead to human diseases (Swartz, 2002). Some serotypes prefer to cause systemic infection in particular hosts. For example, *S.* Typhi and *S.* Paratyphi cause systemic infection only in humans, whereas *S.* Choleraesuis infects pig. Furthermore, some serotypes including *S.* Typhimurium and *S.* Enteritidis are able to cause gastroenteritis in both humans and animals. (Gray and Fedorka-Cray, 2002).

1.2 Diseases caused by *Salmonella* infection

Salmonella infections lead to diseases known as salmonellosis which result in public health concern worldwide. These pathogens are infected by consumption of contaminated raw food or contact with carrier animals directly (Pang *et al.,* 1995). Human diseases caused by these organisms are divided into two major groups. The first known as “non-typhoid salmonellosis or gastroenteritis” which is a localized infection of intestinal epithelium. While the second is systemic infection which known as “typhoid salmonellosis or enteric fever” (Miller *et al.,* 1995). In addition, the manifestation of salmonellosis in human can be divided into four clinical patterns: gastroenteritis, enteric fever, septicemia and asymptomatic carrier (Gray and Fedorka-Cray, 2002). In case of asymptomatic carrier is very important, because of the pathogens can be survive in this host for long periods and result in the potential source for distribution of these pathogens.

Certain serotypes are associated with particular clinical patterns, such as *S.* Typhimurium and *S.* Enteritidis cause gastroenteritis in humans and animals. While enteric fever in humans is affected by *S.* Typhi and *S.* Paratyphi, *S.* Choleraesuis infection cause bacteremia in pig (Gray and Fedorka-Cray, 2002). The outcome of this infection depends on serotype, type and immune status of host, and dose of ingestion. In general, the clinical signs of food-borne illnesses are vomiting, nausea, fever, abdominal pain and diarrhea (Baumler *et al.,* 1998). Typically, gastroenteritis in humans begins 24-48 hours after ingestion with fever, nausea and vomiting, follow with abdominal pain and diarrhea (Gray and Fedorka-Cray, 2002). However, these illnesses are considered as self-limiting infection.

1.3 Transmission and treatment

Contaminated food is a common source of salmonellosis infection for humans. Food-borne illness was most commonly associated with the consumption of contaminated raw food or contact with carrier animals directly (Pang *et al.*, 1995). According to a case-control study performed in England and Wales, humans were infected with *S.* Typhimurium DT104 from eating contaminated food more than contact with ill animals (Hogue *et al.*, 1997). Many reports represented that *Salmonella* were isolated in various food-producing animals such as cattle, pigs and chickens (Gray and Fedorka-Cray, 2002). These data resulted in an important possible transmission of these pathogens to humans.

The primary therapeutic scheme for *Salmonella* gastroenteritis is supportive therapy with fluid and electrolyte replacement (Gray and Fedorka-Cray, 2002). Due to non-typhoid salmonellosis results in a self-limiting diarrhea, hence, antimicrobial therapy is not essential for treatment in this situation. However, severe invasive disease or prolonged illness in suppressed immunucompromise patients can occur. Moreover, in case of new-borne, infant, children and elderly who are at risk of septicemia, antimicrobial therapy is still required (Lee *et al.*, 1994). For humans, the treatment of choice for enteric fever and septicemia is often cetriaxone (a third generation of cephalosporins). While, ampicillin and ciprofloxacin should be used in chronic carrier patients (Gray and Fedorka-Cray, 2002). However, an inappropiate using of antimicrobial agents in both medication and agriculture may result in an emergence of resistant strains. The development of antimicrobial resistance among *Salmonella* is becoming a serious problem, especially the emergence of multi-drug resistant (MDR) *Salmonella* strains (Duijkeren *et al.*, 2003). These MDR strains have led to the increasing therapeutic concern throughout the world.

2. Antimicrobial resistance in *Salmonella*

Antimicrobial agents were implicated that they are capable in bacterial diseases elimination. Although, plenty of antimicrobial drugs are used in medication, these drugs have limited structural diversity and fall into only few classes as shown in Table 1 (Macfarlane and Hancock, 2000). A new chemical class of antimicrobials such quinolones and fluoroquinolones has been used many years ago. However, no new class of antimicrobial agents has been introduced for many years (Macfarlane and Hancock, 2000).

Table 1 Major classes of antimicrobial agents in current medical use.

Drug class	Mechanism of action	Mechanism of resistance
Aminoglycosides	Inhibition of protein synthesis	Enzymatic modification of drug, efflux
	Inhibition of initiation of DNA synthesis	Alterations in energy of uptake, mutation of target
Tetracycline	Inhibition of protein synthesis	Increased efflux
Chloramphenicol	Inhibition of protein synthesis	Enzymatic modification of drug
Macrolides	Inhibition of protein synthesis	Target modification
Fusidans	Inhibition of polypeptide chain elongation	Efflux
β-Lactams	Inhibition of cell wall biosynthesis and assembly, stimulation of autolysins	Enzymatic modification of drug
Glycopeptides	Inhibition of cell wall biosynthesis	Structural modification of peptidoglycan
Quinolones and fluoroquinolones	Inhibition of DNA gyrase	Target modification, decreases uptake due to porin modification
Novobiocin	Inhibition of DNA gyrase	Efflux
Rifamycins	Inhibition of nucleic acid synthesis	Target modification
Polymyxins and colistin	Disruption of outer membrane	LPS modification
Sulfonamides	Inhibition of folic acid synthesis	Target modification, efflux
Trimethoprim	Inhibition of folic acid synthesis	Target modification, efflux

Source: Macfarlane and Hancock (2000)

2.1 The increases of antimicrobial resistance

The emergence of antimicrobial resistant bacteria has become a serious problem worldwide, especially, antimicrobial resistance of food-borne pathogens (White *et al.*, 2002; Tjaniadi *et al.*, 2003). According to the report in 1960, *Salmonella* conferred resistance to several classes of antimicrobial agent such as ampicillin, chloramphenicol, and sulfamethoxazole/trimethoprim (Smith *et al.*, 1984). Furthermore, *Salmonella* Typhimurium DT104 (*S.* Typhimurium DT104) which was known as penta-resistant phenotype, commonly resist to ampicillin, chloramphenicol, streptomycin, sulfonamide and tetracycline (ACSSuT), has been widespread and brought about food-borne diseases in both developed and developing countries (Glynn *et al.*, 1998; Duijkeren *et al.*, 2003). In addition, Carlson and Ferris (2000) reported that *S.* Typhimurium DT104 conferred resistance to new type of antimicrobial agents including amoxicillin and tricarcillin. Chiu *et al.* (2002) and Kristiansen *et al.* (2003) reported that *S.* Choleraesuis and *S.* Typhimurium DT104 was also developed resistance to antimicrobial agents of fluoroquinolones and quinolones respectively. These MDR strains have led to the increasing therapeutic concern around the world.

The major cause of the emerged MDR *Salmonella* strains may result from an inappropiate use of antimicrobial agents in both medical treatment for humans and animals. Especially, antimicrobials were used simultaneously in feeding animals in order to promote growth and prevent from bacterial infection. These situations of antimicrobial agents using could be selected only resistant strains. For example, the trend of antimicrobial resistance strain of *S.* Typhimurium has been raised (Davis *et al.*, 1999). Particularly, *S.* Typhimurium DT104 conferred resistance to penicillin derivatives and has also increased MIC value (Carlson and Ferris, 2000). In addition, resistance to a new drug such as ceftriaxone in *S.* Anatum was also found (Su *et al.*, 2003). These reports suggesting that the adaptive MDR strains were not only capable to resist many classes of drugs but also had survival ability over the increased concentration of antimicrobial agents.

2.2 Mechanisms of *Salmonella* resistance to antimicrobial agents

In general, bacteria possess certain intrinsic properties that provide natural resistance to some classes of drugs (Macfarlane and Hancock, 2000). These trait usually express by chromosomal genes as an inherit features of bacterial species. However, acquired resistance can be occurred by resistant strains emerge from previously sensitive strains. Moreover, acquired resistance can be caused by mutation in chromosomal genes or acquisition by plasmids or transposons. The resistance to antimicrobials is a result of three major mechanisms: destruction or modification of the antimicrobial agents, prevention of antimicrobial agents uptake and alteration of antimicrobial targets (Taylor *et al.*, 2004).

2.2.1 Destruction or modification of the antimicrobial agents

Bacteria produce modifying or hydrolytic enzymes which neutralize particular drugs that have gained access to cell. For example, β-lactamase hydrolyzes a wide range of drugs belonging to β-lactams. This enzyme has been identified in many of species. Enzymes that modify certain classes of aminoglycosides and chloramphenicol are also a part bacteria (Macfarlane and Hancock, 2000; Taylor *et al.*, 2004). Almost genes encoding these enzymes are located on mobile genetic elements such as plasmids or transposons, occasionally, they are also found in chromosome (Hachler and Kayser, 1996).

2.2.2 Prevention of antimicrobial uptake

An important intrinsic resistance of bacteria is made by efflux pump systems. These systems efficiently transport undesired drugs out of cells by active process that obtains energy from the proton motive force. Many efflux pumps have a broad range to transport of antimicrobial agents including detergent (sodium dodecyl sulfate (SDS)) outside. According to Nakamura (1968) report, *E. coli* K-12 resisted to acriflavine and phenethyl alcohol also increased in resistance to SDS by efflux pump mechanism. Resistance to tetracycline, quinolones and macrolides can

be mediated by such systems (Macfarlane and Hancock, 2000). Tetracycline resistance by efflux pump is a common mechanism in gram-negative bacteria, and resistance genes usually encode on plasmid (Taylor *et al.*, 2004).

2.2.3 Alteration of antimicrobial target

This mechanism results from an enzymatic modification of the drug target, therefore, the target can not bind to antimicrobial longer. Resistant strains conferred tetracycline, macrolides, glycopeptides and quinolones are examples of this resistant type (Macfarlane and Hancock, 2000; Taylor *et al.*, 2004). Furthermore, resistance to trimethoprim and sulfonamides was due to acquisition of a foreign gene coding for a new target enzyme. These genes are plasmid mediated (Taylor *et al.*, 2004).

2.3 Dissemination of antimicrobial resistance genes among bacterial pathogens

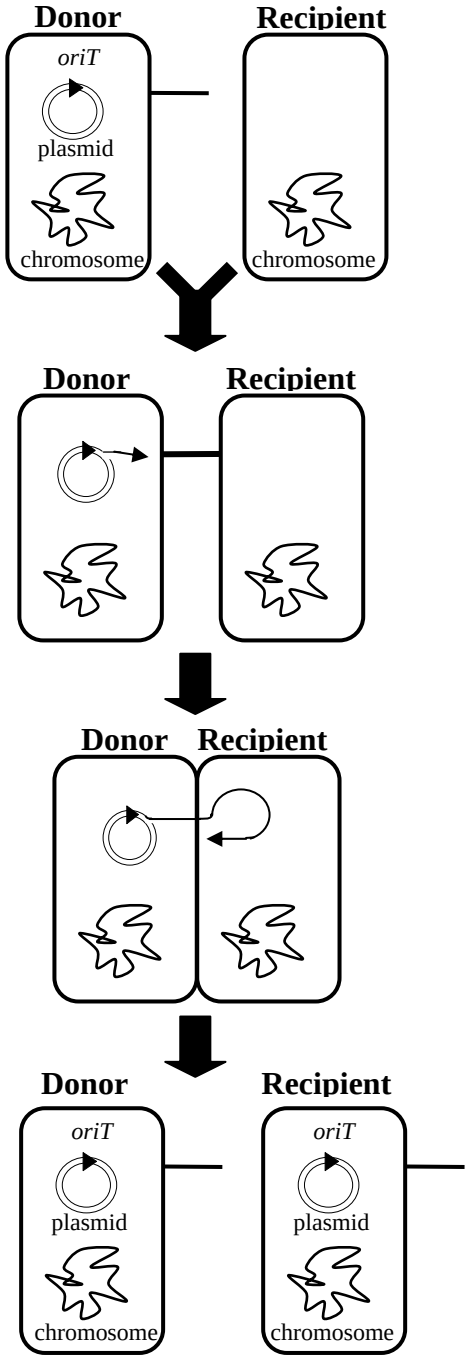
Antimicrobial resistance frequently results from the acquisition of mobile genetic elements carrying resistance genes. Horizontal transferring of these genes are mainly conjugative plasmids. Moreover, a new genetic element has been reported which was described as integrons. The integrons contain resistance gene cassettes that can be moved to other integrons (Fluit and Schmitz, 1999). The integrons have been classified into three classes, of which mostly the class 1 integrons disseminated among gram-negative bacteria. Therefore, in the next issue would be mentioned to the major factors in spreading of MDR strains by resistance genes transferring via conjugative plasmids and class 1 integrons.

2.3.1 Conjugative plasmids

The conjugative plasmids are implicated potentially in dissemination of antimicrobial resistance among gram-negative bacteria that could transfer antimicrobial resistance genes from one to another strain by conjugation (Lawley *et*

al., 2004). Bacterial conjugation is one of the fundamental processes for genes transfer in nature. It was viewed as an interesting process in Enterobacteriaceae that was useful in transfer bacterial genetics. Such process was a reasonable description for the increases in antimicrobial resistant strains. Bacterial conjugation is a property of plasmids which can transfer themselves. This process involves the direct transfer of DNA between cells that come into contact to another one (Lawley *et al.*, 2004).

Conjugative systems have three essential components: I) the transferosome which is responsible for synthesis of conjugative pilus, II) the relaxosome that processes the DNA at the origin of transfer (*oriT*), and III) coupling protein which connects two cells together. DNA transfer is initiated at a strand-specific cleavage site (*nic*) within the *oriT* region. The cleaved strand is transferred into the recipient with 5' to 3' polarity by a rolling-circle mechanism, associated with synthesis of a complementary strand in both donor and recipient cells (Lawley *et al.*, 2004). For example, mechanism of conjugation steps of F-plasmid conjugation was shown in Figure 1. The first step is cell-cell contact that mediated by F-pili then DNA mobilization occurs by nicking at *oriT*. The cleaved single strand of the DNA transfers into the recipient cell by rolling-circle and F plasmid DNA is replicated simultaneously. The second strand of DNA is synthesized in both the donor and recipient cells. After synthesis complete, the DNA is nicked at the *nic* site and two DNA molecules are released and then double-stranded DNA circularize in the recipient cell. After the plasmid is completely transferred, the donor and recipient cells separate. Both donor and recipient cells now have a complete F plasmid and can conjugate with other F^- recipient cells. According to Mirza *et al*. (2000), MDR *S.* Typhi has been reported to be plasmid mediated, as well as, *S.* Typhimurium was reported that conjugative plasmids, IncFI and IncL/M carried multiple antimicrobial resistance genes which could transfer to *Escherichia coli* (*E. coli*) (Tosini *et al.*, 1998).



1) Cell to cell contact mediated by F-pili

2) Mobilization occur by nicking at *oriT*, rolling-circle replication of F plasmid DNA and transfer of a single strand of the DNA into the recipient cell.

3) The second strand of DNA is synthesized in both the donor and recipient cells. After synthesis complete, the DNA is nicked at the *nic* site. Releasing the two DNA molecules. The double-stranded DNA circularizes in the recipient cell.

4) After the plasmid is completely transferred, the cell separate. Both donor and recipient cells now have a complete F plasmid and can conjugate with other F^- recipient cells.

Figure 1 Mechanism of conjugation showing the steps of F-plasmid conjugation.

2.3.2 Class 1 integrons

Integrons are genetic elements that can be carried either by chromosome or plasmids and act as site-specific recombination systems for recognition and acquisition of new resistance gene cassettes (Taylor *et al.*, 2004). The integron itself is defective for self-transposition but they are often found associated with insertion sequences (ISs), transposons and / or conjugative plasmids that can serve as vehicles for the intra and inter-species transmission of genetic material. However, they carry gene cassettes that can be mobilized to other integron or other secondary site. Integrons definition was formulated by Hall and Collis (1995) who described the basic structure of integron. The functional integron consists of the gene for an integrase (*intI*) of the tyrosine-recombinase family and a primary recombination sequence called an *attI* site. The *intI* gene is expressed by P_{int} promoter for adjacent *attI* site. The integrase mediates recombination between the *attI* site and a secondary target called an *attC* site or 59-base elements (59-be) (Taylor *et al.*, 2004). The *attC* site is generally associated with a single ORF in a structure termed a gene cassette, and the gene cassette constitutes the mobile component of the system (Figure 2). Gene cassettes lack a promoter in front of the coding sequence.

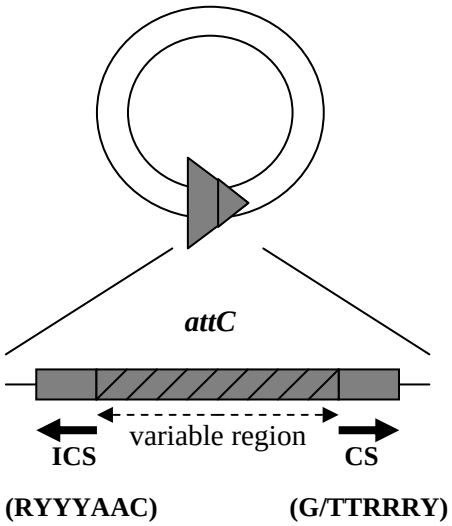


Figure 2 Schematic representation of a circularized cassette. The key features of *attC* sites are the complementary inverse core-site (ICS) and core-site (CS) consensus sequences and the imperfect palindromic variable region.

According to Fluit and Schmitz (1999), at least 59 gene cassettes are known and most of these gene cassettes are antimicrobial resistance (Table 2). The length and sequence of the *attC* sites vary considerably and their similarities are primarily restricted to their boundaries, which correspond to the inverse core site (ICS; RYYYAAC) and the core site (CS; GTTRRRY) recombination point as showed in Figure 2 (Collis *et al.*, 1992; Stokes *et al.*, 1997).

Table 2 Characteristics of class 1 integron gene cassettes.

Gene[a]	Protein	Length of CDS[b] (bp)	Length of *attC* site (bp)	Gene cassette[c] length (bp)	Accession no.
Resistance to β-lactam					
blaP1	PSE-1/CARB-2[d]	915	111	1044	Z18955
blaP2	-	915	111	1044	D13210
blaP3	CARB-4	867	>92	>1023	U14749
bla_{IMP}	IMP-1	741	127	880	D50438
bla_{ESP}	ESP	741	-	880	D78375
bla_{VEB-1}	VEB-1	897	133	1059	AF010416
oxa1	OXA-1	831	90	1004	J02967
oxa2a	OXA-2	828	70	876	M95287
oxa2b	OXA-2b	828	70	876	M95287
oxa3	OXA-3	828	>56	>861	L07945
oxa5	OXA-5	804	106	915	X58272
oxa7	OXA-7	801	65	874	X75562
oxa9	OXA-9	840	69	840	M55547
oxa10	OXA-10 (PSE-2)	801	111	920	U37105
oxa15	OXA-15	-	-	-	U63835
oxa19	OXA-19	-	-	-	AF043381
oxa20	OXA-20	798	117	935	AF024602
oxa21	OXA-21	828	-	-	Y10693
Resistance to aminoglycosides					
aadA1a	AAD(3″)	792	60	856	X12870
aadA1b	AAD(3″)	792	60	856	M95287
aadA2	AAD(3″)	780	60	856	X68227
aadB	AAD(2″)	534	60	591	L06418

Table 2 (continued)

Gene[a]	Protein	Length of CDS[b] (bp)	Length of *attC* site (bp)	Gene cassette[c] length (bp)	Accession no.
aac(6′)-Ia[e]	AAC(6′)-Ia	558	-	>778	M18967
aac(6′)-Ib	AAC(6′)-Ib	555	70	637	M55547
aac(6′)-Id	AAC(6′)-Id	450	72	526	X12618
aac(6′)-Il	AAC(6′)-Il	521	109	720	-
aac(6′)-Iq	AAC(6′)-Iq	551	108	712	AF047556
aacA7	AAC(6′)-I	459	112	591	U13880
aac(6′)-IIa	AAC(6′)-IIa	555	60	628	M29695
aac(6′)-IIb	AAC(6′)-IIb	543	97	653	L06163
aac(3)-Ia	AAC(3)-Ia	465	109	577	X15852
aac(3)-Ib	AAC(3)-Ib	465	>34	>498	L06157
aac(3)-VIa	AAC(3)-VIa	901	-	-	-
Resistance to trimethoprim					
dfrA5	DHFRV	474	87	586	X12868
dfrA7	DHFRVII	474	134	617	X58425
dfrA12	DHFRXII	498	90	584	Z21672
dfrA14	DHFRIb	483	>43	>523	S76821
-	DHFRXV	474	84	593	Z83311
dfrB1	DHFRIIa	237	57	485	U36276
dfrB2	DHFRIIb	237	57	384	J01773
dfrB3	DHFRIIc	237	57	408	X72585
Resistance to chloramphenicol					
catB2	CATB2	633	72	739	M80188
*catB*3	CATB3	633	60	739	U13880
*catB*5	CATB5	633	>25	>677	X82455
*catB*6	CATB6	633	77	730	AJ223604
cmlA	CmlA	1260	70	1549	U12338
cmlA2	CmlA2	1434	68	-	-
cmlB	CmlB	-	-	-	-
Resistance to quarternary compounds (disinfectants and antiseptics)					
qacE	QacE	333	141	587	X72585
qacF	QacF	-	-	-	-
qacG	QacG	333	94	532	AJ223604

Table 2 (continued)

Gene[a]	Protein	Length of CDS[b] (bp)	Length of *attC* site (bp)	Gene cassette[c] length (bp)	Accession no.
Resistance to rifampin					
arr-2	ARR-2	453	114	663	AF078527
Resistance to erythromycin					
ereA	EreA	-	-	-	-
Unidentified ORFs					
orfA	-	435	69	501	J01773/X12869
orfC	-	378	60	507	X17477
orfD	-	291	60	320	M95287
orfE	-	246	60	262	U12338
orfF	-	291	60	320	-
orfN	-	615	77	689	AJ223604

Note: [a] Gene names may differ from the name given in the original publication.

[b] The initiation codon is not always known; in these cases, generally the first initiation codon is assumed functional.

[c] Gene cassette sizes are not always accurate.

[d] The genes for PSE-4 and CARB-3 differ in one nucleotide from the *blaP1* sequence.

[e] The *aac(6')-Ia* gene cassette contains an ORF (orfG) as well, followed by *attC* site.

CDS = coding sequence; - = no data available; ORFs = open reading frames.

Source: Fluit and Schmitz (1999)

Insertion of the gene cassette at the *attI* site, which is located downstream of a resident promoter internal to the *intI* gene (P_{ant} promoter), drives expression of the encoded proteins (Figure 3). Gene cassettes are not necessarily part of the integron, but when integrated, they become part of integron (Fluit and Schmitz, 1999). The integrons have been classified into three classes which are determined by the type of integrase they contain. Class 1 integrons are the most predominant among gram-negative bacteria dissemination (Brown *et al.*, 1996). According to many articles, class 1 integrons played a significant spreading of multi-drug resistance genes in different bacteria including *E. coli, Vibrio chloerae, Yersinia enterocolitica* and

S. Typhimurium (Dalsgaard *et al.*, 2000; Roe *et al.*, 2003; Soto *et al.*, 2003; Majtan *et al.*, 2004). Double class 1 integrons were identified in *S.* Typhimurium DT104 by PCR and sequencing technique. Each of which contained $bla_{PSE\text{-}1}$ and $ant(3^n)\text{-}I\alpha$ gene cassettes that conferred ampicillin and streptomycin resistance respectively (Ridley and Threlfall, 1998; Sandvang *et al.*, 1998). In conclusion, class 1 integron structure is composed of 5' and 3' conserved segment, and internal variable region. The internal variable region is the insertion area of resistance gene cassettes (Hall *et al.*, 1991). The 5' conserved segment contains *int* gene, *attI* recombination site and promoters (P_{int} and P_{ant} promoter), whereas, *qacEΔ1* and *sulI* genes which resist to disinfectants and sulfonamide, usually exist in the 3' conserved segment (Paulsen *et al.*, 1993; Recchia and Hall, 1995).

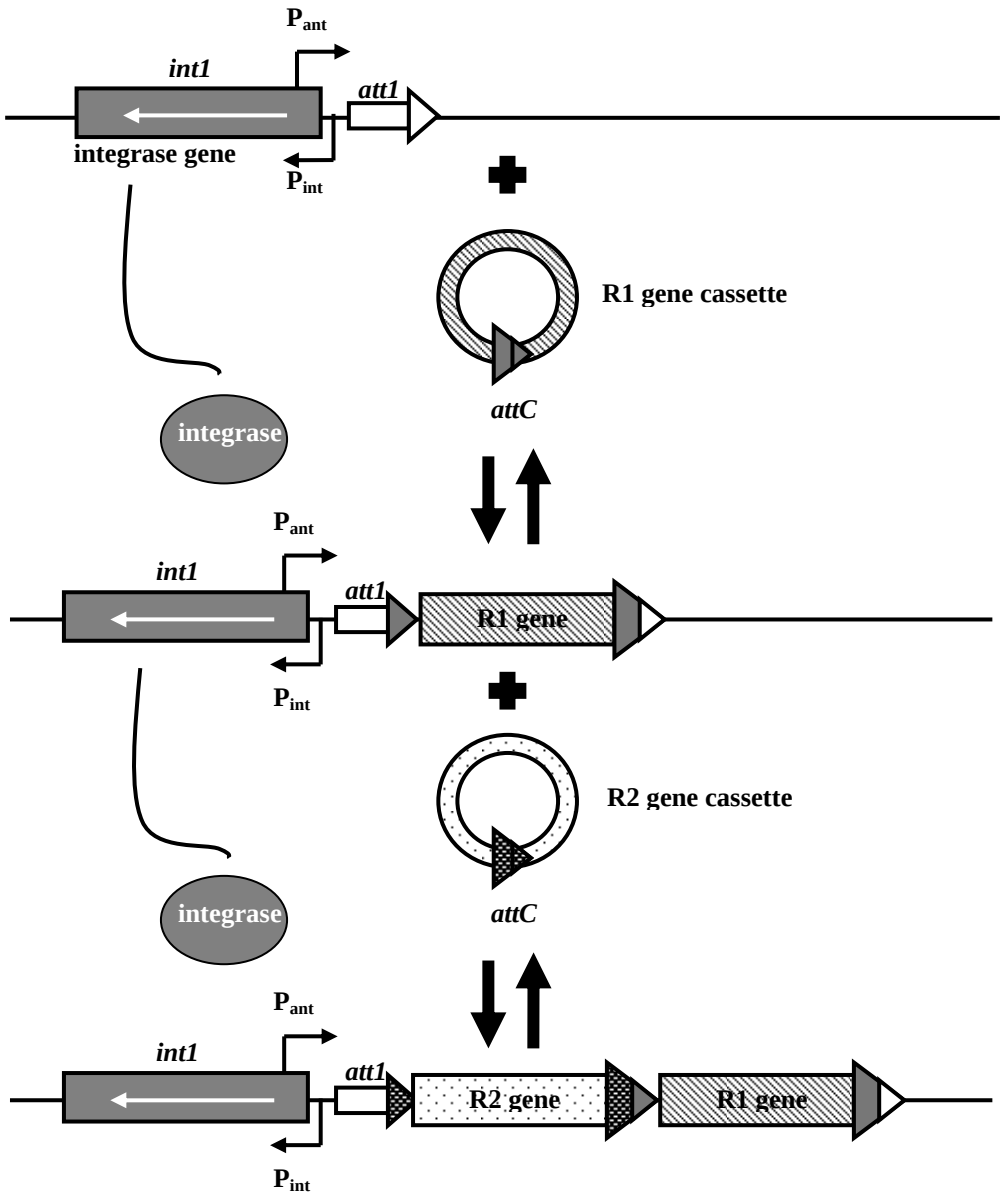


Figure 3 Schematic representation of a model for gene cassette exchange. Outline of the process by which circular antimicrobial resistance gene cassettes (R1 and R2) are repeatedly inserted at the specific *attI* site in a class1 integron downstream of the strong promoter P_{ant}. The triangle arrow of the *attC* represents the recombination point in the CS sequence.

3. Epidemiology of *Salmonella*

It is well known that *Salmonella* are considered as one of the major pathogenic agents. There are many reports of *Salmonella* outbreaks and the incident of particular serotypes in difference areas and period of time. In 2001, Sonja *et al.* had been reported the trends of endemic *Salmonella* serotypes isolated from humans in United States from 1987 to 1997. This report revealed that the isolation rates of *S.* Enteritidis increased whereas *S.* Hadar and *S.* Heidelberg declined in that period. Additionally, outbreaks of *S.* Enteritidis in California frequently had been associated with consumption of contaminated raw or undercooked eggs (Mohle-Boetani *et al.*, 1998). As well as Smith-Palmer *et al.* (2003) had been reported epidemiology of *Salmonella* in Scotland between 1990 and 2001. This report indicated that the incidence of *S.* Typhimurium DT104 infection either in people or animals was highest in 1996, but the number decreased every year from 1996 to 2001. While *S.* Enteritidis PT4 infection in people peaked in 1997 then declined in 2001, whereas in chicken, the incidence of *S.* Enteritidis PT4 peaked in 1998. The epidemiology of *Salmonella* probably changes in various areas. In Western Europe during 1998 to 2003, *S.* Enteritidis PT4 frequently identified in humans in 1998, whereas in 2003 the proportion of PT4 decreased with other strains increasing (Fisher, 2004).

Therefore, the classification of *Salmonella* strains into specific serotypes is essential to epidemiologic studies and tracing the source of outbreaks. Even though serotyping is useful for differentiation of *Salmonella*, phage typing method increases discriminatory power to find out the outbreak strains. Phage typing depends on resistant capability of bacterial cell to bacteriophage infection (Portillo, 2000). However, only some *Salmonella* serotypes are associated with foods of animal origin and consequently lead to human diseases (Swartz, 2002). *S.* Enteritidis and *S.* Typhimurium frequently have been associated with consumption of contaminated raw or undercooked eggs and wide range of animal-producing foods.

Although, the traditional serotyping has been of great value in understanding the epidemiology of *Salmonella* and investigating disease outbreaks, however, production and quality control of the hundreds of antisera required for serotyping is difficult and time-consuming. To circumvent the problems associated with traditional serotyping, many genotyping techniques, such as PFGE, RAPD, RFLP and multiplex PCR (Luk *et al.*, 1993; Chansiripornchai, *et al.*, 2000) have been successfully applied to the determination of *Salmonella* serotypes. According to Liebana (2002), PFGE was determined to be an efficient discriminatory scheme, however, this method is very complicate in practice. Hence, many researches have been improved several new techniques to classify *Salmonella* strains into specific serotypes. Particularly, in RFLP analysis, the RFLP profiles of *fliC* and *fljB* genes, obtained by digestion of each PCR products with restriction endonucleases, have clearly reflected the antigenic variances in the phase 1 and 2 flagellar antigens (Dauga *et al.*, 1998; Hong *et al.*, 2003). This method was proven to be an alternative approach for serotyping *Salmonella* strains.

Many evidences have been reported that RFLP-PCR analysis of *fliC* and *fljB* genes have alternately used as molecular serotyping scheme, because of the variation of the antigen-coding central region of *fliC* and *fljB* genes. Among the different *Salmonella* serotypes, the truncated of amplified *fliC* and *fljB* genes provided the different RFLP patterns (Kilger and Grimont, 1993; Dauga *et al.*, 1998; Hong *et al.*, 2003). While the same RFLP pattern was given in the same *Salmonella* serotype. The study of nucleotide sequences of *fliC* gene among seven *S.* Typhimurium strains were identical (Smith and Selander, 1990). In addition, recent study on the relationship between RFLP-PCR and serotyping for identification of *Salmonella* isolates from chicken in Thailand by Jong *et al.* (2005) has also showed similar result. In this study, twenty serotypes of *Salmonella* from chicken were analyzed RFLP profiles of *fliC* and *fljB* genes by digested with *Mbo*I and *Hha*I. RFLP analysis with *Mbo*I revealed eleven and six discriminated restriction profiles for *fli*C and *flj*B respectively. Nine distinct profiles for *fli*C and six for *flj*B were obtained when using *Hha*I endonuclease. The combination of these two restriction profiles could differentiate over 80% of the total 20 serotypes from one another (Table 3).

Table 3 RFLP profiles of *Salmonella fliC* and *fljB* genes with *Mbo*I and *Hha*I.

Serotype	No. of samples	*fliC* RFLP (*Mbo*I)	*fljB* RFLP (*Mbo*I)	*fliC* RFLP (*Hha*I)	*fljB* RFLP (*Hha*I)
Albany	3	A15	-	M1	-
Bovismorbificans	1	A5	B3	M3	N5
I4,12:d:-	1	A6	-	M14	-
Rissen	1	A8	-	M15	-
Typhimurium	2	A4	B3	M3	N3
Agona	1	A2	-	M15	-
Blockley	2	A3	B9	M18	N10
Mbandaka	1	A4	B4	M4	N2
Virginia	1	A6	B3	M14	N4
Livingstone	1	A6	B5	M1	N9
Hadar	10	A4	B3	M4	N2
Schwarzengrund	4	A6	B2	M14	N8
Orion	1	A16	B4	M17	N2
Newport	4	A1	B3	M19	N4
Weltevreden	2	A5	B8	M14	N9
Virchow	2	A4	B3	M4	N2
Emek	2	A15	-	M1	-
Give	1	A3	B2	M1	N8
Corvallis	4	A7	-	M1	-
Enteritidis	3	A13	-	M6	-

Source: Jong *et al.* (2005)

In order to understand the epidemiology of many infectious diseases, the combination of serotyping and molecular techniques probably provide more powerful epidemiology data, particularly, for identification specific strains and outbreak of salmonellosis (Liebana, 2002).