CHAPTER 5: DISCUSSION

The emergence of MDR *Salmonella* strains that harbored β-lactamase is a global health problem. Extended spectrum cephalosporins (ESC) are drugs of choice to treat invasive salmonellosis. Different β-lactamase genes, such as *bla*$_{TEM,}$ *bla*$_{PSE-1,}$ *bla*$_{SHV,}$ *bla*$_{CTX-M,}$ *bla*$_{CMY}$ and *bla*$_{OXA}$ have been found in *Salmonella* (Uma *et al.*, 2010). Many Gram-negative bacteria are able to express chromosomally-mediated β-lactamases. There is a possibility that the particular gene(s) can be transferred into a plasmid due to environmental selective pressure where it can be disseminated to other bacterial species horizontally (Su *et al*., 2008). The first plasmid-mediated β-lactamase, TEM-1 was described in 1965 (Datta & Kontomichalou, 1965).

In this study, PCR assays were carried out to detect the most prevalent β-lactamases in the selected multidrug resistant (MDR) *Salmonella* strains, including *bla*$_{TEM,}$ *bla*$_{PSE-1,}$ *bla*$_{SHV,}$ *bla*$_{CTX-M,}$ *bla*$_{CMY-2}$ and *bla*$_{OXA-1.}$ These resistance genes can be used for surveillance of the widespread distribution among *Salmonella* serovars. These MDR *Salmonella* strains of human origin were resistant to at least two classes of antimicrobials.

A high resistance rate of the MDR *Salmonella* strains towards β-lactam antimicrobials was observed in this study. Eighteen of 41 strains were resistant to ampicillin. However, only 9 strains harbored *bla*$_{TEM}$ gene, and 5 strains harbored *bla*$_{PSE-1}$ gene. Not all ampicillin-resistant strains harbored *bla*$_{TEM}$, *bla*$_{PSE-1}$ or *bla*$_{OXA-1.}$ Therefore, these genetic elements are not the only one that is responsible for the ampicillin resistance. Thus, the resistance mechanisms should be explored in the future.

On the other hand, *bla*$_{SHV}$ gene was only detected in a strain of *S.* Enteritidis, SE 20/08, which is resistant to cephalothin (Archambault *et al.*, 2006). However, no plasmid

can be detected in this strain, which indicated that this gene might be chromosomally-mediated since many Gram-negative bacteria possess chromosomally-mediated β-lactamase (Bradford, 2001).

Results showed that *bla*$_{\text{CTX-M}}$ gene was the most prevalent gene which has been detected in 12 (33.3%) strains. All these 12 strains were resistant to β-lactam antimicrobials, including ampicillin and cephalosporins. However, only 3 strains were resistant to cefotaxime where the existence of the CTX-M enzyme was usually related to cefotaxime. In 1989, CTX-M-1 gene was designated by Bauernfeind *et al.* (1990), which hydrolyze against cefotaxime and also ceftriaxone. Normally, *bla*$_{\text{CTX-M}}$ gene is located on plasmids that vary in size from 7kb (Cao *et al.*, 2002) to 160kb (Kariuki *et al.*, 2001). Eleven strains that harbored *bla*$_{\text{CTX-M}}$ gene also harbored plasmids that vary in size from 2.2kb to 63.5kb except SE B2, although plasmid extraction was repeated for several times. This may because of the instability of plasmids in this particular strain. Amplicon of a representative strain, SLG B1 was sequenced and demonstrated 99% sequence identity to *bla*$_{\text{CTX-M-15}}$ of *Salmonella* Typhimurium strain B9903 (GenBankk accession no. HM117627.1). According to Archambault *et al.* (2006), *bla*$_{\text{CTX-M-15}}$ gene is located on a 63kb transferable plasmid. However, SLG B1 harbored only a plasmid sized in 20kb. As non-TEM and non-SHV, enzymes of CTX-M have been reported to be the most widespread enzymes since 1995 (Bonnet, 2004). Plasmids that encompass these resistance genes can be also resistant to other antimicrobials, such as tetracycline, aminoglycosides, chloramphenicol, trimethoprim, and sulfonamides (Bonnet, 2004). According to the results, 6 strains that harbored both *bla*$_{\text{TEM}}$ and *bla*$_{\text{CTX-M}}$ gene were also resistant to other antimicrobials, including tetracycline, trimethoprim, aminoglycosides, chloramphenicol and sulfonamides.

AmpC β-lactamase gene, *bla*$_{CMY-2}$, which is plasmid mediated has been associated with resistance to ceftriaxone and ceftiofur (Alcaine *et al.*, 2005). *Salmonella* strains that carried *bla*$_{CMY-2}$ were first isolated from human, animal, and food samples in the U.S. in 1996 (Zhao *et al*., 2001). From the results, there were three strains that harbored *bla*$_{CMY-2}$ gene, where only two strains were resistant to ceftiofur. Besides, these three strains were also resistant to ampicillin, streptomycin, tetracycline, amoxicillin, and chloramphenicol which similar to previous study (Alcaine *et al*., 2005; Whichard *et al.*, 2007). These three strains harbored plasmids vary in size between 2.3kb to 54.0kb. Transferability of the plasmids was not being done in this study. Thus, further study needs to be done.

No *bla*$_{OXA-1}$ gene was detected in the ampicillin resistant strains in this study. In addition, 15 strains that were resistant to β-lactam antimicrobials did not harbor the selected β-lactamase genes. Hence, other resistance mechanisms may be involved, such as changes in bacterial cell wall permeability and energy-dependent removal of antimicrobials via membrane-bound efflux pumps (Barbosa & Levy, 2000; Schwarz & Chaslus, 2001).

On the other hand, resistance to fluoroquinolones among *Salmonella* isolates is on the increase in many countries (Su *et al.*, 2004). Plasmid-mediated *qnr* genes are related to fluoroquinolone resistance. Plasmid-mediated fluoroquinolone resistance associated with *qnrA, qnrB,* and *qnrS* have been described among *Enterobacteriaceae* in Asia, South America, U.S. and several countries in Europe (Robicsek *et al.*, 2006; Minarini *et al.*, 2007; Lavilla *et al.*, 2008; Veldman *et al.*, 2008). In this study, *qnrA* gene was not detected in any MDR *Salmonella* strains, which concurred with other studies (Choi *et al.*, 2005; Gay *et al.*, 2006). Meanwhile, *qnrB* and *qnrS* gene were detected in 18 (75.0%) and 7 (29.2%) strains, respectively. Seven strains harbored both *qnrB* and *qnrS* genes. Results of this study

showed that *qnrB* and *qnrS* genes were detected but not *qnr*A gene. Furthermore, *qnrB* and *qnrS* genes were detected concurrently. However, other resistance mechanisms may be involved, such as membrane permeability and/or efflux pumps over-expression (Cattoir *et al*., 2007).

Concurrent presence of β-lactamases and *qnr* genes is rare. Nevertheless, *qnr* genes have been detected in strains producing plasmid-mediated β-lactamases (Nordmann & Poirel, 2005; Robicsek *et al*., 2006; Poirel *et al.*, 2007; Lavilla *et al*., 2008; Wu *et al*., 2008). In this study, *qnrB* and *qnrS* genes also have been detected simultaneously with β-lactamases including bla_{TEM}, $bla_{\text{CTX-M}}$, and $bla_{\text{CMY-2}}$. These findings were found in 9 strains, including *S.* Lagos (SLG B32), *S*. Enteritidis (SE 16/07, SE 18/07), *S.* Farsta (SFS B5; SFS B36), and *S.* Typhimurium (STM 06/08, STM 07/08, STM 47/07, STM 48/07). *qnrB* gene has been reported on plasmids carrying *bla* genes for CTX-M-15, SHV-12 (Poirel *et al*., 2005) and SHV-30 (Gay *et al.*, 2006). According to Gunell *et al*. (2009), *qnrA1* and *qnrS1* located in plasmids vary in size from 13 to 30kb. Besides, other reports revealed that these common *qnr* genes were identified in plasmids ranging from 54kb to >180kb, which often associated with ESBL genes (Dionisi *et al.*, 2009). In this study, strains that harbored both β-lactamases and *qnr*-like genes also harbored plasmid(s) vary in size from 2.2kb to 63kb.

Determination of class 1 integron was also being carried out in this study. Variable regions of class 1 integron was detected in 9 strains including *Salmonella* serovar of Albany (SAB), Corvallis (SCV), Typhimurium (STM), Paratyphi B (SPB), Limete (SLM), and Bovismorbificans (SBV). Presence of different size of integrons suggests a degree of diversity among these genetic elements. SAB B13 harbored class 1 integron with the *dfrA1* gene that conferred resistance to trimethoprim and orfC, which has unknown function. This

finding has been found previously in *S.* Emek that isolated from humans (Levings *et al.*, 2005). Besides, *S.* Albany (SAB 53/07) harbored a bla_{PSE-1} gene on class 1 integron which confers resistance to ampicillin. Detection of bla_{PSE-1} gene was observed on class 1 integron previously in another research (Guerra *et al*., 2000). The *aadA* gene was the most common resistance gene found in *Salmonella* strains, which confer resistance to aminoglycosides. The *aadA2* gene was observed alone in three strains of *S*. Paratyphi B. The *aadA2* gene was usually present together with *dfrA12* gene, which has been described in *S.* Typhimurium, *S.* Enteritidis, and other bacteria in Asia and Europe (Lindstedt *et al.*, 2003; Huang *et al.*, 2004; Kwon *et al.*, 2002; Kang *et al.*, 2005). From the sequencing results, presence of both *dfrA12* and *aadA2* gene cassettes were observed within class 1 integron in three strains of this study, including *S.* Corvallis (SCV G1), *S.* Typhimurium (STM 06/08), and *S.* Bovismorbificans (SBV 56/07). Besides, gene cassette of *catB3* was identified in *S.* Limete (SLM B37), together with *dfrA1* gene cassette. Gene cassette of *catB3* confers resistance to chloramphenicol, which has been identified previously by Bunny *et al.* (1995). It is interesting to note that the presence of class 1 integron in *S*. Limete (SLM B37) was observed which has not been reported before. Class 1 integron in SLM B37 could have acquired from other bacteria since transfer of integrons between different bacterial species has been documented in the clinical setting. This poses a serious threat to containment of nosocomial infections (Leverstein *et al.*, 2002).

Meanwhile, *Salmonella* Genomic Island 1 (SGI1) was detected in this study because it is an important determinant of multidrug resistant. SGI1 is a 43kb genomic island, which showed 44 coding sequences and most of them encoding hypothetical proteins. SGI1 shows two 18 bp direct repeats at the external boundaries, which suggest

that site-specific recombination events may have driven the insertion of the island within the serovar Typhimurium chromosome (Boyd *et al.*, 2000; 2001). Most of the time, isolates that harbored SGI1 have been described that resistant to a core group of antibiotics, including ampicillin, chloramphenicol/florfenicol, spectinomyicn/streptomycin, sulfonamides and tetracycline (abbreviated as ACSSuT). Besides, some other strains have been identified that resistant to fluoroquinolones and trimethoprim (Daly *et al.*, 2000; Ng *et al.*, 1999; Threlfall *et al*., 1998). From the results, SGI1 was found in two *S.* Albany (SAB B13; SAB 53/07), three *S.* Paratyphi B (SPB 05/08; SPB 06/08; SPB 07/08) and *S.* Limete (SLM B37) which is all class 1 integron-positive. Previously, SGI1 has been identified in *S.* Albany in Thailand (Doublet *et al.*, 2003) and *S.* Paratyphi B in Singapore (Meunier *et al.*, 2002). The typical resistance phenotype (ACCSuT) was only found in SAB B13, which was also resistant to kanamycin, trimethoprim, and nalidixic acid. This finding concurred with other studies. Three strains that harboured SGI1 were also resistant to cephalosporins, including SAB 53/07, SPB 07/08 and SLM B37. Besides, a strain of *S.* Corvallis (SCV 38/07) and *S.* Albany (SAB 57/07) contained only the left junction of the genomic island (SGI1) which did not harbor integron. All six isolates that harbored SGI1 contained integron that harbored gene cassette of *dfrA1*,*bla*$_{\text{PSE-1}}$, and *aadA2*, which had been found previously in variants of SGI1 (Boyd *et al*., 2002; Carattoli *et al.*, 2002; Doublet *et al*., 2003, 2004). SGI1 was identified in *Salmonella* serovar of Typhimurium DT104, Agona (Levings *et al.*, 2005), Kentucky (Levings *et al*., 2007; Doublet *et al*., 2008), Newport (Doublet *et al*., 2004; Cloeckaert *et al*., 2006), Paratyphi B (Meunier *et al*., 2002), Albany (Doublet *et al*., 2003), Meleagridis (Ebner *et al.*, 2004), Newport (Doublet *et al.*, 2004), Cerro, Derby, Dusseldorf, Emek, Infantis and Kiambu (Levings *et al*., 2005). This could be the first report of presence of SGI1 in *S.* Limete. Thus, SGI1 is not confined to just *S.*

Typhimurium DT104 strains but may be transferred to other bacteria via horizontal transfer. However, there is no experimental evidence to demonstrate the molecular mechanism of SGI1 horizontal transfer among *Salmonella* strains, although transduction experiments with a P22-like phage demonstrated a facilitated transduction of resistance genes to susceptible *Salmonella* strains (Schmieger & Schicklmaier, 1999).

Usually, plasmid profiling is used for bacterial typing (Howard and Whitcombe, 1995). However, it is restricted to bacteria that carry plasmids. Moreover, this method is not absolute because the presence of plasmids with the similar approximate molecular weight in two or more strains does not necessarily mean that the strains are epidemiologically related. However, plasmid profiling that carried out in this study was aimed to study association between β-lactamase genes, *qnr* genes and plasmids. In this study, 23 plasmid profiles (PPs) were identified in 30 MDR *Salmonella* strains, and the plasmids ranged from 1.8kb to 65.0kb. According to Taylor *et al*. (1982), *Salmonella* strains can harbor plasmids of different molecular sizes, ranging from 1kb to 200kb. Besides, no plasmid was detected in 11 MDR *Salmonella* strains. Absence of plasmids in resistant strains may be due to the instability of plasmids, which is a common phenomenon among *Salmonella*. During plasmid extraction, shearing of large plasmid DNA and co-precipitation with the chromosomal DNA are often reported to be the causes of plasmid loss (Hansen and Olsen, 1978). Six and nine plasmid-positive strains harbored only β-lactamase gene(s) or *qnr* gene(s), respectively. Nine plasmid-positive strains harbored both β-lactamase gene and *qnr* gene together.

Many studies have reported the rapid development of resistance to β-lactam antibiotics due to the spread of resistance plasmids among *Enterobacteriaceae.* Besides,

coresistance to ESCs and fluoroquinolones may be due to the presence of β-lactamases and *qnr* genes in plasmids of *Salmonella*, which can be disseminated through horizontal transfer from bacteria of nosocomial origin. This phenomenon needs to be concerned because the increasing spread of such strains may reduce therapeutic choices for severe *Salmonella* infections since ESCs and fluoroquinolones were being used to treat such infections.

On the other hand, the simultaneous presence of a resident virulence plasmid and a resistance plasmid also has been reported in *Salmonella* very often. Since most of the resistance genes are localized on transferable elements, resistance determinants were possible to be captured by virulence plasmids. The relationship between resistance and virulence on the same plasmid could contribute to bacterial adaptation and evolution (Carattoli, 2003). Presence of virulence and resistance linked determinants in bacteria will contribute to the selection of antimicrobial resistance. In the meanwhile, antimicrobial resistance pressure will select the virulence traits. Nonetheless, once those determinants have been selected in the bacterial host, they may evolve and finally be transferred to another bacterial population (Martinez and Baquero, 2002). Thus, the correlation between resistance and virulence on the same plasmid should be concerned since many inquiries remain unanswered about mechanisms during the dissemination of plasmids.

A better understanding of the molecular mechanisms of antimicrobial resistance dissemination may facilitate in designing intervention strategies to reduce its progression. Thus, further studies need to be carried out to understand more about the emergence and dissemination of multiple drug resistant *Salmonella* strains in detail.

CHAPTER 6: CONCLUSIONS

Analysis of the β-lactamase genes suggested the strains harbored CTX-M (33.3%), followed by TEM (25.0%), PSE-1 (13.9%), CMY-2 (8.3%) and finally, SHV (2.8%). OXA-1 was not detected in MDR strains of this study. Seven strains were harbored both bla_{TEM} and $bla_{\text{CTX-M}}$ genes. A strain (STM 06/08) was harbored both bla_{TEM} and $bla_{\text{CMY-2}}$ genes and another strain (SFS B36) was harbored three β-lactamase genes together, including bla_{TEM}, $bla_{\text{CTX-M}}$, and $bla_{\text{CMY-2}}$ genes. Besides, 11 strains that resistant to β-lactams but did not harbor any of the selected prevalent β-lactamase genes.

Analysis of the fluoroquinolone resistance genes showed that *qnrB* (75.0%) and *qnrS* (29.2%) were detected. No *qnrA* was detected. *qnrB* and *qnrS* genes were detected more often than *qnrA* gene. Seven strains harbored both *qnrB* and *qnrS* genes together.

Concurrent presence of β-lactamase genes and *qnr* genes were detected in nine strains, including *S.* Lagos, *S.* Enteritidis, *S.* Farsta, and *S.* Typhimurium.

Class 1 integron was detected in nine strains. Five different gene cassettes, namely *aadA2*, *dfrA1-catB3*, $bla_{\text{PSE-1}}$, *dfrA1-orfC*, and *dfrA12-aadA2* were found in the class 1 integron-positive strains which have been described previously. Class 1 integron was detected in *S.* Limete which has not been previously reported.

Analysis of SGI1 indicated that SGI1 was detected in six strains, including *S.* Albany, *S.* Paratyphi B var Java, *S.* Limete.

Twenty three plasmid profiles were identified in 30 MDR *Salmonella* strains, and the plasmids ranged from 1.8kb to 65.0kb. However, 11 MDR strains lacked of visible

plasmids. The predominant plasmid was 20.0kb, which was presented in 9 plasmid-positive strains. Six and nine plasmid-positive strains harbored only β-lactamase gene(s) and *qnr* gene(s) alone, respectively. Nine plasmid positive strains harbored both β-lactamase gene and *qnr* gene. Thus, concurrent resistance to both extended-spectrum cephalosporins and fluoroquinolones was observed in the present study, and this is of concern because it will limit the therapeutic options for severe *Salmonella* infections.