

ANTIMICROBIAL RESISTANCE PROFILE AND GENETIC CHARACTERIZATION
OF *SALMONELLA* CHOLERAESUIS ISOLATED FROM CLINICAL SPECIMENS IN
THAILAND

A THESIS
BY
ORNJIRA TECHAWORARAK

Presented in Partial Fulfillment of the Requirements for the
Master of Science Degree in Biotechnology
at Srinakharinwirot University
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ANTIMICROBIAL RESISTANCE PROFILE AND GENETIC CHARACTERIZATION
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AN ABSTRACT
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The phenomenon of multidrug resistance in *Salmonella* Choleraesuis has become a major clinical and public health problem and causes a serious and systemic infection in human being. The emergence of cephalosporin and fluoroquinolone resistance of *S. Choleraesuis* has important clinical and therapeutic implications. The aim of the present study was to determine the antimicrobial resistance patterns, antimicrobial resistance genes and genotypic relatedness of third generation cephalosporins and/or fluoroquinolone-resistant *Salmonella* Choleraesuis isolated from clinical patients in Thailand. All of sixty two cefotaxime-resistant *S. Choleraesuis* isolates were obtained from sixty one clinical specimens in Thailand from 2010 to 2012. Antimicrobial susceptibility testing was performed using the agar diffusion method, and ESBL production was detected by the combination disc method. A molecular evaluation of *S. Choleraesuis* isolates was performed using PCR and DNA sequencing. Then, a genotypic relatedness study of *S. Choleraesuis* was performed by pulse field gel electrophoresis (PFGE). The highest resistance rate was observed for ampicillin (100%, 62/62) and tetracyclin (100%, 62/62), followed by chloramphenicol (83.87%, 52/62), streptomycin (64.51%, 40/62), amoxicillin-calvulanic acid (48.38%, 30/62) and co-trimoxazole (11.29%, 7/62). The prevalence of resistance of quinolones included nalidixic acid and ciprofloxacin was 14.51% (9/62). No resistance was observed to norfloxacin. All *S. Choleraesuis* isolates were multidrug resistant. Forty-four isolates (44/62, 71.0%) were positive for ESBL phenotypes. Based on the PCR sequencing, 21, 4, 10, 23, 20 and 6 ESBL-producing isolates harboured the ESBL genes *bla*_{CTX-M-14}, *bla*_{CTX-M-15}, *bla*_{CTX-M-55}, *bla*_{CMY-2}, *bla*_{ACC-1} and *bla*_{TEM-1}, respectively. This study also found that nine (9/62, 14.5%) isolates exhibited co-resistance to ciprofloxacin and cefotaxime. All of the co-resistant isolates harboured at least one PMQR gene, the *qnr* genes and the *aac(6')-Ib-cr* gene were detected and found to be the most prevalent genes. The QRDR mutation including the *gyrA* (D87Y and D87G) and *parC* (T57S) genes was also detected. PFGE patterns revealed a high degree of clonal diversity among the ESBL-producing isolates. The multidrug resistance of *S. Choleraesuis* isolates obtained from patients in Thailand showed a high diversity of ESBLs and fluoroquinolone-resistant genes. This is the first report of *bla*_{CTX-M-15} and *bla*_{CTX-M-55} genes in *S. Choleraesuis* isolates obtained from patients with bacteremia. This study emphasizes the importance of actions to control the dissemination of antimicrobial resistances in *S. Choleraesuis*. Antibiotic usage in the production of food animals, such as ceftiofur and enrofloxacin should be tightly monitored in order to limit an emergence and a spread of resistant bacteria. And it only then can this specific serotype be controlled to prevent infection in humans.

รูปแบบการดื้อยาและลักษณะทางพันธุกรรมของเชื้อ *Salmonella Choleraesuis* ที่แยกได้จากตัวอย่าง
สิ่งส่งตรวจทางคลินิกในประเทศไทย



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การดื้อยาแบบหลายกลุ่ม (Multidrug resistance, MDR) ของเชื้อ *Salmonella Choleraesuis* เป็นปัญหาสำคัญทางด้านสาธารณสุขเนื่องจากเชื้อมีความสามารถในการก่อให้เกิดการติดเชื้อในกระแสเลือดโดยเฉพาะการดื้อยาของเชื้อ *S. Choleraesuis* ดื้อยาในกลุ่มเซฟาโลสปอริน (cepharosporin) และกลุ่มฟลูออโรควิโนโลน (fluoroquinolones) ซึ่งยาดังกล่าวจัดเป็นยากลุ่มแรกๆ ที่ถูกเลือกนำมาใช้ในการรักษาการติดเชื้อในมนุษย์ มีผลให้การรักษาโรคเกิดความล้มเหลว ดังนั้นวัตถุประสงค์ของงานนี้คือเพื่อศึกษารูปแบบการดื้อยาและลักษณะทางพันธุกรรมของเชื้อ *S. Choleraesuis* ที่ดื้อยาในกลุ่มเซฟาโลสปอริน และ/หรือ ยาในกลุ่มฟลูออโรควิโนโลน ที่แยกได้จากตัวอย่างสิ่งส่งตรวจทางคลินิกในประเทศไทย จากสิ่งส่งตรวจของผู้ป่วยจำนวน 61 ราย ในระหว่างปี พ.ศ. 2553 – 2555 สามารถแยกเชื้อ *S. Choleraesuis* ที่ดื้อยา cefotaxime ได้ 62 ไอโซเลท โดยศึกษาความไวต่อยาต้านจุลชีพ ตรวจหาพื้นที่ควบคุมการดื้อต่อยาปฏิชีวนะ และจำแนกรูปแบบทางพันธุกรรมของเชื้อด้วยวิธี pulse field gel electrophoresis (PFGE) พบว่าเชื้อดื้อยาสูงสุดคือยา ampicillin (100%, 62/62) และ tetracyclin (100%, 62/62) รองลงมาคือ chloramphenicol (83.87%, 52/62), streptomycin (64.51%, 40/62), amoxicillin - clavulanic acid (48.38%, 30/62) และ co-trimoxazole (11.29%, 7/62) ความชุกของการดื้อยาในกลุ่มควิโนโลน ได้แก่ nalidixic acid และ ciprofloxacin เท่ากับ 14.51% (9/62) และไม่พบการดื้อยา norfloxacin เชื้อ *S. choleraesuis* ที่แยกได้ทั้งหมดสามารถดื้อยาต้านจุลชีพได้มากกว่าหนึ่งกลุ่ม เชื้อที่แยกได้ 44 (71.0%) ไอโซเลท มีความสามารถในการสร้างเอนไซม์เบต้าแลคตาเมสชนิดขยายฤทธิ์ โดยเชื้อที่สร้างเอนไซม์เบต้าแลคตาเมสชนิดขยายฤทธิ์ทั้งหมดพบยีน *bla*_{CTX-M-14}, *bla*_{CTX-M-15}, *bla*_{CTX-M-55}, *bla*_{CMY-2}, *bla*_{ACC-1} และ *bla*_{TEM-1} จำนวน 21, 4, 10, 23, 20 และ 6 ไอโซเลท ตามลำดับ การศึกษานี้ยังพบว่า 9 (14.5%) ไอโซเลท ดื้อยา ciprofloxacin และ cefotaxime ร่วมกัน เชื้อที่มีการดื้อร่วมกันพบว่ามีอยู่ในกลุ่ม PMQR อย่างน้อยหนึ่งยีน ซึ่งยีนกลุ่ม *qnr* และ *aac(6)-Ib-cr* เป็นยีนที่พบมากที่สุด และยังพบการกลายพันธุ์ของยีนในกลุ่ม QRDR ได้แก่ ยีน *gyrA* (D87Y และ D87G) และ *parC* (T57S) นอกจากนี้การจำแนกรูปแบบทางพันธุกรรมของเชื้อ *S. Choleraesuis* ที่สร้างเอนไซม์เบต้าแลคตาเมสชนิดขยายฤทธิ์ด้วยวิธี PFGE พบว่ารูปแบบของลายพิมพ์ดีเอ็นเอที่ได้จากเทคนิค PFGE แสดงความหลากหลายของโคลนจากเชื้อกลุ่มนี้ การดื้อยาคัดหลายกลุ่มของเชื้อ *S. Choleraesuis* ที่แยกได้จากผู้ป่วยในประเทศไทยแสดงความหลากหลายของกลุ่มยีนที่ควบคุมการดื้อยาในกลุ่มฟลูออโรควิโนโลนและที่ควบคุมการสร้างเอนไซม์เบต้าแลคตาเมสชนิดขยายฤทธิ์ การศึกษาในครั้งนี้จัดเป็นรายงานฉบับแรกที่รายงานยีน *bla*_{CTX-M-15} และ *bla*_{CTX-M-55} จากเชื้อ *S. Choleraesuis* ที่แยกได้จากผู้ป่วยที่ติดเชื้อในกระแสเลือด การศึกษาครั้งนี้เน้นถึงความสำคัญของการดำเนินการในการควบคุมการแพร่ระบาดของเชื้อ *S. Choleraesuis* ที่ดื้อต่อยาต้านจุลชีพ การควบคุมการใช้ยาปฏิชีวนะในการผลิตอาหารสัตว์เช่น ceftiofur และ enrofloxacin จึงเป็นเรื่องที่จำเป็นอย่างยิ่งในการที่จะป้องกันการอุบัติใหม่และการแพร่ระบาดของเชื้อแบคทีเรียดื้อยา และจัดเป็นวิธีเดียวที่จะสามารถป้องกันการติดเชื้อในมนุษย์จากเชื้อ serotype นี้

The thesis titled

"Antimicrobial resistance profile and genetic characterization of *Salmonella* Choleraesuis
isolated from clinical specimens in Thailand"

by

Ornjira Techaworarak

has been approved by the Graduate School as partial fulfillment of the requirements for the
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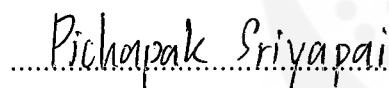
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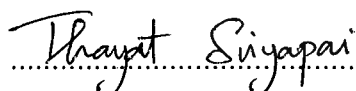
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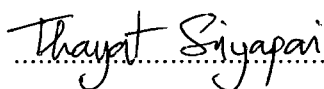
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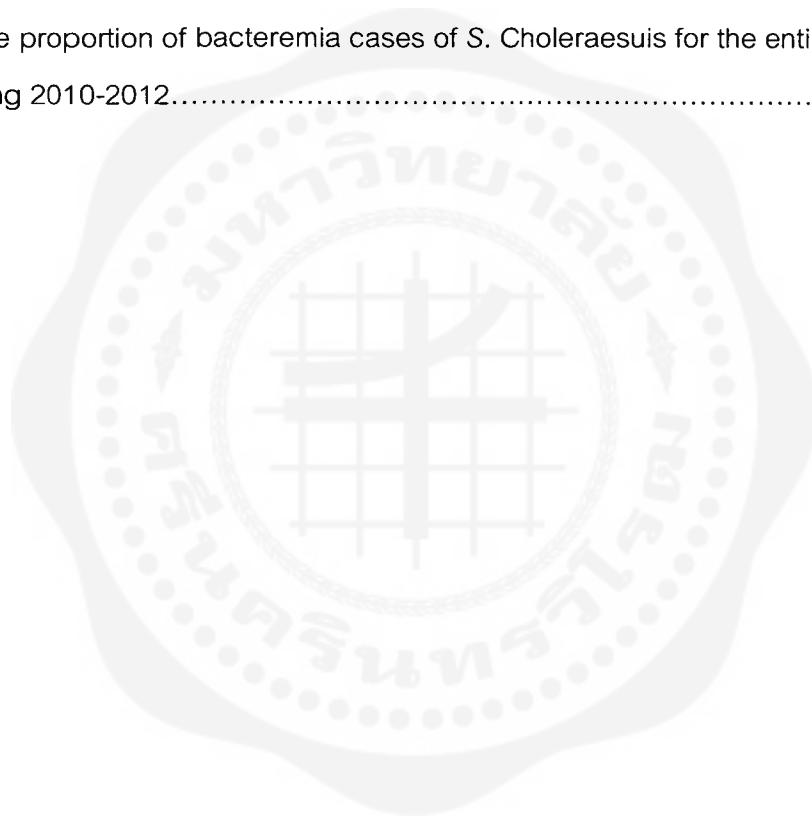
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CHAPTER 1

INTRODUCTION

Rational and Statement of Problem

Salmonella enterica is one of the most common bacteria which is a human pathogen causing 'Salmonellosis' and assorted with cases of gastroenteritis that may be included self-limiting enterocolitis, bacteremia, meningitis, and osteomyelitis ⁽¹⁾. More than 2,600 different serovars of *Salmonella enterica* have been identified. The microorganisms are transmitted by contaminated food and inadequate food hygiene. These infections are generally self-limiting but invasive infections can occur especially due to certain *Salmonella* serotypes such as *S. Enteritidis*, *S. Choleraesuis*, and *S. Dublin* ⁽¹⁶¹⁾. *S. Choleraesuis*, a host-adapted pathogen, usually causes paratyphoid in swine and also frequently causes systemic infection in human with little involvement of the gastrointestinal tract ⁽¹⁶²⁾. A study in nontyphoidal *Salmonella* isolated from Canada, Denmark, and United States have shown an increased risk of hospitalization associated with multidrug resistant nontypoidal *Salmonella* compared with susceptible nontyphoidal *Salmonella* ⁽¹⁰⁻¹⁷⁾. From the collecting data in Southeast Asia and Africa have a high level of multidrug resistant nontyphoidal *Salmonella* and isolates resistant to both the fluoroquinolone ciprofloxacin and the extended-spectrum cephalosporin (ESC) ceftriaxone ⁽¹⁸⁻²²⁾. *S. Choleraesuis* is increasingly recognized as a major cause of systemic salmonellosis in Asian countries; the emergence of *S. Choleraesuis* with comparing to previous susceptibility patterns of among the isolates from Southeast Asia, current nontyphoidal *Salmonella* infections in humans in Thailand are more resistant to fluoroquinolones and ESCs ⁽¹¹²⁾. The incident of co-resistance to both drugs is a serious therapeutic problem in Thailand.

The cephalosporins are a class of β -lactam antibiotics which have different generation based on their antibacterial activities. The Third/fourth generations of cephalosporins have a broad spectrum activity. β -lactam antibiotics work by inhibiting bacterial cell wall synthesis by inactivating peptidoglycan transpeptidases known as

penicillin-binding proteins which catalyze the cross-linking of the peptidoglycan polymerase in the bacterial cell wall. The major cause of resistance to cephalosporins in nontyphoidal *Salmonella* is the production of β -lactamases which destroy β -lactam ring structure of drug, resulting in loss of antibiotic activity. The production of extended-spectrum β -lactamases (ESBLs) are the important resistance mechanisms to extended-spectrum cephalosporins in nontyphoidal *Salmonella* ⁽³²⁾. The resistance to extended-spectrum cephalosporins (ESCs) is the presence of ESBL genes on mobile genetic elements such as plasmid ⁽²⁾. *Salmonellae* have been found to express a wide variety of ESBL genes, including *bla*_{TEM}, *bla*_{CTX-M}, *bla*_{SHV}, *bla*_{ACC}, and *bla*_{CMY} genes ⁽³⁾.

Fluoroquinolone drugs are broad-spectrum antibacterial agents, commonly used both in human and veterinary medicine. Their extensive use has been associated with raising level of fluoroquinolone resistance. To date, the main mechanisms implied in this resistance have been divided in: i) alterations in the targets of the quinolones and ii) decrease in the accumulation of the antibiotic in the bacterial interior by making the membrane impermeable or by expression of active expulsion systems. The mechanisms of quinolone resistance are chromosomally encoded, being either modification of the quinolone targets with changes of DNA gyrase (*gyrA* and *gyrB*) and/or topoisomerase IV (*parC* and *parE*) genes ⁽⁴⁻⁶⁾. A high level of drug resistance arises as a result from the alterations of amino acids on each subunit ⁽⁷⁾. Resistance to quinolones can also be mediated by plasmids that produce the quinolone resistance protein (Qnr). Bacterial DNA are protected from fluoroquinolone lethal inhibition by quinolone resistance protein (Qnr) through competitive binding DNA gyrase and topoisomerase IV. Five classes are currently known Qnr proteins encoded by *qnrA*, *qnrB*, *qnrS*, *qnrC* or *qnrD* genes. The other gene is *aac(6')-Ib-cr* gene and the variation of *aac(6')-Ib* gene also known as genes encoding for aminoglycoside acetyltransferase, responsible for resistance to tobramycin, amikacin, and kanamycin ^(8,9).

According to data obtained in Thailand, *S. Weltevreden*, *S. Stanley*, *S. Anatum*, and *S. Rissen* were the most common serovar in patients offering stool samples. On the other hand, *S. Choleraesuis*, *S. Enteritidis*, *S. Typhimurium*, and *S. Typhi* were observed in clinical samples ⁽¹⁾. In Thailand, the statistic of prevalence has shown increasing in the number of

patients who are infected with *S. Choleraesuis*. From the collecting data statistic reported by National *Salmonella* and *Shigella* Center, increased from 3.4% (n = 116/3426) in 2003 to 7.9% (n= 233/2931) in 2006 and 9.6% (n= 297/3083) in 2008. Furthermore, high rate of ceftriaxone resistance was reported in *S. Choleraesuis* isolated from bacteremia patients at the National *Salmonella* and *Shigella* Center during 2003-2005 ^(1,24). Lee *et al* (2009) found that Thailand and Taiwan demonstrated a frightening high frequency of resistance to fluoroquinolones and 3rd generation cephalosporins ⁽²³⁾. These findings were also supported by Sirichote *et al* (2010) who found cephalosporinases producing *S. Choleraesuis* from Thai patients and a Danish traveler to Thailand harboring ESBL genes ⁽²⁵⁾. However, few studies have reported data on antimicrobial resistance to fluoroquinolones and third-generation cephalosporins in *S. Choleraesuis* isolated in Thailand. Therefore, this study was evaluated antimicrobial resistance patterns, antimicrobial resistance genes and genotypic patterns of *S. Choleraesuis* isolated from clinical specimens in Thailand.

OBJECTIVE

General Objective

To study antimicrobial resistance profile and genetic characterization of *Samonella* Choleraesuis isolated from clinical specimens in Thailand.

Specific Objective

1. To determine antimicrobial resistance of *Salmonella* serovar Choleraesuis isolated from clinical samples.
2. To determine resistance genes of ESBL-producing *S. Choleraesuis* isolated from clinical samples.
3. To determine resistance genes of resistance to fluoroquinolones in *S. Choleraesuis* isolated from clinical samples.
4. To determine genotype relatedness of fluoroquinolones and/or ESBL-producing *S. Choleraesuis* isolated from clinical samples using pulsed-field gel electrophoresis (PFGE).

Benefit of Research

The results in this study will provide the useful information of antimicrobial resistance patterns, antibiotic resistance genes, and PFGE relatedness of *S. Choleraesuis* isolates obtained from clinical patient samples in Thailand. In addition, this study also presents the molecular epidemiology of this phenotype and mechanisms responsible for its decreased susceptibility to fluoroquinolones and extended-spectrum cephalosporins (ESCs).

CHAPTER 2

LITERATURE REVIEWS

1. General characteristics of *Salmonella enterica* and *Salmonella enterica* serovar Choleraesuis

Salmonellae are Gram-negative rod with 0.7-1.5 μm width and 2-5 μm length. They are non-spore forming, noncapsulated, facultative anaerobic bacteria, which classified in the family *Enterobacteriaceae*. Most strains of *Salmonella* are motile with peritrichous flagella except *S. Gallinarum* and *S. Pullorum*. *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).

Salmonella is generally identified as being non-lactose fermenting (NLFs) and oxidase negative. D-glucose is fermented with the production of acid and usually gas. They are chemo-organotrophs, obtaining their energy from oxidation and reduction reactions using organic sources. Most species produce hydrogen sulfide except *S. Paratyphi A* and *S. Choleraesuis*. Other carbohydrates usually fermented are L-arabinose, maltose, D-mannitol, D-mannose, L-rhamnose, D-sorbitol, trehalose, D-xylose, and dulcitol. All species (100%) negative for indole, Voges Proskauer (VP) tests, and urea (except *S. arizonae* appear positive on prolonged incubation). The biochemical reactions for identification of *Salmonella* are shown in Table 1 and Table 2.

The genus *Salmonella* consists of only two species including *Salmonella enterica* and *Salmonella bongori*. Six subspecies are differentiated within *S. enterica* based on their biochemical and genomic characteristics, these six subspecies are follows: *S. enterica* subsp. *enterica* (I), *S. enterica* subsp. *salamae* (II), *S. enterica* subsp. *arizonae* (IIIa), *S. enterica* subsp. *diarizonae* (IIIb), *S. enterica* subsp. *houtenae* (IV) and *S. enterica* subsp. *indica* (VI). *S. bongori* is formerly called *S. enterica* subsp. *bongori* (V). Subspecies *enterica* is commonly isolated from humans and warm-blooded animals. Serogroups belonging to this

subspecies *enterica* are A, B, C1, C2, D and E ⁽²⁷⁾. For the other subspecies and *S. bongori* are associated with the environment and cold-blooded animals but rarely from humans ⁽²⁸⁾.

Salmonella serotype is a definitive typing method used for epidemiological characterization of bacteria. Serotyping of *Salmonella* enteric strains is carried out by identification of surface antigens (LPS, O-antigens) and flagella antigens (proteins, H-antigens). Most common strains of *Salmonella* express two phases of H-antigens but aphasic, monophasic and triphasic variants are known. Some salmonellae have capsular (K) antigens, referred to as Vi, which may interfere with absorbed antisera and are associated with invasiveness. Agglutination tests with absorbed antisera for different O and H antigens forms the basis for serological classification of *S. Choleraesuis* in accordance with the scheme of Kauffman and White ⁽²⁷⁾ and shown in Table 3.

Among more than 2,600 serotypes, such as serotypes Typhi and Paratyphi, are highly adapted to humans and have no other known natural host ⁽¹⁶⁹⁻¹⁷⁰⁾. Other, such as serotypes Typhimurium and Enteritidis, have a broad host range and can infect a wide variety of animals ⁽¹⁷¹⁾. However, some serovars such as *Choleraesuis* (swine paratyphoid) ⁽¹⁶³⁻¹⁶⁴⁾, Dublin (abortion in cattle) ⁽¹⁷²⁾, and *S. enterica* subsp. *arizonae* (reptiles) ⁽¹⁷³⁻¹⁷⁴⁾, are most highly adapted to specific animal species but occasionally infect humans. These nontyphoid *Salmonella* serotypes can cause protean manifestations in humans, including gastroenteritis, bacteremia, and extraintestinal localized infections involving many organs.

S. enterica serovar *Choleraesuis* shares the antigenic formula with Paratyphi C. It is partly defined by its host range; serotype *Choleraesuis* is a host-adapted pathogen that causes swine paratyphoid fever, but it is also capable of causing disease in humans, can also cause septicemic disease with little involvement of the intestinal tract ⁽¹⁶³⁻¹⁶⁴⁾. Invasive bacterial infections are often associated with underlying diseases in the patient ^(111, 152). The annual number of serotype *Choleraesuis* infections of humans reported to the Centers for Disease Control and Prevention was approximately 80 in 1990 to 1996. There are reports from Taiwan, the USA and England & Wales of invasive indexes ranging from 52% to 74%, indicating that infection with *Choleraesuis* often leads to systemic disease in human ^(152,165-167). In Thailand,

during 1988 to 1993, serotype Choleraesuis was the 10th most common serotype that caused salmonellosis in humans ⁽¹⁶⁸⁾.

2. Pathogenesis

Salmonella infection can cause diseases ranging from gastroenteritis, enteric fevers and bacteremia in humans and animals. Particular serovars show a strong propensity to produce a particular syndrome such as *S. Typhi*, *S. Paratyphi A*, and *S. Schottmuelleri* produce enteric fever; *S. Choleraesuis* produce bacteremia or focal infection; *S. Typhimurium*, and *S. Enteritidis* produce gastroenteritis. In general, more serious infections occur in infants, in adults over the age of 50, and in subjects with debilitating illnesses ⁽²⁹⁾. The infecting inoculum required to cause symptomatic disease in healthy adults must be relatively high (more than 10⁵ colony forming units), although it can be lower when protected by food ⁽³⁰⁾.

Salmonella infections can cause severe problem depend on the immune status of the host and virulence factors such as *Salmonella* pathogenicity islands (SPIs) and plasmids ⁽³¹⁾. The bacterial cells can penetrate into the host cells and lead to increasing of intracellular free calcium and the cytoplasm rearrangement. The invasion of bacterial cell can also make the cell membrane disorder (Figure 1). Salmonellosis can cause diarrhea, fever, abdominal cramps, nausea and vomiting. In mild cases diarrhea may be non-bloody, occur several per day and not to be very voluminous. On the other hand, in severe cases, it may be more frequent with bloody and mucus in high volume. Especially infected by nontyphoidal *Salmonella*, the symptoms can be resolved without treatment within 5-7 days after infection. The severity can be found in dehydrated patients or infected by antibiotic resistant strains. Bacteremia can be characterized by infection in tissue surrounding the brain and spinal cord and infection in the bloodstream or sepsis.

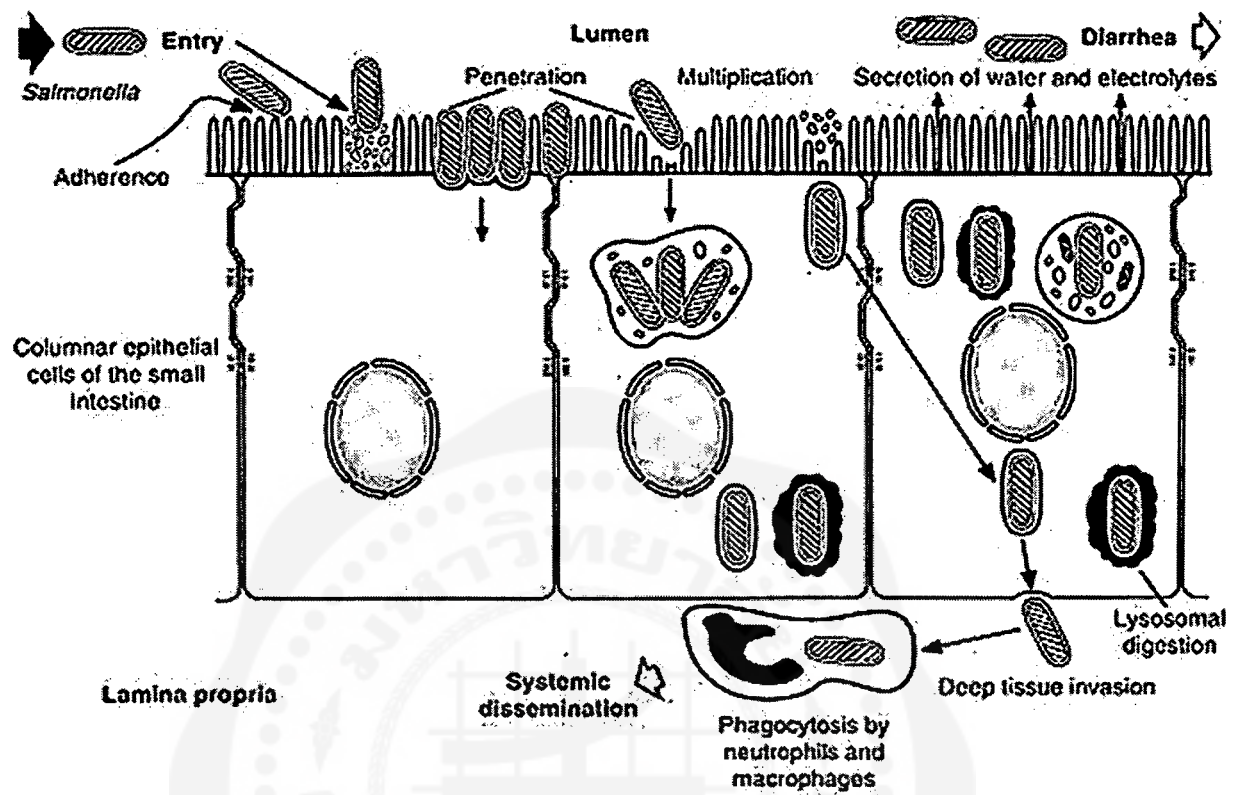


Figure 1 Invasion of intestinal mucosa by *Salmonella* ⁽¹¹³⁾.

Table 1 Differential characters of *Salmonella* species and subspecies

Species	<i>S. enterica</i>						<i>S. bongori</i>
Subspecies	<i>enterica</i>	<i>salamae</i>	<i>arizonae</i>	<i>diarizonae</i>	<i>houtenae</i>	<i>indica</i>	
Characters							
Dulcitol	+	+	-	-	-	d	+
ONPG (2 h)	-	-	+	+	-	d	+
Malonate	-	+	+	+	-	-	-
Gelatinase	-	+	+	+	+	+	-
Sorbitol	+	+	+	+	+	-	+
Growth with KCN	-	-	-	-	+	-	+
L(+)-tartrate ^(a)	+	-	-	-	-	-	-
Galacturonate	-	+	-	-	+	+	+
γ-glutamyltransferase	+ ^(b)	+	-	-	+	+	+
β-glucuronidase	d	d	-	-	-	d	-
Mucate	+	+	+	-(75%)	-	+	+
Salicine	-	-	-	-	+	-	-
Lactose	-	-	-(75%)	+(75%)	-	d	-
Lysed by phage O1	+	+	-	+	-	+	d
Usual habitat	Warm-blooded animals		Cold-blooded animals and environment				

^a α-tartrate; ^b Typhimurium d, Dublin -; + = 90% or more positive reactions, - = < 10%, d = 10-89%

Table 2 The main biochemical reactions of *Salmonella* sp.

Test or substrate	<i>Salmonella</i> Typhi	<i>Salmonella</i> Paratyphi	<i>Salmonella</i> Choleraesuis	Other
Oxidase	-	-	-	-
TSI	K/A gas+	K/A gas+	K/A gas+	K/A gas+
H ₂ S	+	+	-	+
Motility	+	+	+	+
Indole	-	-	-	-
Urease	-	-	-	-
Citrate	d	-	-	+
Lysine	+	+	-	+
Ornithine	+	-	+	+

K = purple alkaline reaction

A = yellow acid reaction

+ = positive within one or two days incubation.

- = no reactions.

d = different reactions; + or -

Table 3 Antigenic Schema for *Salmonella* Choleraesuis Formular**Group O:7 (C₁)**

Strains in this group can be lysogenized by phage 14 (O:6,7—> O:6,7,14, former group C4).

Type	Somatic (O) antigen	Flagella (H) antigen		
		Phase 1	Phase 2	Other
Sanjuan	6,7	a	1,5	
II	6,7, <u>14</u>	a	1,5	
Umhlali	6,7	a	1,6	
Austin	6,7	a	1,7	
Oslo	6,7, <u>14</u>	a	e,n,x	
Denver	6,7	a	e,n,z ₁₅	
Coleypark	6,7, <u>14</u>	a	l,w	
Damman	6,7, <u>14</u>	a	z ₆	
II	6,7	a	z ₆	
II	6,7	a	Z ₄₂	
Brazzaville	6,7	b	1,2	
Edinburg	6,7, <u>14</u>	b	1,5	
Adime	6,7	b	1,6	
Koumra	6,7	b	1,7	
Lockleaze	6,7, <u>14</u>	b	e,n,x	
Georgia	6,7	b	e,n,z ₁₅	
II	6,7	b	[e,n,x]	z ₄₂
Ohio	6,7, <u>14</u>	b	l,w	[z ₅₉]
Leopoldville	6,7, <u>14</u>	b	z ₆	
Kotte	6,7	b	z ₃₅	
II	6,7	b	z ₃₉	
Hissar	6,7, <u>14</u>	c	1,2	
Paratyphi C ¹	6,7,[Vi]	c	1,5	
Choleraesuis ¹	6,7	c	1,5	
Typhisuis ¹	6,7	c	1,5	

3. Transmission and Treatment

Salmonella infection can transmit from animal to animal, animal to human and human to human. Nowadays, there are not only many findings about the transmission to human via food chain but also found most of them were antibiotic resistant strains. The transmission usually occurred by fecal-oral route. The transmission can prevent by cooking all kind of food thoroughly and drink only pasteurized milk.

Salmonella infections usually can be resolved in 5 to 7 days and most cases require no treatment by antibiotics unless the patients become more severely dehydrated or the infection spreads. The patients with severe diarrhea require only rehydration treatment.

Antibiotic treatment is not required for *Salmonella* gastroenteritis but is essential for enteric fever, invasive salmonellosis, and for patients at risk of extra-intestinal disease. Salmonellosis can be generally treated with the β -lactam antibiotics amoxicillin and cefotaxime or ceftriaxone for 'susceptible' isolates and imipenem/meropenem for ESBL infections, together with fluoroquinolones (ciprofloxacin) are the mainstay for the treatment of invasive salmonellosis. The length of treatment varies depending on the patient's illness and can range from 14 days for enteric fever to 6 weeks for bacteremia. Some of *Salmonella* bacteria can develop antibiotic resistance mechanisms. The use of extended-spectrum cephalosporins (cefotaxime or ceftriaxone) and fluoroquinolones (ciprofloxacin), are widely use as drug of choice for salmonellosis treatment. Because of this reason, the increasing of resistance rate for extended-spectrum cephalosporins and fluoroquinolones in nontyphoidal *Salmonella* has become clinical concern.

4. Epidemiology of extended-spectrum cephalosporin and fluoroquinolone resistant

Salmonella is one of the leading cause serious diseases in both humans and animals, and infections with non-typhoidal *salmonella* are a significant cause of illness and death worldwide ⁽²⁶⁾. The Center for Disease Control and Prevention (CDC) has estimated that 9.4 million food borne illnesses, 55,961 hospitalizations and 1,351 deaths occur in the United

State each year ⁽³³⁾. Non-typhoidal *Salmonella* cause an estimated 1 million illnesses with approximately 20,000 hospitalizations and approximately 378 deaths each year ⁽³³⁾. *S. Choleraesuis* is among the non-typhoidal *Salmonella* serotypes that cause bacteremia. It is a host adapted pathogen that is commonly isolated from porcine sources causing swine paratyphoid which is highly pathogenic to humans. It is uncommon in the United States as compared to Taiwan, where it ranks second only to serotype Typhimurium as the most frequently isolated *Salmonella* serotype from human sources ⁽²⁸⁾. In Thailand, during 1988 to 2008, *S. Choleraesuis* was the 10th most common serotype that caused salmonellosis in human. In 2008 alone, a total of 3,083 cases of *Salmonella* were reported of which only 295 cases were of *S. Choleraesuis*: 279 cases were isolated from human blood, 5 cases were isolated from human pus, and 11 cases were isolated from other human specimens ⁽²⁹⁾.

Extended-spectrum cephalosporins (ESCs), especially ceftriaxone, are commonly used to treat invasive infections and severe diarrhea caused by nontyphoidal *Salmonella*. However, ceftriaxone resistant non-typhoidal *Salmonella* in human has frequently been reported worldwide in recent years, including United States, Europe, South America, and Asia ^(10-12, 25). The prevalence of ESC resistance among nontyphoidal *Samonella* in Asia is shown in Table 4.

In three-year period study, 2003-2005, in seven Asian countries (Philippines, Hongkong, Singapore, Sri Lanka, Korea, Thailand and Taiwan) shown that 3.0% were resistant to ceftriaxone ⁽²⁵⁾. A study in Taiwan, the 3,592 nontyphoidal *Salmonella* isolated in 1999-2003 which were examined ⁽³⁰⁾. The results showed that, 0.6% (22/3,592) of isolates were resistant to ceftriaxone and rate of resistance increased in several serogroups (0.8-2.1%; average, 1.5%). Moreover, the ceftriaxone resistance increased significantly from 0.8% in 1999 to 1.5% in 2003. All 22 ceftriaxone resistant isolates were associated with CMY-2 in 14 (63.64%) isolates and with SHV-12 (1 isolate). In 2004, the prevalence of resistance to ESC in nontyphoidal *Salmonella* in Taiwan were increased to be 3.3% (20/600) and 17.8% high rate resistance were detected in *S. Choleraesuis*. All ESC-resistant isolates produced CMY-2 ⁽³¹⁾.

Fluoroquinolones have been used for treatment of salmonellosis. The development of resistance in clinical isolates of *Salmonella* results in failure of ciprofloxacin therapy. The

reports during 1995-1999 demonstrated the reduction of fluoroquinolones susceptibility in *Salmonella enterica* serotypes in travelers resulting from Southeast Asia ⁽⁵²⁾. Moreover, nalidixic acid resistance in *Salmonella* from diarrheal patients in Vietnam and Thailand ⁽⁵³⁾ increased from less than 1% in 1992, 4% in 1993-1994, 9% in 1995 and 21% in 1996-1999 but ciprofloxacin resistance was less than 1%. Nalidixic acid resistance in *Salmonella* was also reported in contaminated food from Thailand ⁽⁵²⁾. The uses of antibiotic not only in treatment but also as a growth promoter in animal caused the increasing of the resistant strains ^(107,108). Other study in nontyphoidal *Salmonella* isolated from bacteremic patients in Thailand during 2003 to 2005 showed high frequency drug resistance. All isolates of *S. Choleraesuis* were ceftriaxone resistance and also showed high level resistance of nalidixic acid and most of them showed reduced susceptibility to ciprofloxacin ⁽²⁴⁾. Recently, nontyphoidal *Salmonella* infections in humans are more resistant to fluoroquinolone and cephalosporins ⁽¹⁴²⁾. The incidence of co-resistance to fluoroquinolones and extended-spectrum cephalosporins may limit the therapeutic options available for their treatment. Decreased susceptibility to both drug classes was identified in Thailand in 1993 (serovar Anatum, Derby, Enteritidis, Typhimurium, Weltevreden, and I.4,5,12:i:-) ⁽¹²⁶⁾, the United Kingdom in 1998 (serovar Senftenberg, Typhimurium, and Virchow) ⁽¹²⁷⁾, Belgium as early as 2001 (serovar Virchow) ⁽¹²⁸⁾, India in 2002 (serovar Typhi) ⁽¹²⁹⁾, the United States in 2002 (serovar Mbandaka) ⁽¹³⁰⁾, France in 2003 ⁽¹²⁸⁾, and Taiwan in 2004 (serovar Choleraesuis, Cairo, and Kaduna) ⁽¹¹⁸⁾. In the United States, 27 (4.6%) of 588 *Salmonella* serovar Typhimurium isolates obtained from food animals in 1999 were resistant to ceftiofur and nalidixic acid.

Table 4 The prevalence of ESCs resistance among nontyphoidal *Salmonella* in Asia

Country/ region	Year (collected)	No. of test isolates	ESC resistance (%)	Serotypes	Mechanism of ESC resistance	Reference
<u>Asia</u>						
Taiwan	1997-2000	384	6 (1.6%)	<i>S. Typhimurium</i>	CMY-2	(114)
Northern Taiwan	1999	549	2 (0.36%)	<i>S. Typhimurium</i>	CMY-2	(110)
	2000	870	13 (1.49%)	<i>S. Senftenberg</i>	CTX-M-14	
	2001	893	7 (0.78%)	<i>S. Panama</i>		
	2002	715	9 (1.26%)	<i>S. Derby</i>		
Taiwan	1999-2003	3,592	22 (0.6%)	<i>S. Typhimurium</i> <i>S. Enteritidis</i> <i>S. Choleraesuis</i> Others	CMY-2 CTX-M-3 SHV-2a SHV-12	(115)
Taiwan	2004	600	20 (3.3%)	<i>S. Choleraesuis</i> <i>S. Stanley</i> Others	CMY-2	(118)
Korea	2002-2004	130	1 (0.8%)	<i>Salmonella</i> spp.	DHA-1	(119)
Asia	2003-2005	400	12 (3%)	<i>S. Typhimurium</i> <i>S. Panama</i>	ND	(23)
Singapore	2003-2006	640	15 (2.3%)	<i>S. Typhimurium</i> <i>S. Enteritidis</i> Others	SHV-5 CMY-2 group CTX-M-9 group CTX-M-1 group DHA	(117)

Table 4 The prevalence of ESCs resistance among nontyphoidal *Salmonella* in Asia (cont)

Country/ region	Year (collected)	No. of test isolates	ESC resistance (%)	Serotypes	Mechanism of ESC resistance	Reference
China	2006	221	7 (3%)	S. Derby	CTX-M-14	(116)
				S. Agona	CTX-M-83	
				Others	CTX-M-84	
					CTX-M-85	
					CTX-M-86	
China	2007-2009	62	44 (27.4%)	S. Typhimurium	CTX-M-14	(142)
					CTX-M-15	
					CTX-M-55	
Thailand	2002-2008	253	66 (4.5%)	S. Kedougou	CTX-M-63	(143)
					CMY-2	
					TEM-1b	
Thailand	2003,2007- 2008	24	13 (54.16%)	S. Choleraesuis	CTX-M-14 CMY-2 TEM-1b	(25)

5. Extended-spectrum cephalosporins

5.1 β -Lactam antibiotics and cephalosporins

Hydrolysis of β -lactam antibiotics by β -lactamases is the most common mechanism of resistance for this class of antibacterial agents in clinically important Gram-negative bacteria. Because penicillins, cephalosporins, and carbapenems are included in the preferred treatment regimens for many infectious diseases, the presence and characteristic of these enzymes play a clinical role in the selection of appropriate therapy. The development of extended-spectrum cephalosporins into clinical practice in the early 1980s was heralded as a major breakthrough in the fight against β -lactamases-mediated bacterial resistance to antibiotic⁽¹²⁰⁻¹²²⁾. The first cephalosporin pharmaceutical, cephalothin, was introduced for clinical use in 1964. There are more than 20 cephalosporin antibiotic in use today. The cephalosporin class is among the most widely prescribed antimicrobial classes because of its broad spectrum of activity, low toxicity, ease of administration, and favorable pharmacokinetic profile. Most of the available cephalosporins are semisynthetic derivatives of cephalosporin C. The basic structure of the cephem nucleus includes a β -lactam ring fused to a six-member sulfur-containing dihydrothiazine ring (Figure 2). Chemical modifications of the basic cephem structure by substitution of constituents at position C1, C3, and C7 led to the various cephalosporin compounds in use today^(123,124).

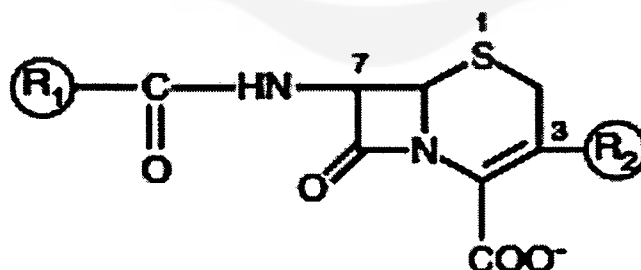


Figure 2. Basic cephalosporin nucleus⁽¹²⁴⁾.

There are several microbiologic and pharmacology differences that could serve as a basis for classification among the cephalosporin drug class. The most widely accepted classification includes five divisions, or generation, based loosely on the microbial spectrum of activity including:

- 1) the first-generation cephalosporins exhibit activity focused primarily on gram-positive bacteria;
- 2) the second-generation drugs have enhanced activity against gram-negative bacilli but maintain varying degrees of activity against gram-positive cocci;
- 3) the third-generation cephalosporins have markedly increased potency against gram-negative bacilli; however, for some compounds in this group, activity against gram-positive cocci is reduced;
- 4) the fourth-generation has the widest spectrum of activity of the five groups;
- 5) the fifth-generation is referred to as the methicillin-resistant *Staphylococcus aureus* (MRSA)-active cephalosporins.

Both of third- and fourth-generation drugs are also called the extended-spectrum cephalosporins (ESCs). The production of extended-spectrum β -lactamases (ESBLs) are the important resistance mechanisms to extended-spectrum cephalosporins in bacteria. In general, ESBLs are capable of hydrolyzing penicillins (e.g. ampicillin and piperacillin), first, second, third and fourth generations of cephalosporins, and the monobactam aztreonam (but not the cephamycin or carbapenams) ^(37,83). In contrast, ESBLs (particularly TEM and SHV family derivative) are readily inhibited by the commercially available beta-lactamase inhibitors (i.e. clavulanic acid, tazobactam, or sulbactam). This unique property serves as an important phenotypic test that is conveniently exploited to identify ESBLs in bacteria. Two systems are commonly used to classify beta-lactamase: the Ambler scheme and the Bush-Medeiros-Jacoby system ^(37,41). They are summarized in Table 2.5. Both systems are used interchangeably in the literature: ESBLs belong to group 2b in the Bush-Medeiros-Jacoby system and to class A in the Ambler system.

Table 5 Classification of β -Lactamases⁽¹²⁵⁾

Ambler Molecular Class	Major Subtypes	Preferred Substrates	Inhibitor	Main Genetic Localization	Representative Enzyme (s)
A	Gram-positive β -lactamase 2a	Penicillins	Clavulanic acid	Chromosome or plasmid	PC1
	Gram-negative β -lactamase 2b	Penicillins, early cephalosporins	Clavulanic acid	Chromosome or plasmid	TEM-1, SHV-1
	Extended-spectrum β -lactamase 2be	Penicillins, extended-spectrum cephalosporins, aztreonam	Clavulanic acid	Plasmid	TEM-24, SHV-12, CTX-M-15
	Inhibitor-resistant TEM β -lactamase 2br	Penicillins	Clavulanic acid*	Plasmid	TEM-30, SHV-10
	Carbenicillin-hydrolyzing β -lactamase 2c	Carbenicillin	Clavulanic acid*	Plasmid	PSE-1, CARB-3
	Cephalosporins-hydrolyzing β -lactamase 2e	Extended-spectrum cephalosporins	Clavulanic acid	Chromosome	CepA
	Carbapenem-hydrolyzing β -lactamase 2f	Carbapenems	Clavulanic acid*	Chromosome or plasmid	KPC-2, SME-1
B	Metallo- β -lactamase 3a	All β -lactams except monobactam	EDTA, divalent cation chelators	Chromosome or plasmid	IMP-1, VIM-2, NDM-1
C	AmpC-type β -lactamase 1	Cephalosporins	Cloxacillin	Chromosome or plasmid	AmpC, CMY-2
D	Oxacillin-hydrolyzing β -lactamase 2d	Oxacillin	Clavulanic acid*	Chromosome or plasmid	OXA-1, OXA-10
	Extended-spectrum β -lactamase 2de	Extended-spectrum cephalosporins	Clavulanic acid*	Plasmid	OXA-11, OXA-15
	Carbapenem-hydrolyzing β -lactamase 2df	Carbapenems	Clavulanic acid*	Plasmid	OXA-23, OXA-40

* Indicates relatively weaker inhibition.

5.2 Mechanism of action of β -lactam drugs

β -Lactam antibiotics interfere with the final stage of cell wall (peptidoglycan) synthesis⁽⁷¹⁾ by inhibiting bacterial enzymes called penicillin-binding proteins (PBPs). PBPs are bifunctional enzymes present in the cytoplasmic membrane of bacteria. They catalyze the synthesis of peptidoglycan in bacterial cell walls by linking N-acetyl glucosamine to muramic acid (transglycosidase function) and through transpeptidase activity mediate cross-linking of peptidoglycan polymers by means of interlinking pentapeptide side chains. Peptidoglycan has a net-link structure (Figure 3) composed of saccharide chains cross-linked by peptides⁽⁷¹⁾ and protected the organism from osmotic rupture⁽⁷²⁾. β -Lactamases are largely retained in the periplasmic space between the cytoplasmic and outer membranes (Figure 4). The efficacy of β -lactam antibiotics, specifically against gram-negative bacteria, is dependent on accessibility to its targets, the degree of resistance to enzymatic inactivation by β -lactamases present in the periplasm, and their ability to inhibit the target PBPs. Any alteration of these parameters may result in resistance⁽⁷³⁾.

The major cause of resistance to extended-spectrum cephalosporins in nontyphoidal *Salmonella* is production of β -lactamases which destroy β -lactam ring structure of drug, resulting in loss of antibiotic activity. The production of ESBLs and plasmid-mediated AmpC β -lactamases are the important resistance mechanisms to extended-spectrum cephalosporins in nontyphoidal *Salmonella*^(32,34).

5.3 Definition of extended-spectrum β -lactamases (ESBLs)

Many genera of gram-negative bacteria can express chromosome-mediated β -lactamases, which are a major resistance mechanism to β -lactam antibiotics. The first plasmid-mediated β -lactamases to be discovered, TEM-1, was described in 1965 by Datta and Kontomichalou⁽³⁵⁾. The subsequently discovered TEM-2 was derived from TEM-1 with a similar hydrolytic spectrum⁽³⁶⁾. The later, SHV-1 was discovered, is another common plasmid mediated β -lactamases. Its hydrolytic spectrum of activity was similar to that of TEM-1, but it had better activity against ampicillin⁽³⁷⁾. These early plasmid-mediated β -lactamases are broad-spectrum β -lactamases, which are capable of hydrolyzing both penicillins and narrow-spectrum cephalosporins⁽³⁷⁾. Over the last 20 years, many new β -lactams have been

developed that were purposely designed to be resistant to the hydrolytic action of β -lactamases. One of these new classes was the oxyimino-cephalosporins (ceftazidime, cefotaxime, and ceftriaxone) in third-generation cephalosporins, which became extensively used for the treatment of serious infections caused by gram-negative bacteria in the 1980s. Not surprisingly, in 1985, Kliebe *et al.* described the first plasmid-mediated β -lactamase, termed SHV-2 in *Klebsiella ozaenae* isolated in Germany and it was able to hydrolyze the extended-spectrum cephalosporins. Thus, this enzyme was called extended-spectrum β -lactamase⁽³⁸⁾. Then, other extended-spectrum β -lactamases (ESBLs) have been discovered and increasingly described in several species of Enterobacteriaceae^(32, 39).

By definition, ESBLs are molecular class A or D β -lactamases, which (i) are able to hydrolyze oxyimino, (ii) have an active-site serine, and (iii) are inhibited by β -lactamase inhibitors such as clavulanic acid^(37,40). ESBLs contain a number of mutations that allow them to include its criteria. The major of ESBLs are molecular class A β -lactamases such as TEM, SHV, and CTX-M-type derivatives⁽³²⁾. Class A β -lactamases commonly found four motifs including Ser70-X-X-LYS73 (X is any amino acid), Ser130-X-Asn, Asp133-Lys234-Thr/Ser-Gly, and Ω -loop (Glu166-Pro-Glu-Leu-Asn) which Ser70 was the main catalytic residue in their active site (Ambler numbering⁽⁴¹⁾)^(42,43) (Figure 4). The OXA-type β -lactamases are molecular class D β -lactamases and contain an active site serine. In the Bush functional classification scheme, ESBLs are placed in two subgroups of group 2 which are inhibited by clavulanate, including subgroups 2be for mainly TEM and SHV-derived ESBLs and 2d for OXA-derived ESBLs⁽³⁷⁾.

ESBLs have been reported frequently in *Escherichia coli* and *Klebsiella* species⁽³⁹⁾. Furthermore, these enzymes have been found in other bacteria species including *Salmonella* spp., *Pseudomonas aeruginosa*, and *Proteus mirabilis*^(32,44). The early predominance of TEM and SHV-type variants among ESBLs appears to reflect the widespread distribution of their plasmid-mediated ancestor enzyme (TEM-1 and SHV-1) in the 1970s. On the other hand, the CTX-M-type β -lactamases have increased dramatically in recent years and are the most rapidly growing group of ESBLs⁽³²⁾.

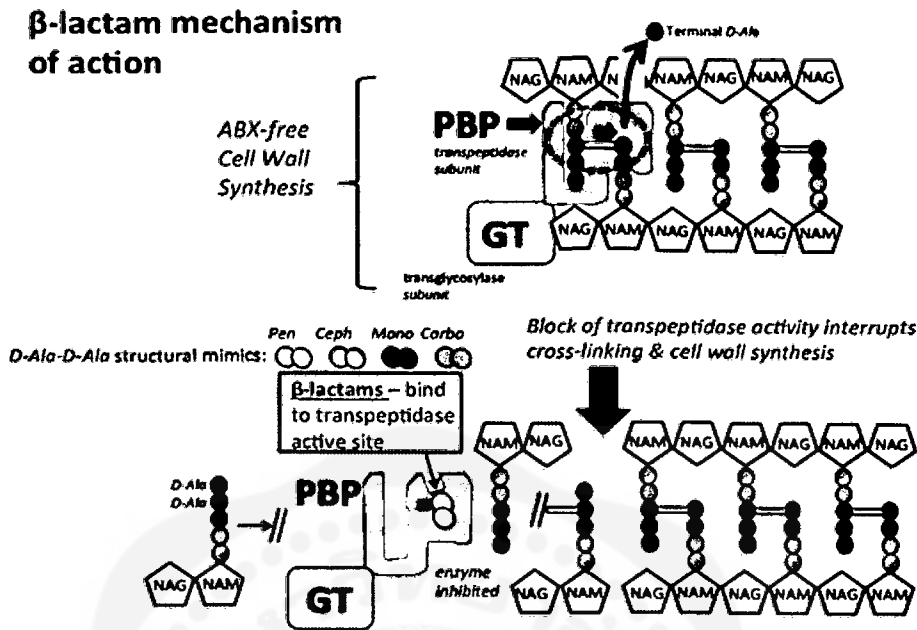


Figure 3 Mechanism of action of beta-lactam antibiotics ⁽⁷³⁾.

Omega loop															
TEM-1	Leu 21	Gln 39	Ala 42	Leu 51	Gly 92	Glu 104	His 153	Arg 164	Met 182	Gly 218	Ala 237	Gly 238	Glu 240	Thr 265	Ser 268
SHV-1	His 8	Leu 35	Arg 43	Leu 51	Leu 122	Met 129	Ser 130	Asn 158	Leu 173	Asp 179	Ala 187	Arg 205	Gly 238	Glu 240	
CTX-M-1								Pro 167					Ser 237	Asp 240	Arg 278
Element 1 S-X-X-K ABL 70-74				Element 2 S-D-N ABL 130-132				Element 3 E-P-E-L-N ABL 166-170				Element 4 D-K-T-G ABL 233-236			

Figure 4 The key amino acid positions in molecular class A β-lactamases TEM, SHV, and CTX-M. The gray shaded areas represent the evolutionary conserved structural elements that limit the active site and the numbering is according to the scheme of Ambler *et al.* ⁽⁴¹⁾.

5.4 Type of ESBLs

ESBLs may be divided into four groups: oxyimino TEM or SHV -derived enzymes and non-TEM or non-SHV enzymes (OXA-derived ESBLs)⁽⁷⁴⁾. More than 150 different natural ESBL variants are known at present. SHV and TEM ESBLs belong to class A enzymes and have serine as the main catalytic residue in their active site and are penicillinases and cephalosporinases usually found on plasmids or transposons^(41,71,75).

5.4.1 TEM

TEM-1 was discovered from *E.coli* isolate from a Greek patient named Temoniera, thus termed TEM⁽⁴⁵⁾. The TEM-1 is able to hydrolyze penicillins and first-generation cephalosporins and spread worldwide. It is now the most common mechanism of β -lactam resistance in gram-negative bacilli. In *E.coli* up to 50-60% of ampicillin-resistant was due to the production of TEM-1⁽⁴⁰⁾. The TEM-type ESBLs are derivative of TEM-1 and TEM-2. In the years since that first report, over 90 additional TEM derivatives have been described (for amino acid sequences for TEM, SHV, and OXA extended-spectrum and inhibitor-resistant β -lactamases, see <http://www.lahey.org/studies/webt.htm>). Some of these β -lactamases are inhibitor resistant enzymes, but the majority of the new derivatives are ESBLs. The amino acid substitutions that occur within TEM variants occur at a limited number of positions. The combinations of these amino acid changes result in various alterations in the ESBL phenotypes, such as the ability to hydrolyze specific oxyimino-cephalosporins such as ceftazidime and cefotaxime, or a change in their isoelectric points, which range from a pI of 5.2 to 6.5^(46, 48). TEM-type ESBLs are most often found in *E.coli* and *K. pneumonia* and have been reported in *Enterobacteriaceae* such as *Morganella morganii*, *Proteus mirabilis*, and *Salmonella* spp.^(49,50). Furthermore, TEM-type ESBLs have been found in non-*Enterobacteriaceae* gram-negative bacteria such as TEM-42 β -lactamase which was found in a strain of *P. aeruginosa*⁽⁵¹⁾.

5.4.2 SHV

SHV β -lactamase appears to be derived from *Klebsiella* spp. The progenitor of SHV enzymes, SHV-1, is commonly found in *K. pneumonia* within the chromosome, so it may

be that the gene for SHV-1 β -lactamase was evolved as a chromosomal gene in *Klebsiella* and was later incorporated into a plasmid which has spread to other bacterial species ⁽⁴⁰⁾. SHV-1 confers resistance to penicillins and narrow-spectrum cephalosporins such as cephalothin and cephaloridine but not to the oxyimino-cephalosporins ⁽⁴⁰⁾. Then, SHV-2 with the same pl, the first derivative of SHV-1 was demonstrated to be the first plasmid-mediated resistance mechanism for oxyimino-cephalosporins. Therefore, the first reported ESBL belonging to the SHV family, SHV-2, differs from SHV-1 by a single amino acid, Gly238Ser ⁽³⁸⁾. The amino acid substitution resulted in an enhanced affinity for oxyimino-cephalosporins, with a significant increase in the MIC to cefotaxime and a more limited increase in the MIC to ceftazidime ⁽³⁸⁾.

5.4.3 CTX-M

The new family of plasmid-mediated ESBLs, called CTX-M, that preferentially hydrolyzes cefotaxime, has become particularly widespread in recent years. The first CTX-M (CTX-M-1) was found in a clinical isolate of *E.coli* from Germany in 1989 ^(76,77). CTX-M-2 was described from an isolated of multidrug-resistant *Salmonella enterica* serovar Typhimurium and had 84% amino acid identity to CTX-M-1 ^(77,78). In recent years, the numbers of CTX-M derivatives have been described from CTX-M-1 to CTX-M-92 but amino acid sequences of CTX-M-14, CTX-M-18, CTX-M-55 and CTX-M-57 are identical ⁽⁴⁸⁾. CTX-M-type enzymes are divided into five groups, namely CTX-M-1, CTX-M-2, CTX-M-9, CTX-M-8, and CTX-M-25, according to the similarity of their amino acid sequences ^(48,79). CTX-M derivatives were: the CTX-M-1 group, including CTX-M-1, CTX-M-3, CTX-M-10, CTX-M-11, CTX-M-12, CTX-M-15, CTX-M-22, CTX-M-23, CTX-M-29, CTX-M-30, CTX-M-32, CTX-M-33, CTX-M-28, CTX-M-36, and CTX-M-54; the CTX-M-2 group, including CTX-M-2, -4, -6, -7, -20, -31, CTX-M-44, and Toho 1; the CTX-M-9 group, including CTX-M-9, -13, -14, -16, -17, -19, -24, -27, -45, -46, -47, -48, -49, and CTX-M-50; the CTX-M-8 group, including CTX-M-8 and CTX-M-40; the CTX-M-25 group, including CTX-M-25, -26, -39, and CTX-M-41 ⁽⁷⁹⁾. CTX-M-producing strains are resistant to cefotaxime, but they often appear to be susceptible to ceftazidime. The crystal structure of CTX-M enzymes has shown that the active sites of CTX-M enzymes are not large enough to recognize ceftazidime, which is larger than cefotaxime ⁽⁸⁰⁾. Moreover, most of CTX-

M enzymes hydrolyze cefepim effectively and MIC values of cefepim for bacteria producing CTX-M enzymes tend to be higher than those for bacteria producing other types of ESBLs⁽⁸¹⁾. The capabilities of the CTX-M enzymes to hydrolyze extended-spectrum cephalosporins⁽³⁹⁾.

The major organisms producing TEM-type and SHV-type ESBLs are identified from hospitalized patients especially in strain of *Klebsiella* spp., however a growing number of infections caused by CTX-M producing organisms in the community setting have been reported typically in *E.coli* since the late 1990s⁽⁸²⁾. Moreover, these enzymes have commonly been found in strains of *Salmonella* spp. In recently years, and have also been described in other species of Enterobacteriaceae⁽⁴⁸⁾.

Recently, it is interesting that an endemic situation is dominant in most countries in Europe, Asia and South America (Figure 5)⁽⁴⁶⁾. In the USA, there are only sporadic reports have been published⁽⁸³⁾. Different enzymes are not similarly represented in all geographic areas (Figure 6)⁽⁴⁶⁾. The enzymes from the CTX-M-9 group are well represented in the countries surrounding the Mediterranean Sea^(84,85). The CTX-M-2 has been mainly isolated in South America and Japan^(86,87) while CTX-M-15 is spread nearly worldwide^(88,89). CTX-M enzymes have been reported in many outbreaks worldwide, e.g. in China (CTX-M-3, CTX-M-9, CTX-M-13, and CTX-M-14)^(90,91), Vietnam (CTX-M-14 and CTX-M-17)⁽⁹²⁾, Taiwan (CTX-M-3 and CTX-M-14)⁽⁵¹⁾, Korea (CTX-M-14)⁽⁹³⁾, Poland (CTX-M-3 and CTX-M-15)^(94,95). This suggests that they are widely dispersed. There is a concern that CTX-M β -lactamases confer resistance to all cephalosporins, but are not detectable by detection tests which are based on using only ceftazidime.

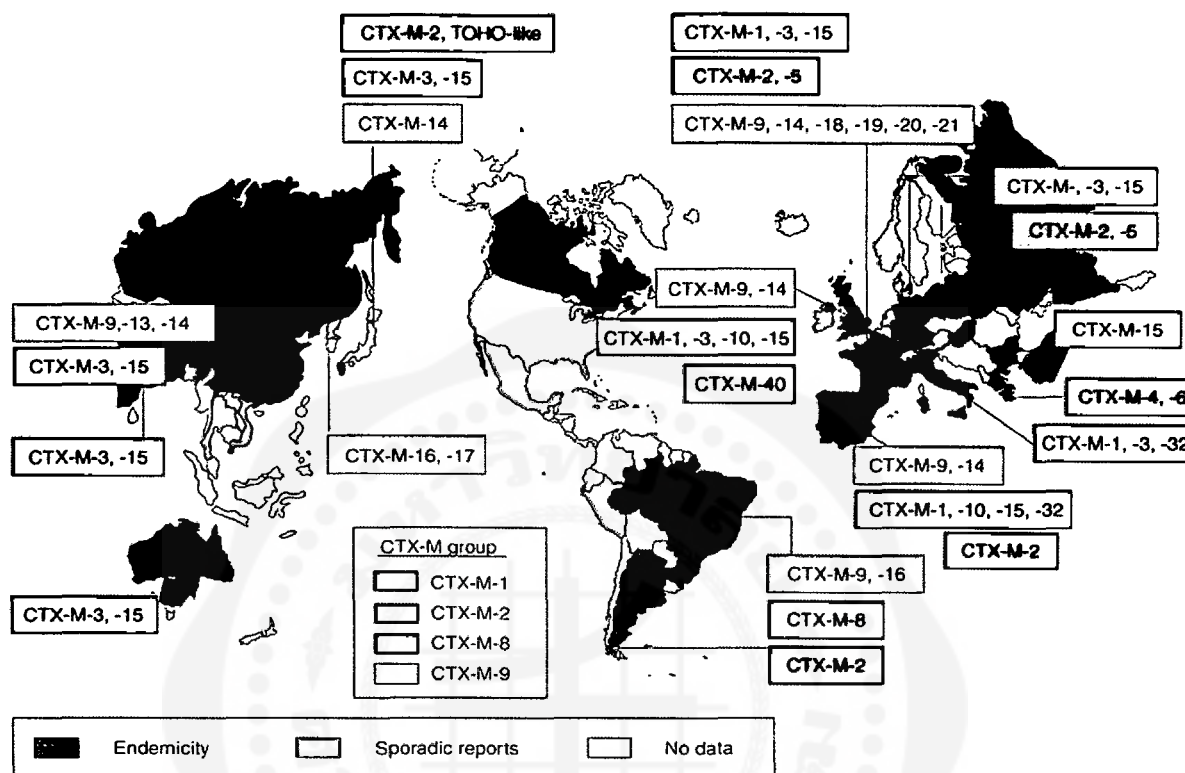


Figure 5 The current situation of CTX-M type ESBLs in different geographic areas ⁽⁴⁶⁾

6. Plasmid-mediated AmpC β -lactamases

AmpC β -lactamases, verified to be chromosomally mediated since 1981, have been described in many genera of bacteria such as *Acinetobacter* spp., *Aeromonas* spp., *Chromobacterium violaceum*, *C. freundii*, *Enterobacter* spp., *E.coli*, *Hafnia alvei*, *Lysobacter lactamgenus*, *Morganella morganii*, *Ochrobactrum anthropic*, *Proteus rettgeri*, *Providencia stuartii*, *P. aeruginosa*, *Psychrobacter immobilis*, *Rhodobacter sphaeroides*, *S. marcescens*, and *Yersinia enterocolitica* ^(96,97).

Until 1989, Bauernfeind *et al.* demonstrated *K. pneumonia* isolate from South Korea that could transfer resistance to penicillins, cefoxitin, cefotetan, oxyimino-cephalosporins, and monobactams to *E.coli*. The enzyme was identified as plasmid-mediated AmpC β -lactamases, termed CMY-1 which showed 82% identity to chromosomal *ampC* genes from *Aeromonas hydrophila* ^(98,99). Lately, the plasmid-mediated AmpC β -lactamases have been reported in several bacterial species that lack chromosomal AmpC enzymes, such as *K. pneumonia*, *K. oxytoca*, *Salmonella* spp., and *P. mirabilis* as well as *E. coli*, which has a low level expression of chromosomal AmpC enzymes ^(96,100).

AmpC enzymes were classified to class C in the Ambler structural classification of β -lactamases ⁽¹⁰¹⁾ and were assigned to group 1 in the functional classification scheme of Bush *et al.* ⁽³⁷⁾. AmpC enzymes were consistently resistant to penicillins and provided resistance to cephalosporins in the oxyimino-cephalosporins (ceftazidime, cefotaxime, ceftriaxone) and the cephamycins (cefoxitin, cefotetan) ⁽⁹⁶⁾. MICs were usually higher for ceftazidime than for cefotaxime and for cefoxitin than for cefotetan ⁽⁹⁶⁾. These enzymes were also resistant to the monobactam and aztreonam and were poorly inhibited by β -lactamases inhibitors such as clavulanic acid, sulbactam, and tazobactam ⁽⁹⁴⁾. However, the enzymes were susceptible to defepime, cefpirome and carbapeneme (imipenem, meropenem) ⁽⁹⁶⁾. The plasmid AmpC β -lactamases are classified base on amino acid sequences into six families that are closely related to chromosomal-mediated AmpC β -lactamases ^(96,102) including:

1. **MOX**: the MOX-type β -lactamases included CMY-1, CMY-8 to CMY-11, CMY-19, MOX-1 to MOX-8 ^(48,96,102);
2. **CIT**: most of AmpC β -lactamases were derivatives of the CIT-type β -lactamases, including LAT-1, CMY-2 to CMY-7, CMY-12 to CMY-18, and CMY-20 to CMY-52 ^(48,96,102);
3. **DHA**: the DHA-type β -lactamases including DHA-1 ^(103,104) and DHA-2 ⁽¹⁰⁵⁾;
4. **ACC**: the ACC-type β -lactamases including ACC-1 to ACC-4 ⁽⁴⁸⁾;
5. **EBS**: the EBS-type β -lactamases including ACT-1 to ACT-8 and MIR-1 to MIR-5 ⁽⁴⁸⁾;
6. **FOX**: the FOX-type β -lactamases including FOX-1 to FOX-7 ⁽⁴⁸⁾.

Variations in β -lactam MICs resulted from substitution of amino acid in different type of plasmid-mediated AmpC β -lactamases and level of AmpC expression ⁽¹⁰⁰⁾. Most *ampC* genes on plasmids were considered to be noninducible because of the constitutively high-level expression of AmpC enzymes ⁽⁹⁶⁾. Thus, these enzymes were resistant to all cephalosporins except the fourth-generation cephalosporins. Most of chromosomal AmpC were inducible enzymes, which were not resistant to the third-generation cephalosporins unless the AmpC β -lactamases was induced to be a high-level expression ⁽¹⁰⁶⁾.

Recently, nontyphoidal *Salmonella* isolates have been reported worldwide to have both inducible and noninducible plasmid-mediated AmpC β -lactamases including CMY-2, CMY-4, CMY-7, ACC-1, and DHA-1 ^(103,107-109). CIT derivatives especially CMY-2 has been commonly reported to be responsible for ceftriaxone resistance in nontyphoidal *Salmonella* ⁽¹¹⁰⁾. CMY producers were found in several serovars of *Salmonella enterica*, with *S. Typhimurium* and *Newport* being the most common ^(32,44).

7. Quinolones and fluoroquinolones resistance

7.1 Development of quinolones

Quinolones are broad spectrum antibiotics and commonly used in both clinical and veterinary medicine. Quinolone was derived from quinine. In the early 1960s, (Figure 6) nalidixic acid was the first quinolone to be developed. Many different derivatives groups were synthesized and evaluated for their antibacterial activity. The modifications in the basic structure of quinolones by replacing hydrogen with fluorine at position 6, substituting a diamine residue residue at position 7 and adding new residues at position 1 of the quinolone ring, have led to enhance antibacterial efficacy. The main use for all these agents is the treatment of urinary tract infection. Quinolones can be classified into four groups following the developing of quinolone (Figure 7) according to clinical use, potency, pharmacokinetics and pharmacodynamics. The first group consists of urinary agents (Group A) that are the older drugs developed before 1985. The second group includes the anti-Gram-negative systemic agents (Group B) that can be used for Gram negative infection. The third group has broad

spectrum activity (Group C) in Gram negative and Gram positive. And the fourth group includes the respiratory agents (Group D) that mainly uses in respiratory tract infection.

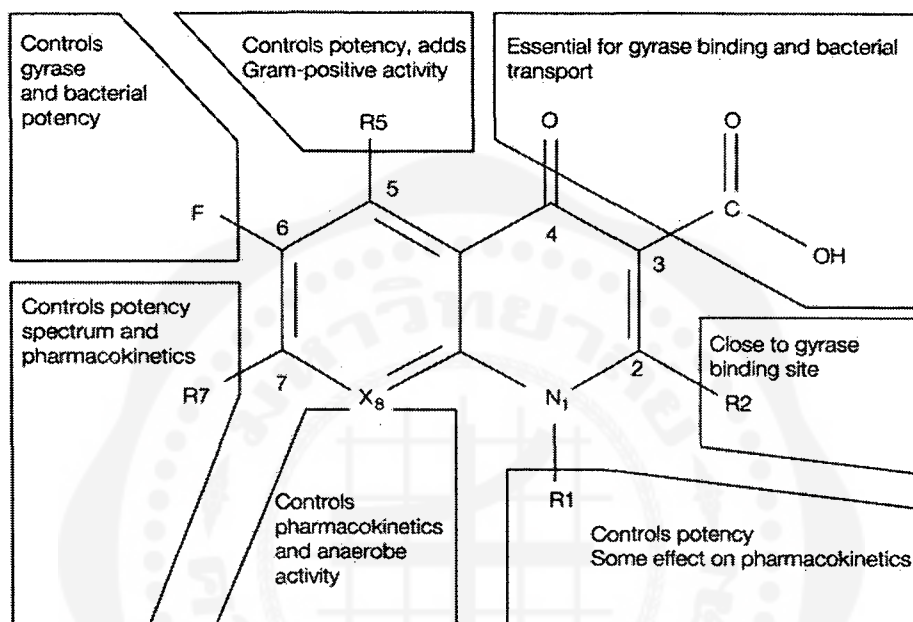


Figure 6 Structure of the quinolone and naphthyridone molecule. In molecules where X is a carbon atom, the molecule is a quinolone (cinoxacin, norfloxacin, ofloxacin, ciprofloxacin, temafloxacin, sparfloxacin, grepafloxacin, levofloxacin, clinafloxacin, moxifloxacin, gatifloxacin). Where X is a nitrogen atom the molecule is a naphthyridone (nalidixic acid, enoxacin, tosufloxacin, trovafloxacin, gemifloxacin). Adapted from Domagala (1994) ⁽⁵⁴⁾.

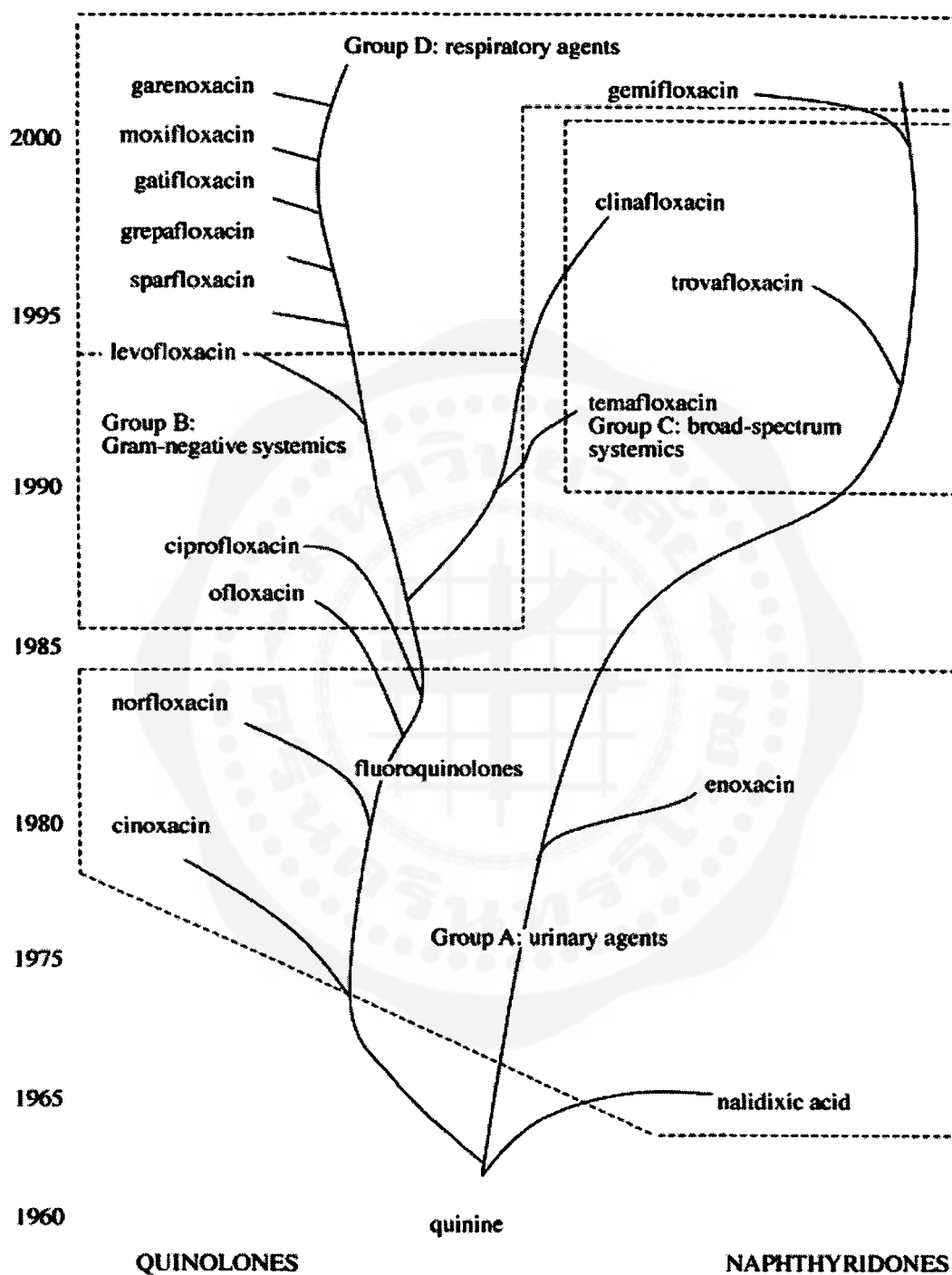


Figure 7 Development of quinolones from 1960 to 2000 ⁽⁵⁴⁾.

The majority of quinolones in clinical use belong to the subset fluoroquinolones, which have a fluorine atom attached to the central ring system, typically at the 6 position or C-7 position and gave the first indication that modification of the basic chemical structure could improve Gram positive activity⁽⁵⁴⁾. Fluoroquinolones are widely used for treatment. The example of fluoroquinolone drugs are norfloxacin (Figure 8) and ciprofloxacin (Figure 9).

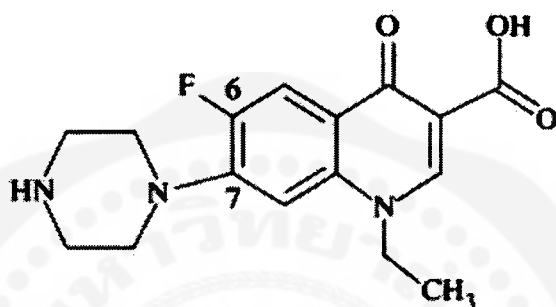


Figure 8 Modifications of quinolone structures: norfloxacin. In all subsequent molecules, except garenoxacin, fluorine was retained at position 6. A six-membered ring at position 7 is found in ciprofloxacin, temafloxacin, sparfloxacin, ofloxacin and levofloxacin⁽⁵⁴⁾.

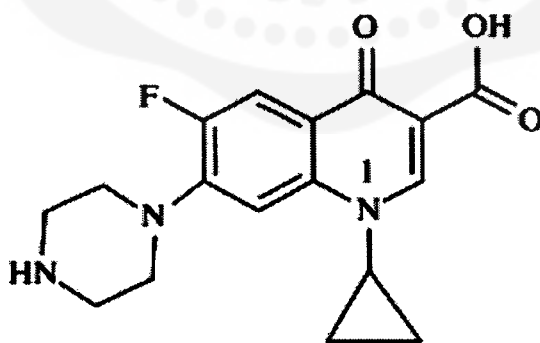


Figure 9 Modification of quinolone structures: ciprofloxacin. A cyclopropyl group at position 1 is found in sparfloxacin, grepafloxacin, clinafloxacin, gatifloxacin, moxifloxacin and garenoxacin⁽⁵⁴⁾.

7.2 Mechanism of resistance to fluoroquinolones

7.2.1 Chromosomal-mediated quinolone resistance mechanisms

7.2.1.1 Gene target of fluoroquinolones

DNA gyrase is a bacterial enzyme that introduces negative supercoil into DNA. The protein binds to DNA as a tetramer in which two A and two B subunits wrap DNA into a positive supercoil. Then one region of duplex DNA is passed through another via DNA breakage and rejoining⁽⁵⁵⁾. The two subunits of gyrase are encoded by *gyrA* and *gyrB* genes, respectively (Figure 10). *gyrA* and *gyrB* genes are located at the quinolone resistance determining region (QRDR). Gyrase plays at least four roles in chromosome functions. First, it maintains particular level of negative supercoiling. Second, it facilitates the movement of replication and transcription complex through DNA. Third, it acts on the removal of knots from DNA and fourth, it helps to bend and fold DNA⁽⁵⁶⁾.

Topoisomerase IV is a homologue of DNA gyrase comprising of four subunits two of C and two of E subunit, encoded by *parC* and *parE* genes, respectively (Figure 10). The topoisomerase IV locus was described in 1990⁽⁵⁷⁾. However, a number of quinolone resistance markers had already been described and mapped to this locus. The mechanism of topoisomerase IV action is similar to gyrase but topoisomerase IV binds to DNA crossover rather than wrapping DNA. Topoisomerase IV is primarily involved in decatenation, the unlinking of replicated daughter chromosomes^(55, 58).

7.2.1.2 Gene target alteration

Quinolones and fluoroquinolones are chemotherapeutic bactericidal drugs, eradicating bacteria by interfering with DNA replication. Other drugs used to treat bacterial infections, such as penicillin and cephalosporin inhibit cell wall biosynthesis, thereby causing bacterial cell death, as opposed to the interference with DNA replication as seen within the fluoroquinolone class of drugs. Quinolones can enter cells easily via porins and, therefore, are often used to treat intracellular pathogens, such as *Legionella pneumophila* and

Mycoplasma pneumonia. For many Gram-negative bacteria, DNA gyrase is the target, whereas topoisomerase IV is the target for many Gram-positive bacteria ⁽⁵⁹⁾.

Complexes between gyrase and DNA are trapped by the quinolones in a reversible reaction that blocks DNA synthesis and cell growth. The inhibition of DNA synthesis and cell growth due to gyrase mutation may lead into two pathways including 1) the putative trapped complex removal system releases lethal double strand DNA breaks from the complexes which can be blocked by chloramphenicol and rifampin treatment and 2) fluoroquinolones at high concentration may stimulate gyrase subunit disassociation, which releases lethal double strand breaks ⁽⁵⁶⁾.

Alterations in DNA gyrase are the major cause of quinolones and fluoroquinolones resistance. The reconstruction by mutations of DNA gyrase generated the holoenzyme with decreased affinity to quinolone caused quinolones resistance. The mutations detected in DNA gyrase mostly occur in the quinolone resistance determining region (QRDR), located at amino acid position Ala67 to Gln106 in *gyrA* ^(62,63). Mutation mostly found at amino acid position 83 and 87 which are located near the active sites of DNA gyrase ^(62,64,65). This mutation may alter the binding of quinolones to the site and lead to reduce susceptibility or resistance to quinolone. A single mutation in *gyrA* of *Salmonella* spp. can be sufficient to cause resistance to nalidixic acid but not in fluoroquinolone resistance ⁽⁶²⁾. The mutations mostly occur at amino acid position Ser83 and Asp87 in QRDR. Mutation in *gyrB* of *Salmonella* spp. is rarely detected. The evidence of *gyrB* mutation has been identified that located outside of QRDR (amino acid, 426-447). The *gyrB* mutation provides to decrease susceptibility to quinolone less than *gyrA* mutation.

The topoisomerase IV is not as sensitive to quinolone as DNA gyrase ^(57,66) because it is a secondary target for quinolones in Gram-negative bacteria. The mutations in topoisomerase IV will not affect unless the sensitivity of the DNA gyrase to fluoroquinolones has been reduced by DNA gyrase mutation ⁽⁶⁶⁾. Due to the homology between the subunits of DNA gyrase and topoisomerase IV, QRDR located at amino acid position Ala64 to Gln103 in *parC* (homology to Ala67 to Gln106 in *gyrA*) and Asp420 to Lys441 in *parE* (homology to Asp426 to Lys447 in *gyrB*) ⁽⁶³⁾. Mutations in *parC* of *Salmonella* spp. do not play an important

role in quinolone resistance or it may be required to achieve high-level resistance^(67,68). The *parC* mutations have been detected in strains that also carry mutation in *gyrA*⁽⁶⁹⁾. Moreover, the mutations outside of the QRDR of *parC* have been identified such as substitution of Thr57Ser. As the Thr57Ser mutation occurs outside of the QRDR it may result in different mechanism of quinolone resistance other than mutations within the QRDR⁽⁶⁶⁾.

7.2.1.3 Decrease permeability or over expression of efflux pump

The decrease accumulations of drugs are found in two main mechanisms, i) decrease permeability of the membrane and ii) over expression of efflux pump systems. Quinolones may cross the outer membrane in two different ways, through specific porins or by diffusion through the phospholipid bilayer. The degree of diffusion of a quinolone is greatly dependent on its level of hydrophobicity. All quinolones may cross the outer membrane through the porins, but only those with a greater level of hydrophobicity may diffuse through the phospholipid bilayer. Thus the alterations in membrane permeability are usually associated with decreased expression of porins. In lipopolysaccharide-defective mutants, increased susceptibility to hydrophobic quinolones has been described, without alterations in the level of resistance to the hydrophilic quinolones^(60,61).

7.2.1.4 Transferability of quinolone resistance

Recently, there are the reports of the new plasmid containing quinolone resistance genes called *qnr*. In 1998, the plasmid-mediated quinolone resistance had been first reported in *Klebsiella pneumonia* isolated from a urine culture collected in 1994 in Birmingham, Alabama, USA⁽¹⁰⁹⁾. This strain carried plasmid pMG252 which coding for *qnr* gene and has been renamed to *qnrA* and later *qnrA1* after discovery of other variants. After that there were many studies reported about the new variants of *qnr* genes. In 2005, *qnrS* genes was discovered on plasmid pAH0376 in *Shigella flexneri* 2b, which was an outbreak strain in food poisoning in 2003 in Japan⁽¹¹⁰⁾. This plasmid conferred low level resistance to nalidixic acid and fluoroquinolones. In 2006, *qnrB* was first identified from *Klebsiella pneumonia* isolated from blood culture in 2002 from South India and this strain also produced

CTX-M-15 β -lactamases on the same plasmid⁽¹¹¹⁾. QnrC has been first identified in 2008 in china⁽¹¹²⁾. The *qnrC* genes was found in *Proteus mirabilis* which caused urinary tract infection, the susceptibility testing showed that this organism was susceptible to most antibiotic except amoxicillin, cotrimoxazole and showed reduced susceptibility to ciprofloxacin⁽¹¹²⁾. The real mechanism of plasmid-mediated quinolone resistance is still unknown.

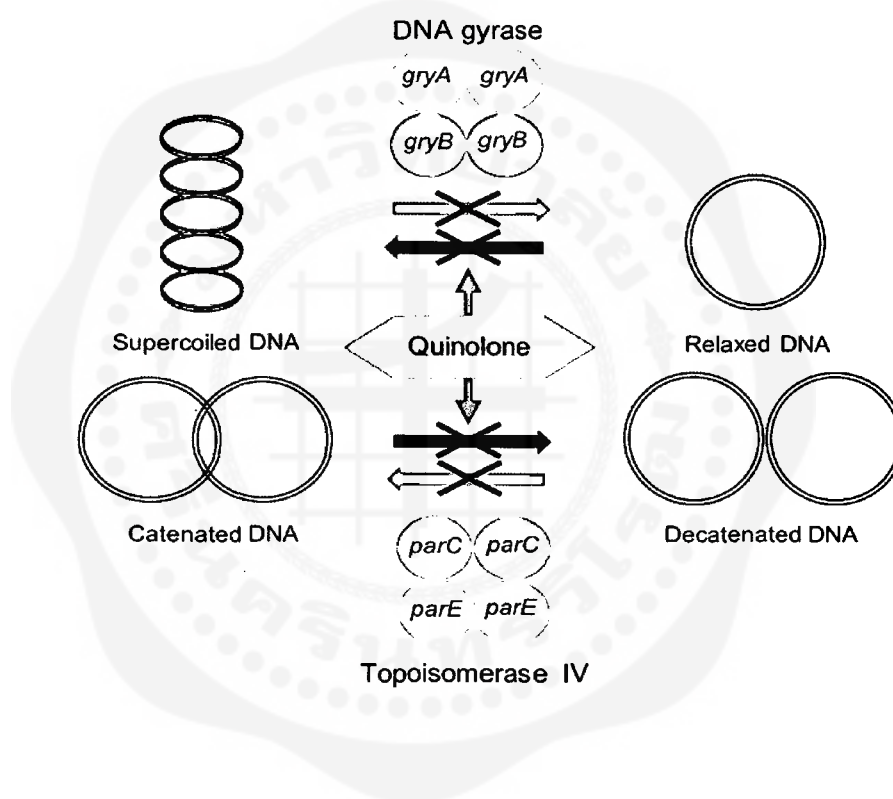


Figure 10 Structure and principle function of DNA gyrase and topoisomerase IV⁽⁵⁸⁾.

CHAPTER 3

MATERIALS AND METHODS

1. Study Design and Bacterial Strains

Sixty-two cefotaxime-resistant *S. Choleraesuis* were obtained from the WHO National *Salmonella* and *Shigella* Center in Thailand, which collected from 174 *S. Choleraesuis* isolated on all regions of Thailand during this 3-year period (2010-2012). The stock culture of *S. Choleraesuis* isolates were re-subcultured for checking the purity and were confirmed serovar according to the Kauffmann-White antigen schema. Antimicrobial susceptibility testing was carried out using the Kirby-Bauer agar diffusion method and ESBLs production were detected by synergy disc diffusion technique. ESBL-producing and ciprofloxacin resistance *S. Choleraesuis* isolates were detected ESBL genes, Quinolone Resistance Determining Region (QRDR) genes and Plasmid-Mediated Quinolone Resistance (PMQR) genes by using PCR method and ciprofloxacin resistant mechanism were checked expression of efflux pump system by using real-time PCR method. ESBL and ciprofloxacin resistance isolates were determined minimal inhibitory concentrations (MICs) against fluoroquinolones and third generation cephalosporin drugs by E-test method. Then, epidemiological study of *S. Choleraesuis* was also performed by pulse field gel electrophoresis (PFGE).

2. Reconfirmed *S. Choleraesuis* Isolates by Biochemical Test

All of 62 *S. Choleraesuis* isolates were streaked onto Xylose Lysine Deoxycholate (XLD) agar and incubated at 37°C for 18-24 h. The colonies of *S. Choleraesuis* on XLD agar appeared colorless without black center. The biochemical test; Lysine Indole Motility (LIM) and Triple Sugar Iron (TSI) agar were used for preliminary identification of *S. Choleraesuis* and biochemical results were confirmed according to Edwards and Ewing's identification of *Enterobacteriaceae* ⁽¹³²⁾ (Table 6). The LIM results of *S. Choleraesuis* were showed lysine

decarboxylase and motility positive but indole negative. TSI results were presented as alkaline (red) slant and acid butt (yellow), gas positive, and hydrogen sulfide negative. All isolates expected as *S. Choleraesuis* from presumptive biochemical test were subcultured on Trypticase Soy Agar (TSA) and kept them in semi-solid stock culture medium and were kept at room-temperature for further analysis.

3. Antimicrobial Susceptibility Test

All of 62 *S. Choleraesuis* isolates were tested for susceptibility to 19 antimicrobial agents using the agar disk diffusion method (Kirby-Bauer test) following the guidelines and criteria of the Clinical and Laboratory Standards Institute (CLSI) ⁽¹³³⁾. Furthermore, *Escherichia coli* ATCC 25922 was used for quality control.

3.1 Antimicrobial agents

Nineteen antimicrobial agents were used for susceptibility test of *S. Choleraesuis*, including ampicillin (Amp; 10 µg), amoxicillin-calvulanic acid (AMC; 20/10 µg), aztreonam (ATM; 30 µg), cefepime (FEP; 30 µg), ceftazidime (CAZ; 30 µg), cefoxitin (FOX; 30 µg), cefotaxime (CTX; 30 µg), cefpodoxime (CPD; 10 µg), cephalothin (KF; 30 µg), cefuroxime (CXM; 30 µg), ceftriaxone (CRO; 30 µg), ceftiofur (EFT; 30 µg), ciprofloxacin (CIP; 5 µg), chloramphenicol (CHL; 30 µg), nalidixic acid (NA; 30 µg), norfloxacin (NOR; 10 µg), streptomycin (S; 10 µg), tetracyclin (TET; 30 µg), and trimethoprim/sulfamethoxazole (STX; 25 µg).

3.2 Antimicrobial susceptibility test

All of 62 isolates were subcultured on Tryptic Soy Agar (TSA) and incubated at 37°C for 18-24 h. Three to five colonies on TSA were picked up and transferred into 5 ml of 0.85% normal saline (NaCl) solution. Bacterial suspension in 0.85% NaCl was adjusted to a turbidity equivalent to the McFarland standard No. 0.5 (approximately 1×10^8 CFU/ml). Agar disk diffusion assay was performed on Muller-Hinton Agar (MHA) by drying MHA plate at room temperature. The bacterial suspension was mixed by using a vortex and a sterilized cotton swab was soaked into suspension. The surface of MHA plate was streaked across the plate

in 3 planes by using the swab inoculum. Then, plate was evenly streaked in several planes for making the confluent growth. After inoculated on MHA plate, the antimicrobial disks were placed the surface of MHA plate by a sterile dispenser. Each disk was pressed down and then incubated at 37°C for 16-18 h.

The inhibition zone was measured with a Vernier caliper. The zone diameters were recorded in mm. The designation of tested isolates as susceptible or resistance were compared with the interpretive chart of CLSI, 2015⁽¹³³⁾ and classified as resistant, intermediate and susceptible (Table 7). Quality of media, antibiotic disks and E-test strips were controlled with *Escherichia coli* ATCC 25922.

4. Detection of ESBL Producing Isolates by Combination Disk test

All 62 *S. Choleraesuis* isolates were screened for ESBLs producer by combination disk method (CLSI, 2015). An overnight culture of isolate, which was adjusted to 0.5 McFarland (1×10^8 CFU/ml) in 0.85% NaCl was inoculated on MHA plate by using a sterilized cotton swab. A double combination disk test containing 30 µg cefotaxime with 10 µg clavulanic acid, and 30 µg ceftazidime with 10 µg clavulanic acid were placed on the opposite sides of the same inoculated plate. Inhibition zones were measured following incubation at 37°C for 16-18 h. A difference in diameters exhibited ≥ 5 mm for either antimicrobial agent tested in combination with clavulanic acid was determined as a positive ESBL result (Table 8).

5. MIC of Fluoroquinolones and β -Lactams Resistance by E-test Method

Minimal inhibitory concentrations (MICs) were determined using the E-test method, according to guideline of Clinical and Laboratory Standards Institute (CLSI), 2015⁽¹³³⁾. The antibiotics used in this study were ciprofloxacin (32–0.002 µg/mL), cefotaxime (256–0.016 µg/mL), ceftazidime (256–0.016 µg/mL), ceftriaxone (256–0.016 µg/mL), and cefpodoxime (256–0.016 µg/mL).

S. Choleraesuis isolates that positive ESBL and ciprofloxacin resistance were grown on TSA. MICs value were determined on MHA inoculums were prepared from an overnight culture on TSA and the turbidity was adjusted to 0.5 McFarland (1×10^8 CFU/ml) in 0.85% NaCl was inoculated on MHA plate by using a sterilized cotton swab. Strip were applied to agar surface using forceps. The strip with the 'E end' were placed at the edge of the plate and with the scale visible. The plates were incubated at 37°C for 16-18 h. The MIC was determined to the lowest concentration of antimicrobial agent at which there is no visible growth.

Table 6 The main biochemical reactions of *Salmonella Choleraesuis*.

Biochemical test	<i>Salmonella Choleraesuis</i>
Triple Sugar Iron agar (TSI)	K/A Gas+ , H ₂ S -
Dextrose	+ , Gas+
Lactose	-
Mannitol	+
Sucrose	-
Citrate	-
Urea	-
Lysine Indole Motility medium (LIM)	LDC + , LDA - , H ₂ S - , Indole - , Motile +

- K = red alkaline reaction.
 A = yellow acid reaction.
 + = positive within one or two days incubation.
 - = no reaction.

Table 7 Zone diameter interpretive standards for *Salmonella* sp. (CLSI, 2015) ⁽¹³³⁾

Class/Antibiotic(μ g)	Zone diameter breakpoint (mm)			
	R	I	S	<i>E.coli</i> ATCC 25922
PENICILINS				
Ampicillin (10 μ g)	≤ 13	14-16	≥ 17	16-22
β-LACTAM/β-LACTAMASE INHIBITOR COMBINATIONS				
Amoxicillin-calvulanate (20/10 μ g)	≤ 13	14-17	≥ 18	18-24
MONOBACTAMS				
Aztreonam (30 μ g)	≤ 17	18-20	≥ 21	28-36
CEPHALOSPORINS				
Cefepime (30 μ g)	≤ 14	15-17	≥ 18	31-37
Ceftazidime (30 μ g)	≤ 17	18-20	≥ 21	25-23
Cefoxitin (30 μ g)	≤ 14	15-17	≥ 18	23-29
Cefotaxime (30 μ g)	≤ 22	23-25	≥ 26	29-35
Cefpodoxime (10 μ g)	≤ 17	18-20	≥ 17	23-28
Cephalothin (30 μ g)	≤ 14	15-17	≥ 18	15-21
Cefuroxime (30 μ g)	≤ 14	15-22	≥ 23	20-26
Ceftriaxone (30 μ g)	≤ 19	20-22	≥ 23	29-35
Ceftiofur (30 μ g)	≤ 17	18-20	≥ 21	26-31
FLUOROQUINOLONES				
Ciprofloxacin (5 μ g)	≤ 20	21-30	≥ 31	30-40
Norfloxacin (10 μ g)	≤ 12	13-16	≥ 17	28-35
QUINOLONES				
Nalidixic acid (30 μ g)	≤ 13	14-18	≥ 19	22-28
AMINOGLYCOSIDES				
Streptomycin (10 μ g)	≤ 11	12-14	≥ 15	12-20
PHENICOLS				
Chloramphenicol (30 μ g)	≤ 12	13-17	≥ 18	21-27
TETRACYCLINS				
Tetracyclin (30 μ g)	≤ 11	12-14	≥ 15	18-25
FOLATE PATHWAY-INHIBITOR				
Trimethoprim/sulfamethoxazole (25 μ g)	≤ 10	11-15	≥ 16	23-29

Table 8 Screening and confirmatory tests for ESBLs in *Salmonella* isolates (CLSI, 2015)⁽¹³³⁾

Test	Initial screen test	Phenotypic confirmatory test
Antimicrobial (concentration)	Cefotaxime (30 µg)	Ceftazidime (30 µg) Ceftazidime-clavulanic acid (30/10 µg)
	Ceftriaxone (30 µg)	
	Ceftazidime (30 µg)	
Results	Cefotaxime ≤ 22 mm Ceftriaxone ≤ 19 mm Ceftazidime ≤ 17 mm	A ≥ 5 mm increase in a zone diameter for either antimicrobial agent tested in combination with clavulanic acid vs the zone diameter of the agent when tested alone = ESBL (eg, ceftazidime zone = 16; ceftazidime-clavulanic acid zone = 21).

6. Characterization of ESBL, QRDR, and PMQR Genes of *S. Choleraesuis*

S. Choleraesuis isolates resistant to extended-spectrum cephalosporins with ESBL phenotype were investigated for the presence of ESBL genes, including *bla*_{TEM}, *bla*_{SHV}, *bla*_{CTX-M}, *bla*_{ACC}, and *bla*_{CMY-2} by PCR method. All isolates resistant to ciprofloxacin were tested for the presence of mutations in QRDR of *gyrA* and *parC* genes and screening for PMQR, including the *qnrA*, *qnrB*, *qnrS*, and *aac(6)-Ib-cr* genes, were amplified by PCR.

6.1 Chromosomal DNA extraction

DNA templates of all the *S. Choleraesuis* isolates were extracted by modified method of Kieser et al (2000). The *S. Choleraesuis* isolates were grown in Luria Bertani (LB) broth for 18-24 h at 37°C with agitation at 200 rpm. Then, the cell suspension was centrifuged at 11,000×g for 10 min to collect the cell pellet. The cell pellet was resuspended with 750 µl of TES buffer pH 8.0, then 50 µl of lysozyme were added and incubated at 37°C with agitation at 200 rpm for 2 h. After incubated 800 µl of 50 mM MgCl₂ solution was added, and centrifuged at the same speed. The pellet was collected and was resuspended with 800 µl of HTE buffer pH 8.0. Then, 50 µl of 20% SDS was added. After addition of 400 µl of solution

III the mixture was incubated for 30 min at 65°C, and placed on ice immediately for 45 min. The cell debris was discarded after centrifugation at 11,000×g for 10 min. The supernatant containing genomic DNA was removed to a new Eppendorf tube and 800 µl of isopropanol was added. Then, the suspension was incubated at room-temperature for 5 min, and centrifuged at 11,000×g for 5 min. Next, the genomic DNA was washed with 70% ethanol, and centrifuged at the same speed. The genomic DNA was resuspended in 30 µl of deionized water and stored at -20°C for further analysis.

6.2 Plasmid DNA extraction

The plasmid DNA was extracted using nucleospin plasmid quick pure (GmbH & Co. KG, Germany) according to the manufacturers. The positive ESBL producing and ciprofloxacin resistance of *S. Choleraesuis* isolates were cultured in Luria Bertani (LB) broth for 18-24 h at 37°C with agitation at 200 rpm. Then, the cell suspension was centrifuged at 11,000×g for 30 sec to collect the cell pellet. The cell pellet was resuspended with 600 µl of buffer A1 (resuspension solution) and mixed by vortexing. The 600 µl of buffer A2 (lysis solution) was added to the sample and mixed gently by inverting 6-8 times. The 700 µl of buffer A3 (neutralizing solution) was added to the sample and mixed gently by inverting 6-8 times. Next, the sample was centrifuged at 11,000×g for 10 min. The supernatant containing the plasmid DNA was removed to a plasmid mini column, and centrifuged at 11,000×g for 1 min. The filtrate was discarded from the tube and was replaced into the same tube. The 500 µl of buffer AW (wash solution) was added to the column, and centrifuged at the same speed. The buffer AW was discarded. The 600 µl of buffer A4 (precipitate solution) was added and centrifuged at the same speed. The buffer A4 was discarded. The column was replaced into the same tube and was centrifuged for 1 additional minute to remove residual solution. The column was transferred to a 1.5 ml microcentrifuge tube and the 30 µl of deionized water was added and incubated at 70°C for 2 min. After that, the column was centrifuged at 11,000×g for 1 min to elute the plasmid DNA. The plasmid DNA was stored at -20°C for further analysis.

6.3 Oligonucleotide primers for PCR and sequencing

The ESBL genes, including *bla*_{TEM}, *bla*_{SHV}, *bla*_{CTX-M}, *bla*_{ACC}, and *bla*_{CMY-2}, were amplified by PCR. The mutations in QRDR of *gyrA* and *parC* genes and screening for PMQR,

including the *qnrA*, *qnrB*, *qnrS*, and *aac(6')-Ib-cr* genes were amplified by PCR. All primers used for this study are listed in Table 9.

6.4 PCR amplification of the ESBL, QRDR, and PMQR genes

The PCR amplification was performed individually in a final volume of 25 µl. The PCR mixture, including 2.5 µl of 10X Ex buffer, 1.5 µl of dNTP mixture, 0.2 µl of *Tag* polymerase (Takara Bio Inc., Shiga, Japan), 2 µl forward and reverse primer, and 2 µl of DNA template. The final volume was adjusted to 25 µl with deionized water. Amplification conditions for the *bla_{SHV}*, *bla_{CTX-M}*, *bla_{ACC}*, and *bla_{CMY-2}* genes were standardized as follows: an initial denaturation at 95°C for 5 min, followed by 30 cycles of denaturation at 98°C for 10 sec, annealing at 55°C for 1 min and DNA extension at 72°C for 1.30 min, with a final extension at 72°C for 10 min. An annealing temperature of 50°C was used for the amplification of *bla_{TEM}*. For confirmation, the CTX group was amplified and sequenced by the *bla_{CTX-M-1}* group, the *bla_{CTX-M-2}* group, the *bla_{CTX-M-8/25}* group, the *bla_{CTX-M-9}* group and the *bla_{CTX-M-15}* primer. The PCR program consisted of an initial denaturation at 95°C for 5 min, followed by 30 cycles of denaturation at 98°C for 10 sec, annealing at 55°C for the *bla_{CTX-M-1}*, *bla_{CTX-M-2}*, *bla_{CTX-M-9}* groups and *bla_{CTX-M-8/25}* groups, and 50°C for *bla_{CTX-M-15}* for 1 min with a DNA extension at 72°C for 1.30 min, followed by a final extension at 72°C for 10 min.

Amplification conditions for the mutations in QRDR of *gyrA* and *parC* genes and screening for PMQR, including the *qnrA*, *qnrB*, *qnrS*, and *aac(6')-Ib-cr* genes were standardized as follows: an initial denaturation at 95°C for 5 min, followed by 30 cycles of denaturation at 98°C for 10 sec, annealing at 55°C (*parC*, *qnrA*, *qnrS*, and *aac(6')-Ib-cr*) and 50°C (*gyrA* and *qnrB*) for 1 min and DNA extension at 72°C for 1.30 min, with a final extension at 72°C for 10 min.

6.5 Analysis of amplified DNA and purification of PCR products

The PCR products were analyzed on 1.5% gel electrophoresis. PCR products were mixed with 6X of loading dry buffer. The electrophoresis was carried out for 30 min at 100 volt using 0.5X TAE running buffer. The gel was stained with 0.5 µg/ml of ethidium bromide for 5 min and de-stained in distilled water for 10 min. The amplified products were visualized and photographed under UV light. A 100-bp ladder (Gibco BRL, Ontario) was used

as an amplicon size marker. The PCR product sizes of entire *bla*_{TEM}, *bla*_{CTX-M}, *bla*_{SHV}, *bla*_{ACC}, *bla*_{CMY-2} genes were 964 bp, 593 bp, 854 bp, 818 bp, and 758 bp, respectively. The PCR product sizes of entire *gyrA*, *parC*, *qnrA*, *qnrB*, *qnrS*, and *aac(6')-lb-cr* genes were 312 bp, 261 bp, 579 bp, 263 bp, 427 bp and 482 bp, respectively. The PCR products were purified using nucleospin gel extraction (GmbH & Co. KG, Germany) according to the manufacturers.

6.6 Sequence analysis

PCR products were sequenced using four primer sets, including *bla*_{TEM}, *bla*_{ACC}, *bla*_{CTX-M}, *bla*_{CMY-2}, *gyrA*, *parC*, *qnrA*, *qnrB*, *qnrS*, and *aac(6')-lb-cr* genes. The reacted products were sequenced with an applied biosystems model 3730XL automated DNA sequencing system by Macrogen Inc. (Seoul, Korea). Nucleotide sequence analysis was compared with the published sequence using NCBI blast search available at the National Center for Biotechnology Information website (<http://www.ncbi.nlm.nih.gov>). The mutation of multiple sequence alignment of *gyrA* and *parC* sequences were analyzed by ClustalW2 (<http://www.ebi.ac.uk/Tools/msa/clustalo>).

Table 9 Primers used in this study for determination of *bla* genes, QDRD, and PMQR genes of *S. Choleraesuis*

Gene	Sequence (5' to 3')	T _{Annealing} (°C)	Amplicon Size (bp)	Reference
ESBL genes				
<i>bla</i> _{TEM}	F: GCGGAACCCCTATTTG R: ACCAATGCTTAATCAGTGAG	50	964	134
<i>bla</i> _{SHV}	F: TTCGCCTGTGTATTATCTCCCTG R: TTAGCGTTGCCAGTGYTCG	55	854	109
<i>bla</i> _{CTX-M}	F: ATGTGCAGYACCAGTAARGTKATGGC R: TGGGTRAARTARGTSACCAGAAAYCAGCGG	50	593	144
AmpC β-lactamases genes				
<i>bla</i> _{ACC}	F: AGCCTC AGCAGCCGGTTAC R: GAAGCCGTTAGTTGATCCGG	55	818	109
<i>bla</i> _{CMY-2}	F: GCACTTAGCCACCTATACGGCAG R: GCTTTTCAAGAATGCGCCAGG	55	758	109
Sequencing for identification ESBL genes				
<i>bla</i> _{CTX-M-1} gr.	F: GAATTAGAGCGGCAGTCGGG R: CACAACCCAGGAAGCAGGC	56	588	145
<i>bla</i> _{CTX-M-9} gr.	F: GTGCAACGGATGATGTTTCGC R: GAAACGTCTCATCGCCGATC	56	475	145
<i>bla</i> _{CTX-M-2} gr.	F: ATGTGAGYACCAGTAARGTRATGGC R: CCGTGGGTTACGATTTTCGC	55	684	146
<i>bla</i> _{CTX-M-8/25} gr.	F: ATGTGAGYACCAGTAARGTRATGGC R: AACCGTCGGTGACRATTYTS	55	695	146
<i>bla</i> _{CTX-M-15}	F: TTCCAGAATAAGGAATCCCATGG R: CCGTTTCCGCTATTACAAACCG	50	907	147
Quinolone resistance determining region (QRDR) genes				
<i>gryA</i>	F: TACCGTCATAGTTATCCACGA R: GTACTTTACGCCATGAACGT	50	312	137
<i>parC</i>	F: CTATGCGATGTCAGAGCTGG R: TAACAGCAGCTCGGCCTATT	55	261	137
Plasmid mediated quinolone resistance (PMQR) genes				
<i>qnrA</i>	F: GGGTATGGATATTATTGATAAAG R: CTAATCCGGCAGCACTATTA	55	579	138
<i>qnrB</i>	F: GGMATHGAAATTCGCCACTG R: TTGCGYGYCGCCAGTCGAA	50	263	136
<i>qnrS</i>	F: GCAAGTTCATTGAACAGGGT R: TCTAAACCGTCGAGTTCGGCG	55	427	136
<i>aac(6')-Ib-cr</i>	F: TTGCGATGCTCTATGAGTGGCTA R: CTCGAATGCCTGGCGTGTTT	55	482	140

T_{Annealing} : Annealing temperature

7. Pulse Field Gel Electrophoresis (PFGE)

Pulse Field Gel Electrophoresis (PFGE) was performed according to the standardized PulseNet of *E.coli* O157:H7, *E.coli* non-O157 (STEC), *Salmonella* serovar, *Shigella sonnei*, and *Shigella flexneri* (CDC, 2013) ⁽¹⁶³⁾.

7.1 Preparation of agarose plugs

ESBL-producing *S. Choleraesuis* isolates were grown in TSA at 37°C for 14-18 h. Colonies were picked and suspended into 2 ml of cell suspension buffer (CSB; 100 mM Tris: 100 mM EDTA, pH 8.0) and adjusted concentration of cell suspension by BioMérieux Vitex colorimeter at the 17-18% transmittance (10^8 cells). Next, 200 µl of each cell suspension were transferred into 1.5 ml-microcentrifuge tube and 10 µl of proteinase K (20 mg/ml stock) was added to each tube and mixed gently by pipetting up and down. The 200 µl of melted 1% SeaKem Gold (SKG) agarose containing 1% SDS was added to 200 µl of cell suspension and mixed gently by pipetting up and down for several times. Then, melted agarose gel was kept in a warm water-bath (55-60°C). Immediately, the cell suspensions were dispensed into wells of plug molds. Plugs were allowed to solidify at room temperature for 10-15 min.

7.2 Lysis of cells in agarose plugs

Agarose plugs containing cell suspensions were removed from plug molds and transferred into individual tubes (two plugs/tube). The cell lysis buffer containing proteinase K (5 ml: 25 µl) was added into a 50 ml-tube prepare for one sample. The tubes were incubated at 54°C in a water-bath with constant shaking for 2 h.

7.3 Washing of agarose plugs

After cell lysis, the plugs were removed from the water-bath and lysis buffer was carefully discarded. The plugs were washed two times with Sterile Ultrapure water (CLRW). Sterile Ultrapure water (CLRW) was pre-heated at 54°C before each washing step. Then, 15 ml of pre-heated CLRW was added to each individual tube. The tubes were incubated at 54°C for 15 min in a shaking water-bath. CLRW was discarded from the plugs and repeated washing step. Then, the plugs were washed four times with sterile TE buffer (10 mM Tris : 1 mM EDTA, pH 8.0). Sterile TE buffer was pre-heated at 54°C before each washing step. The

15 ml of pre-heated sterile TE buffer was added to each individual tube. The tubes were incubated at 54°C for 15 min in a shaking water-bath. TE buffer was carefully discarded from the plugs. The washing step with TE buffer was repeated thrice. After the last wash, the TE buffer was discarded and 5 ml of fresh TE buffer was added to each tube and stored at 4°C until assay.

7.4 Restriction digestion with *Xba*I

Each agarose plug was cut into 2 mm-wide slice with a scalpel blade and transferred to labeled 1.5 ml-microcentrifuge tubes containing 200 µl of diluted 1X H buffer (prepared from 10X H buffer as shown in Table 3.7), and incubated at 37°C in a water-bath for 30 min. Then, diluted 1X H buffer was removed by pipette tip, and 200 µl of *Xba*I restriction enzyme mixture (Table 10) was added into each individual tube, and was incubated at 37°C in a water-bath for 5 h.

7.5 Loading restricted plug slices on the comb

The restricted plug slices were removed from the water-bath, and enzyme buffer mixture from restricted plug slices were discarded and 200 µl of 0.5X TBE was added, then incubated at room temperature for 5 min. Gel form was set by put comb on bench top and was loaded restriction plug slices on the bottom of the comb teeth. The *Salmonella* Braenderup H9812 standards was loaded on teeth (lanes) 1, 7, 15 (for 15-well gel) and samples on remaining teeth. The excess buffer was removed with tissue and plug slices was air-dried on the comb for 3-5 min. 1% of Seakem Gold (SKG) agarose was melted at 56°C and was carefully poured into the gel form. Gel was allowed to be solidified for 30-45 min. Then, comb was removed.

In parallel, the preparation of electrophoresis chamber and freshly prepared 0.5X TBE was added and the cover of electrophoresis chamber was closed. The power supply was turned on pump by set 1 liter/min of a flow rate (approximately 70) and cooling module (14°C) for 30 min. The conditions of PFGE on the CHEF-DR III electrophoresis (CHEF-DR III, Biorad) shown in Table 11.

7.6 Staining and documentation of PFGE agarose gel

After electrophoresis run was over, equipment was turned off. The gel was removed and stained gel with ethidium bromide containing 40 µl of ethidium bromide stock solution (10 mg/ml) in 400 ml of distilled water. The gel was placed in a covered container for 20-30 min. After staining, the agarose was changed every 20 min. The gel was captured image by using Gel Doc and converted a 0.1sec file to a *.tif file for analysis with the BioNumerics software program.

8. Data Analysis

The prevalence of *Salmonella* Cholearesuis and antimicrobial resistance, and detection of ESBL genes were presented as percentage. PFGE profile was analyzed by BioNumerics software version 3.0 (Biolabs, England) with a position tolerance of 1.0% and 0.5% optimization.

Table 9 Preparation of 1X H buffer

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

Table 10 Preparation of 1X H buffer containing restriction enzyme*

Reagent	μl / Plug slice	μl / N x Plug slice
Sterile Milli Q water	175	N x 175
H buffer	20	N x 20
Enzyme ^a (10 u/μl)	5	N x 5
Total volume	200	N x 200

* CDC PulseNet, 2013

^a *Xba*I restriction enzyme for *Salmonella*

Table 11 The conditions of PFGE on the CHEF DR III electrophoresis*

Condition	μl / N Plug slice
Cooling temperature	14°C
Initial switch time ^a	2.2 s
Final switch time ^a	63.8 s
Voltage gradient	6 V /cm
Included angle	120°
Actual current	0
Run time	19 h

* CDC PulseNet (2013) ⁽¹⁶³⁾.

^a Run times may be different in laboratory and will have to be optimized for gels so that the lowest band in the *S. Braenderup* H9812 standard migrates 1.0-1.5 cm from the bottom of the gel.

CHAPTER 4

RESULTS

1. Bacteria strains

A total of 62 *Salmonella* Choleraesuis isolated from clinical specimens were used in this study. All of *S. Choleraesuis* in this study were obtained from WHO National *Salmonella* and *Shigella* Center during 2010 to 2012. Each strains was from different patient. Fifty Two (83.87%) strains were from blood, followed by 2 (3.22%) strains from joint, stool and pus, and only one each (1.61%) strain from urine, knee joint aspiration, ovary swab and swab material. A majority of *S. Choleraesuis* isolates in this study were recovered from blood specimens which were accounted for 83.87% of all strains. The results are shown in Table 13 and Table 14.

Table 13 Types of clinical specimen of *S. Choleraesuis* 62 strains

Specimen types	No. of strains	Total (%)
Blood	52	83.9
Joint	2	3.2
Stool	2	3.2
Pus	2	3.2
Swab material	1	1.6
Urine	1	1.6
Knee joint aspiration	1	1.6
Ovary swab	1	1.6
Total	62	

Table 14 Epidemiological data of *S. Choleraesuis* 62 strains from clinical specimens in Thailand during 2010-2012

Lab ID code	Specimen	Patient Age(Sex)	Province
SH1544/10	Blood	61Y(M)	Saraburi
SH2539/10	Blood	53Y(F)	Nan
SH495/10	Blood	28Y(F)	Bangkok
SH1660/10	Blood	12Y(M)	Nakhon Phanom
SH1407/10	Blood	23Y(F)	Saraburi
SH1208/10	Blood	39Y(M)	Sa Kaeo
SH3124/10	Blood	71Y(M)	Sa Kaeo
SH1007/10	Blood	25Y(M)	Saraburi
SH2788/10	Blood	35Y(F)	Sa Kaeo
SH862/10	Blood	39Y(M)	Sa Kaeo
SH2223/10	Blood	38Y(M)	Nan
SH2789/10	Blood	35Y(F)	Bangkok
SH2821/10	Blood	31Y(F)	Bangkok
SH1036/10	Blood	38Y(M)	Nakhon Phanom
SH2543/10	Blood	46Y(F)	Bangkok
SH2609/10	Blood	1Y(M)	Nakhon Phanom
SH2347/10	Blood	14Y(F)	Saraburi
SH3141/10	Blood	49Y(F)	Ratchaburi
SH2736/10	Blood	46Y(M)	Ratchaburi
SH787/10	Blood	27Y(F)	Bangkok
SH876/10	Joint	75Y(M)	Sa Kaeo
SH564/10	Blood	59Y(F)	Udon Thani
SH735/10	Blood	49Y(F)	Bangkok
SH737/10	Blood	1Y(M)	Sa Kaeo
SH598/10	Blood	29Y(F)	Surat Thani
SH1364/10	Blood	9M(M)	Bangkok
SH809/10	Blood	63Y(M)	Sa Kaeo
SH218/10	Joint	71Y(M)	Ratchaburi
SH1038/10	Blood	39Y(M)	Bangkok
SH1086/10	Blood	54Y(M)	Nakornpanom
SH2132/10	Blood	52Y(M)	Bangkok

Table 14 Epidemiological data of *S. Choleraesuis* 62 strains from clinical specimens in Thailand during 2010-2012 (continued)

Lab ID code	Specimen	Patient Age(Sex)	Province
SH2653/10	Blood	36Y(M)	Sa Kao
SH2737/10	Blood	6Y(M)	Saraburi
SH3000/10	Blood	58Y(M)	Bangkok
SH3003/10	Blood	36Y(M)	Bangkok
SH3230/10	Pus	61Y(M)	Ratchaburi
SH370/10	Blood	62Y(F)	Payoi
SH704/10	Blood	26Y(M)	Phetchaboon
SH734/10	Blood	49Y(F)	Bangkok
SH785/10	Blood	59Y(F)	Nan
SH791/10	Blood	38Y(M)	Bangkok
SH2653/10	Blood	36Y(M)	Sa Kao
SH2737/10	Blood	6Y(M)	Saraburi
SH3000/10	Blood	58Y(M)	Bangkok
SH3003/10	Blood	36Y(M)	Bangkok
SH3230/10	Pus	61Y(M)	Ratchaburi
SH646/11	Blood	30Y(M)	Sa Kao
SH293/11	Blood	25Y(M)	Surat Thani
SH1421/11	Urine	62Y(M)	Bangkok
SH265/11	Blood	36Y(F)	Sa Kao
SH962/11	Swab ovary	38Y(M)	Ratchaburi
SH143/11	Blood	67Y(M)	Ratchaburi
SH1631/11	Blood	78Y(M)	Bangkok
SH1450/12	Blood	59Y(M)	Ratchaburi
SH359/12	Blood	26Y(M)	Buriram
SH1504/12	Stool	30Y(M)	Bangkok
SH120/12	Blood	46Y(F)	Chonburi
SH1155/12	Blood	18Y(F)	Bangkok
SH575/12	Blood	40Y(M)	Bangkok
SH1456/12	Stool	45Y(M)	Ratchaburi
SH1697/12	Blood	ND(M)	Nakhon Ratchasima
SH1423/12	Blood	53Y(M)	Bangkok

2. Antimicrobial Susceptibility Test

2.1 Antimicrobial susceptibility test

A total of 62 *S. Choleraesuis* strains were isolated from patients with systematic infection in Thailand during 2010-2012. All strains were tested for their susceptibility to 19 antimicrobial agents using the disc diffusion method (Figure 11). Multidrug resistance (resistance to at least three different classes of antimicrobials) was observed in all strains. All *S. Choleraesuis* strains showed reduced susceptibility to 18 antibiotics except for norfloxacin. All strains were 100% resistant to ampicillin, tetracycline, nalidixic acid and third-generation cephalosporin drugs (ceftriaxone, cefotaxime, and cefalothin). The susceptibility to antimicrobials was as follows: cefpodoxime (98.4%), ceftiofur (96.8%), cefuroxime (85.5%), chloramphenicol (83.9%), streptomycin (64.5%), ceftazidime (62.9%), amoxicillin/clavulanate (48.4%), aztreonam (43.5%), cefoxitin (40.3%), ciprofloxacin (14.6%), trimethoprim-sulfamethoxazole (11.3%) and cefepime (8.1%).

Forty-four *S. Choleraesuis* (44/62 strains, 71.1%) strains were positive for ESBL phenotypes. All ESBL-producing strains were resistant to ampicillin, and 100% were cross-resistant to cefotaxime and cefpodoxime. In total, 18.7% (12/62 strains) were resistant to all 9 cephalosporin antimicrobials (cefepime, cefoxitin, ceftazidime, cefotaxime, cefpodoxime, cefalothin, cefuroxime, ceftriaxone and ceftiofur). Additionally, 100% (62/62 strains) and 14.6% (9/62 strains) were resistant to (fluoro)quinolones, including nalidixic acid, and ciprofloxacin, respectively (Table 15).

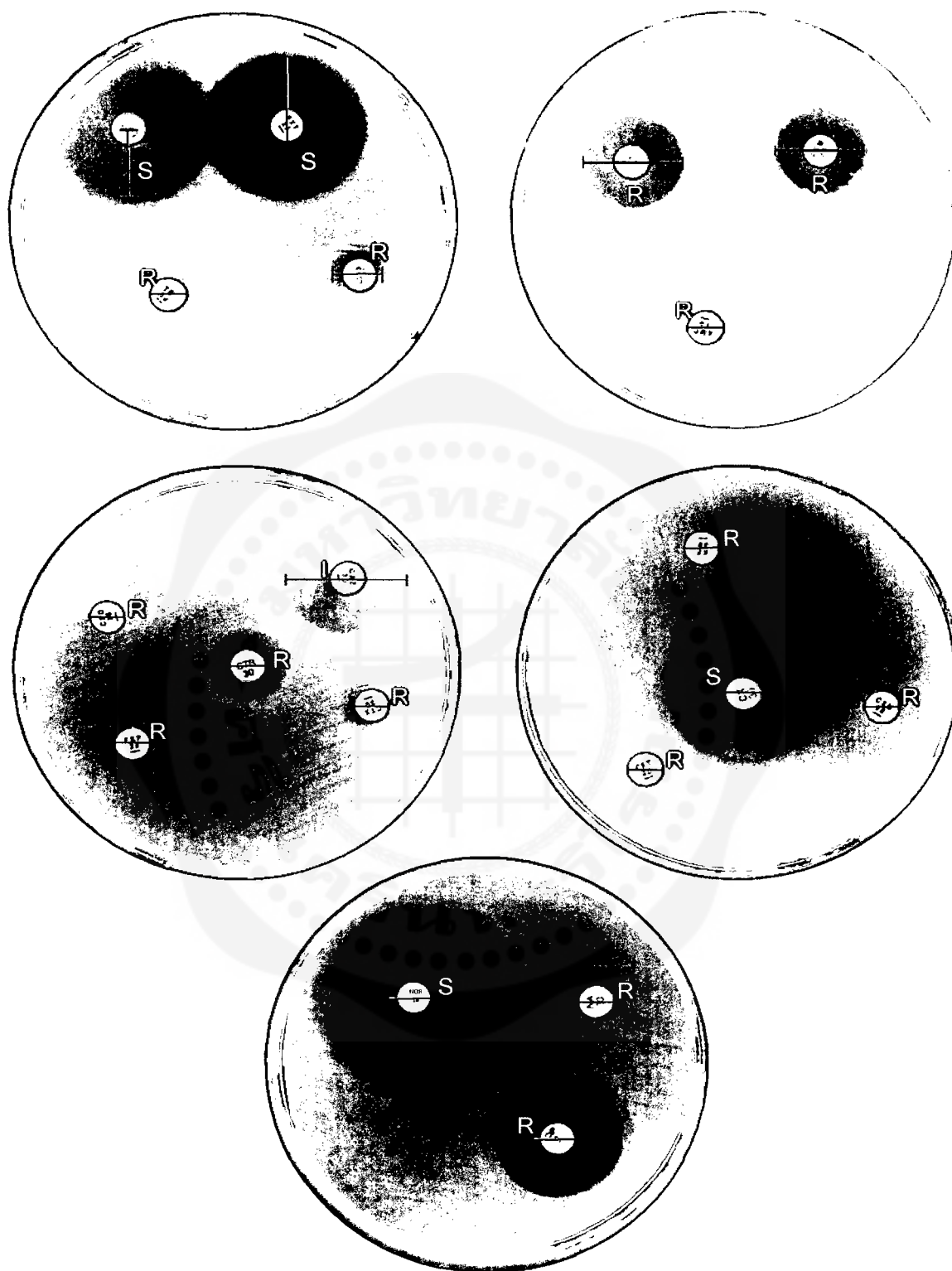


Figure 11 Antimicrobial susceptibility test of *S. Choleraesuis* strain SH1423; S = Sensitive, I = Intermediate, and R = Resistant

Table 15 Antimicrobial susceptibilities of *S. Choleraesuis* 62 strains from clinical specimens

Antimicrobial		Group	% Resistant (strains)	% Intermediate (strains)	% Sensitive (strains)
Ampicillin	(AMP)	Penicillin	100 (62)	0	0
Amoxicillin/ calvulanic acid	(AMC)	β -Lactam/ β -lactam inhibitor combination	48.4 (30)	0	51.6(32)
Aztreonam	(ATM)	Monobactam	43.5 (27)	12.9(8)	43.5(27)
Cefepime	(FEP)	Cephalosporin	8.1 (5)	21.(13)	71.0(44)
Ceftazidime	(CAZ)	Cephalosporin	62.9 (42)	6.4(4)	25.8(16)
Cefoxitin	(FOX)	Cephalosporin	40.3 (25)	4.8(3)	54.8(34)
Cefotaxime	(CTX)	Cephalosporin	100.0 (62)	0	0
Cefpodoxime	(CPD)	Cephalosporin	98.4 (61)	0	0
Cefalothin	(KF)	Cephalosporin	100 (62)	0	0
Cefuroxime	(CXM)	Cephalosporin	85.5 (53)	12.9(8)	1.6(1)
Ceftriaxone	(CRO)	Cephalosporin	100.0 (62)	0	0
Ceftiofur	(EFT)	Cephalosporin	96.8 (60)	3.2(2)	0
Ciprofloxacin	(CIP)	Fluoroquinolone	14.6 (9)	67.7(42)	17.7(11)
Chloramphenicol	(CHL)	Phenicol	83.9 (52)	9.7(6)	6.4(4)
Nalidixic acid	(NAL)	Quinolone	100.0 (62)	0	0
Norfloxacin	(NOR)	Fluoroquinolone	0.0 (0)	0	100(62)
Streptomycin	(S)	Aminoglycoside	64.5 (43)	19.3(12)	11.3(11)
Tetracycline	(TET)	Tetracycline	100.0 (62)	0	0
Co-Trimoxazole	(STX)	Folate Pathway inhibitor	11.3 (7)	1.61(1)	87.1(54)

2.2 Detection of ESBL phenotype

All of 62 *S. Choleraesuis* strains were screened for ESBL phenotype by the combination disk method by CLSI⁽¹³³⁾. Forty-Four of *S. Choleraesuis* strains, were positive for ESBL phenotype by combination disk test (Figure 12). Of the 44 strains, 95.5% (42/44) showed positive results with cefotaxime and cefotaxime/clavulanic acid disk and 75% (33/42) showed positive results with ceftazidime and ceftazidime/clavulanic acid disk. All strains with ESBL phenotype showed that inhibition zone of cefotaxime/clavulanic acid were ≥ 5 mm larger than that of cefotaxime disk alone and showed a difference of 5 to 27 mm zone size. Two strains showed a difference in zone diameter of 5 mm for cefotaxime and cefotaxime/clavulanic acid disk and had negative result with ceftazidime and ceftazidime/clavulanic acid disk. A total of 33 strains with positive results for ceftazidime and ceftazidime/clavulanic acid disk showed a difference of 5 to 20 mm zone size. Eighty strains showed negative results with ESBL phenotype.

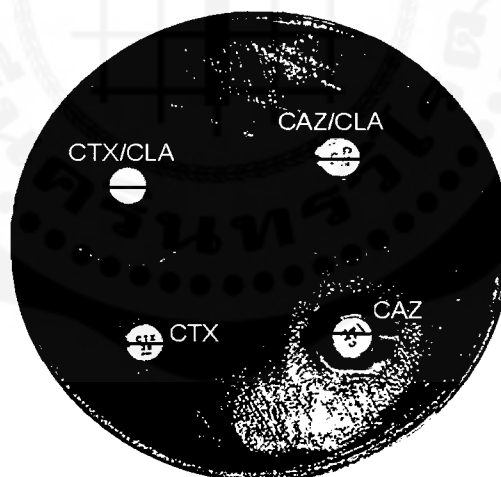


Figure 12 The combination disk test: cefotaxime (CTX), cefotaxime/clavulanic acid (CTX/CLA) and ceftazidime (CAZ), ceftazidime/clavulanic acid (CAZ/CLA): The strain was positive for both cefotaxime and ceftazidime combination disk test.

The different antimicrobial resistance patterns of *S. Choleraesuis* were found amongst the ESBL (n = 44) and non-ESBL (n = 18) strains. There was a difference between the level of antimicrobial resistance in the ESBL-positive and ESBL-negative isolates (Table 16). A total of 30 different resistance patterns were observed amongst the ESBL and non-ESBL producing *S. Choleraesuis* strains, which were multidrug-resistant to 9 to 16 antimicrobial agents (Table 16). The most common resistance patterns were AMP-AMC-NA-CHL-S-TET-KF-FOX-CXM-CAZ-EFT-CRO-CPD-CTX (4 ESBL strains and 4 non-ESBL strains, 12.9%) and AMP-AMC-NA-ATM-CHL-S-TET-KF-FOX-CXM-CAZ-EFT-CRO-CPD-CTX (2 ESBL strains and 6 non-ESBL strains, 12.9%), followed by AMP-NA-CHL-TET-KF-CXM-EFT-CRO-CPD-CTX (6 ESBL strains, 9.7%) and AMP-NA-CHL-S-TET-KF-CXM-EFT-CRO-CPD-CTX (4 ESBL strains, 6.4%).

The antimicrobial susceptibility testing and MICs of 9 *S. Choleraesuis* co-resistance to third generation cephalosporin and ciprofloxacin are summarized in Table 17. Based on the MICs of the tested cephalosporins, the MIC values of fluoroquinolone and ESC-resistant isolates for cefotaxime, ceftazidime, ceftriaxone and cefpodoxime ranged from 64 to ≥ 256 $\mu\text{g/ml}$, 1 to 48 $\mu\text{g/ml}$, ≥ 256 $\mu\text{g/ml}$ and 32 to ≥ 256 $\mu\text{g/ml}$, respectively. In addition, 4 strains isolated from the blood samples that showed high MIC levels for ciprofloxacin (MIC 2 $\mu\text{g/ml}$). Only one strain in a 40-year-old patient, SH575/12, showed high MIC levels for both ciprofloxacin (MIC 2 $\mu\text{g/ml}$) and cephalosporins (cefotaxime MIC ≥ 256 $\mu\text{g/ml}$, ceftazidime MIC 48 $\mu\text{g/ml}$, ceftriaxone MIC ≥ 256 $\mu\text{g/ml}$ and cefpodoxime ≥ 256 $\mu\text{g/ml}$). MIC distribution of the third generation cephalosporin resistant *S. Choleraesuis* strains are shown in Figure 13 – Figure 15.

Table 16 Antimicrobial resistance patterns of *S. Choleraesuis* 62 strains from clinical specimens

Antimicrobial resistance patterns	No. of strains (%)	
	ESBL	Non-ESBL
9 antimicrobial resistance		
AMP-NA-TET-KF-CXM-EFT-CRO-CPD-CTX	3 (4.8)	-
10 antimicrobial resistance		
AMP-NA-CHL-TET-KF-CXM-EFT-CRO-CPD-CTX	6 (9.6)	-
AMP-NA-CHL-S-TET-KF-CXM-EFT-CPD-CTX	1 (1.6)	-
11 antimicrobial resistance		
AMP-NA-CHL-S-TET-KF-CXM-EFT-CRO-CPD-CTX	4 (6.4)	-
AMP-NA-ATM-CHL-TET-STX-KF-EFT-CRO-CPD-CTX	1 (1.6)	-
AMP-NA-TET-KF-FEP-CXM-CAZ-EFT-CRO-CPD-CTX	1 (1.6)	-
AMP-NA-ATM-TET-KF-CXM-CAZ-EFT-CRO-CPD-CTX	1 (1.6)	-
AMP-NA-ATM-CHL-TET-KF-CXM-EFT-CRO-CPD-CTX	1 (1.6)	-
AMP-AMC-NA-CHL-TET-KF-CXM-EFT-CRO-CPD-CTX	1 (1.6)	-
AMP-CIP-NA-TET-STX-KF-CXM-EFT-CRO-CPD-CTX	1 (1.6)	-
AMP-AMC-NA-S-TET-KF-FOX-CAZ-CRO-CPD-CTX	-	1 (1.6)
12 antimicrobial resistance		
AMP-CIP-NA-ATM-CHL-TET-KF-CXM-EFT-CRO-CPD-CTX	1 (1.6)	-
AMP-AMC-NA-CHL-S-TET-KF-FOX-CAZ-CRO-CPD-CTX	1 (1.6)	-
AMP-NA-S-TET-KF-FEP-CXM-CAZ-EFT-CRO-CPD-CTX	1 (1.6)	-
AMP-AMC-NA-CHL-S-TET-KF-CAZ-EFT-CRO-CPD-CTX	-	2 (3.2)
AMP-AMC-NA-TET-KF-FOX-CXM-CAZ-EFT-CRO-CPD-CTX	-	1 (1.6)
13 antimicrobial resistance		
AMP-NA-ATM-CHL-TET-STX-KF-CXM-CAZ-EFT-CRO-CPD-CTX	2 (3.2)	-
AMP-NA-ATM-CHL-S-TET-KF-CXM-CAZ-EFT-CRO-CPD-CTX	2 (3.2)	-
AMP-NA-ATM-S-TET-STX-KF-CXM-CAZ-EFT-CRO-CPD-CTX	1 (1.6)	-
AMP-CIP-NA-ATM-CHL-S-TET-KF-CXM-EFT-CRO-CPD-CTX	1 (1.6)	-
AMP-AMC-NA-CHL-S-TET-KF-FOX-CAZ-EFT-CRO-CPD-CTX	-	3 (4.8)

Table 16 Antimicrobial resistance patterns of *S. Choleraesuis* 62 strains from clinical specimens (continue)

Antimicrobial resistance patterns	No. of strains (%)	
	ESBL	Non-ESBL
14 antimicrobial resistance		
AMP-AMC-NA-CHL-S-TET-KF-FOX-CXM-CAZ-EFT-CRO-CPD-CTX	4 (6.4)	4 (6.4)
AMP-CIP-NA-ATM-CHL-S-TET-KF-CXM-CAZ-EFT-CRO-CPD-CTX	4 (6.4)	-
AMP-AMC-NA-ATM-CHL-S-TET-KF-FOX-CAZ-EFT-CRO-CPD-CTX	1 (1.6)	-
15 antimicrobial resistance		
AMP-AMC-NA-ATM-CHL-S-TET-KF-FOX-CXM-CAZ-EFT-CRO-CPD-CTX	2 (3.2)	6 (9.6)
AMP-AMC-NA-CHL-S-TET-STX-KF-FOX-CXM-CAZ-EFT-CRO-CPD-CTX	1 (1.6)	-
AMP-NA-ATM-CHL-S-TET-STX-KF-FEP-CXM-CAZ-EFT-CRO-CPD-CTX	1 (1.6)	-
AMP-AMC-NA-ATM-CHL-S-TET-KF-FEP-CXM-CAZ-EFT-CRO-CPD-CTX	-	1 (1.6)
16 antimicrobial resistance		
AMP-AMC-CIP-NA-ATM-CHL-S-TET-KF-FOX-CXM-CAZ-EFT-CRO-CPD-CTX	1 (1.6)	-
AMP-AMC-CIP-NA-ATM-CHL-S-TET-KF-FEP-CXM-CAZ-EFT-CRO-CPD-CTX	1 (1.6)	-

AMP, amoxicillin; AMC, amoxicillin + clavulanic acid; CIP, Ciprofloxacin; NAL, nalidixic acid; ATM, Aztreonam; CHL, Chloramphenicol; S, streptomycin; TET, tetracycline; SXT, sulfamethoxazole; KF, Cefalothin; FOX, Cefoxitin; FEP, Cefepime; CXM, Cefuroxime; CAZ, ceftazidime; CTX, cefotaxime; CRO, ceftriaxone; CPD, cefpodoxime; EFT, Ceftiofur.

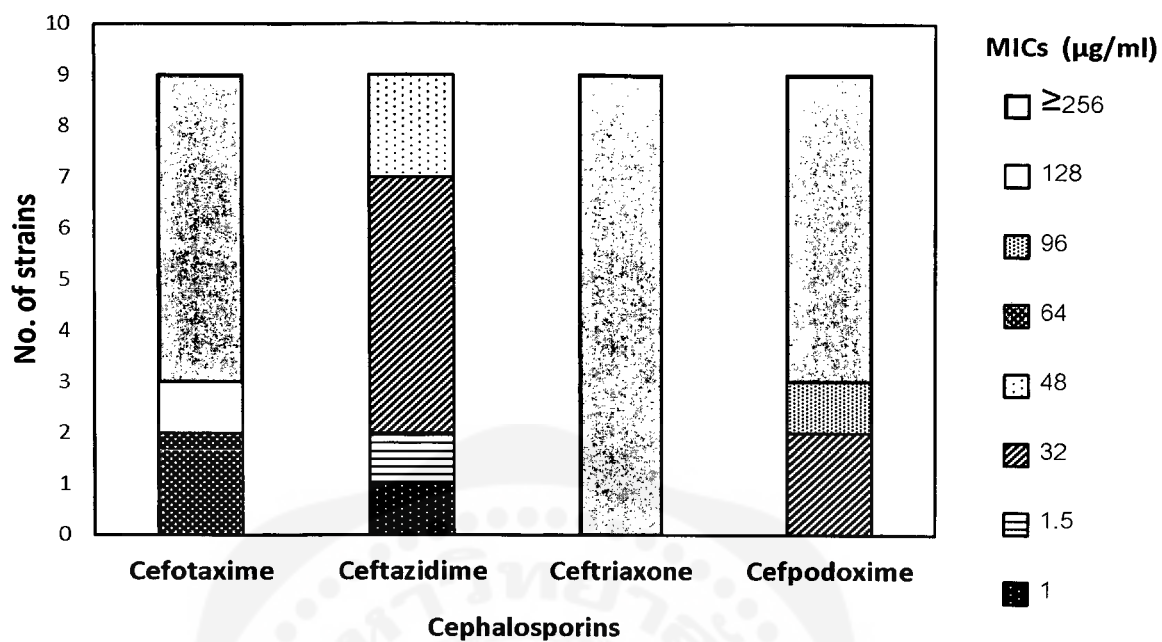


Figure 13 Distribution of MICs for cefotaxime, ceftazidime, ceftriaxone, and cefpodoxime of 9 ciprofloxacin resistant *S. Choleraesuis* strains.

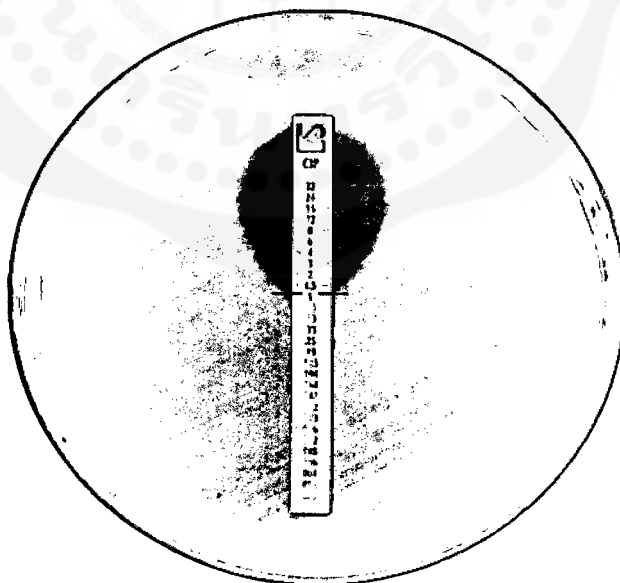


Figure 14 MIC of Ciprofloxacin (1 µg/ml) of *S. Choleraesuis* strain SH1423

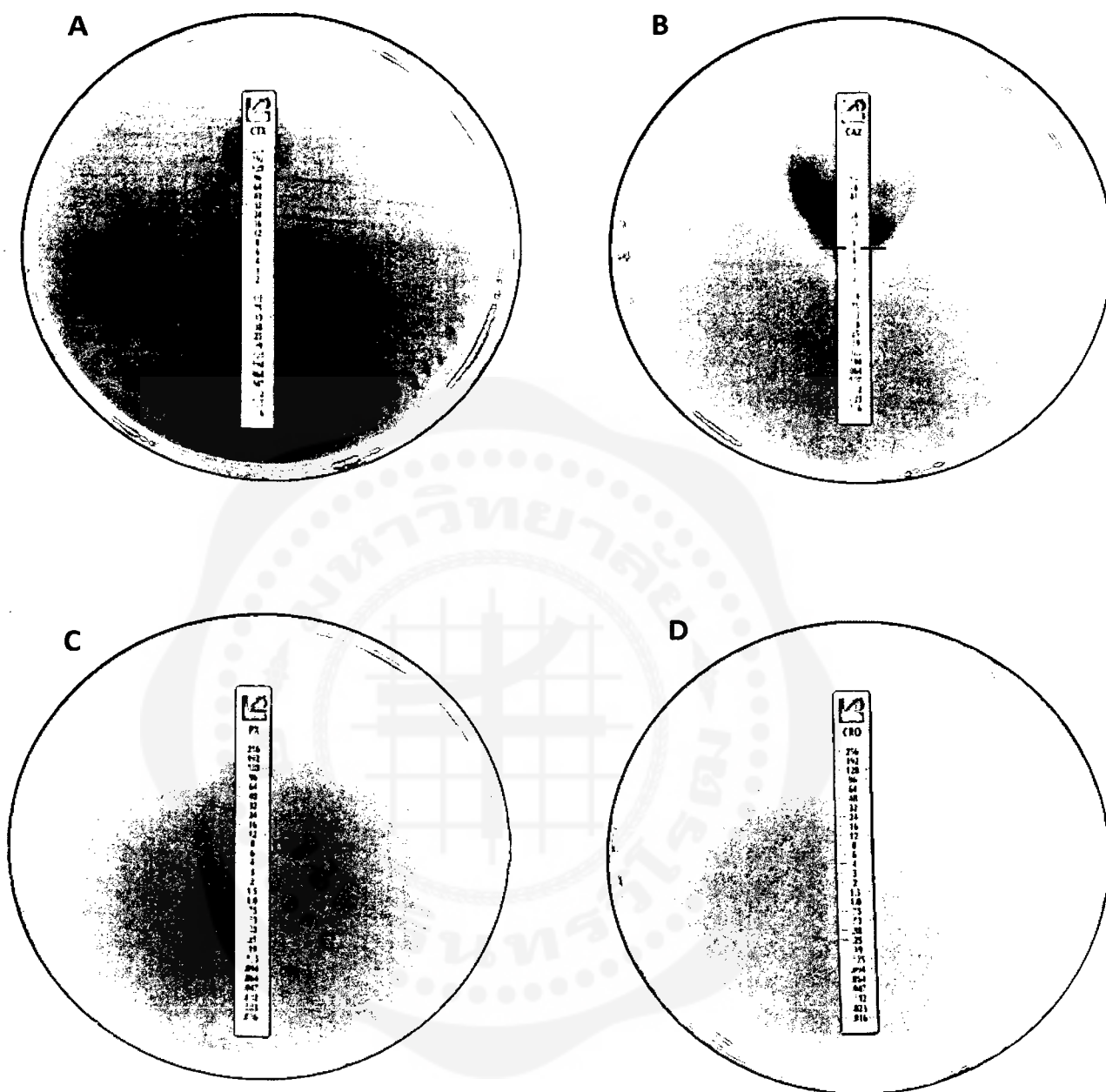


Figure 15 MIC of cephalosporin of *S. Cholereasuis* strain SH1423; (A) MIC Cefotaxime = ≥ 256 µg/ml. (B) MIC Ceftaxidime = 6 µg/ml. (C) MIC Ceftriaxone = ≥ 256 µg/ml. and (D) MIC Cefpodoxime = ≥ 256 µg/ml.

Table 17 The susceptibility of co-resistance to cephalosporins (cefotaxime, ceftazidime, ceftriaxone, and cefpodoxime) and ciprofloxacin of 9 *S. Choleraesuis* strains

Lab ID code	MIC CIP (32–0.002µg/mL)	QRDR mutation in :		PMQR genes	MIC Cephalosporin (256-0.016 µg/mL)				ESBL genes
		gryA	parC		CTX	CAZ	CRO	CPD	
SH1007/10	0.25	D87Y	T57S	<i>qnrS1, qnrA</i>	128	1.5	≥256	96	<i>bla</i> _{CTX-M-14} , <i>bla</i> _{ACC-1} , <i>bla</i> _{CMY-2}
SH2609/10	2	D87G	T57S	<i>qnrS1, qnrB, aac(6')-lb-cr</i>	≥256	32	≥256	≥256	<i>bla</i> _{CTX-M-15}
SH2789/10	0.5	D87Y	T57S	<i>qnrS1, qnrA, qnrB, aac(6')-lb-cr</i>	64	1	≥256	32	<i>bla</i> _{CTX-M-14} , <i>bla</i> _{ACC-1} , <i>bla</i> _{CMY-2}
SH735/10	2	D87Y	T57S	<i>qnrS1, aac(6')-lb-cr</i>	64	32	≥256	32	<i>bla</i> _{ACC-1} , <i>bla</i> _{CMY-2}
SH575/12	2	D87G	T57S	<i>qnrS1, qnrA, qnrB, aac(6')-lb-cr</i>	≥256	48	≥256	≥256	<i>bla</i> _{CTX-M-55} , <i>bla</i> _{TEM-1}
SH1423/12	1	D87G	T57S	<i>qnrS1, qnrB, aac(6')-lb-cr</i>	≥256	6	≥256	≥256	<i>bla</i> _{CTX-M-15}
SH577/12	2	D87G	T57S	<i>qnrS1, qnrA, qnrB,</i>	≥256	32	≥256	≥256	<i>bla</i> _{CTX-M-55} , <i>bla</i> _{TEM-1}
SH1424/12	0.12	D87G	T57S	<i>qnrS1, qnrA, qnrB, aac(6')-lb-cr</i>	≥256	48	≥256	≥256	<i>bla</i> _{CTX-M-15}
SH1456/12	0.25	D87G	T57S	<i>qnrS1, qnrA, aac(6')-lb-cr</i>	≥256	32	≥256	≥256	<i>bla</i> _{CTX-M-15}

CTX, cefotaxime; CAZ, ceftazidime; CRO, ceftriaxone; and CPD, cefpodoxime. D, aspartic acid; G, glycine; Y, tyrosine; T, threonine; and S, serine.

3. Characterization of ESBL, QRDR and PMQR genes

3.1 Screening for the presence of *bla*_{TEM}, *bla*_{SHV}, *bla*_{ACC}, *bla*_{CMY-2}, *bla*_{CTX-M}, and group of *bla*_{CTX-M} genes

A total of 44 *S. Choleraesuis* strains with positive for ESBL phenotype were screened for the presence of ESBL genes including, *bla*_{TEM} (664 bp), *bla*_{SHV} (854 bp), *bla*_{ACC} (818 bp), *bla*_{CMY-2} (758 bp), and *bla*_{CTX-M} (593 bp) by PCR. All *S. Choleraesuis* strains carrying *bla*_{CTX-M} were investigated for the group of *bla*_{CTX-M} genes encoding CTX-M β -lactamase, including CTX-M-1, CTX-M-2, CTX-M-9, and CTX-M-8/25 groups. The expected PCR product sizes of CTX-M-1, CTX-M-2, CTX-M-9, and CTX-M-8/25 groups were 588, 684, 907 and 695 bp, respectively (Figure 16). Based on PCR sequencing, 35, 23, 20 and 6 ESBL-producing strains harbored the ESBL genes encoding *bla*_{CTX-M}, *bla*_{CMY-2}, *bla*_{ACC} and *bla*_{TEM-1}, respectively. Within the CTX-M family, the CTX-M-9 group is more prevalent than the CTX-M-1 group. The CTX-M-1 and CTX-M-9 groups were represented by CTX-M-55 (10 strains, 22.7%) or CTX-M-15 (4 strains, 9.1%) and CTX-M-14 (21 strains, 47.7%), respectively. The CTX-M-2 group and CTX-M-8/25 group were no positive result. . All ESBL-producing strains have 13 patterns of β -lactamase-resistant genes. The most common ESBL gene patterns were *bla*_{CTX-M-14}-*bla*_{ACC}-*bla*_{CMY-2} (8 strains, 18.2%), followed by *bla*_{ACC}-*bla*_{CMY-2} (6 strains, 13.6%), *bla*_{CTX-M-14}-*bla*_{CMY-2} (4 strains, 9.09%), *bla*_{CTX-M-55}-*bla*_{TEM-1} (2 strains, 4.5%), *bla*_{CTX-M-55}-*bla*_{ACC}-*bla*_{CMY-2} (2 strains, 4.5%), *bla*_{CTX-M-55}-*bla*_{ACC}-*bla*_{TEM-1} (2 strains, 4.5%), *bla*_{CTX-M-14}-*bla*_{ACC} (1 strain, 2.3%), *bla*_{CMY-2}-*bla*_{TEM-1} (1 strain, 2.3%) and *bla*_{TEM-1}-*bla*_{CMY-2}-*bla*_{ACC} (1 strain, 2.3%). The *bla*_{OXA} and *bla*_{SHV} gene was also targeted, but there were no positive results (Figure 17).

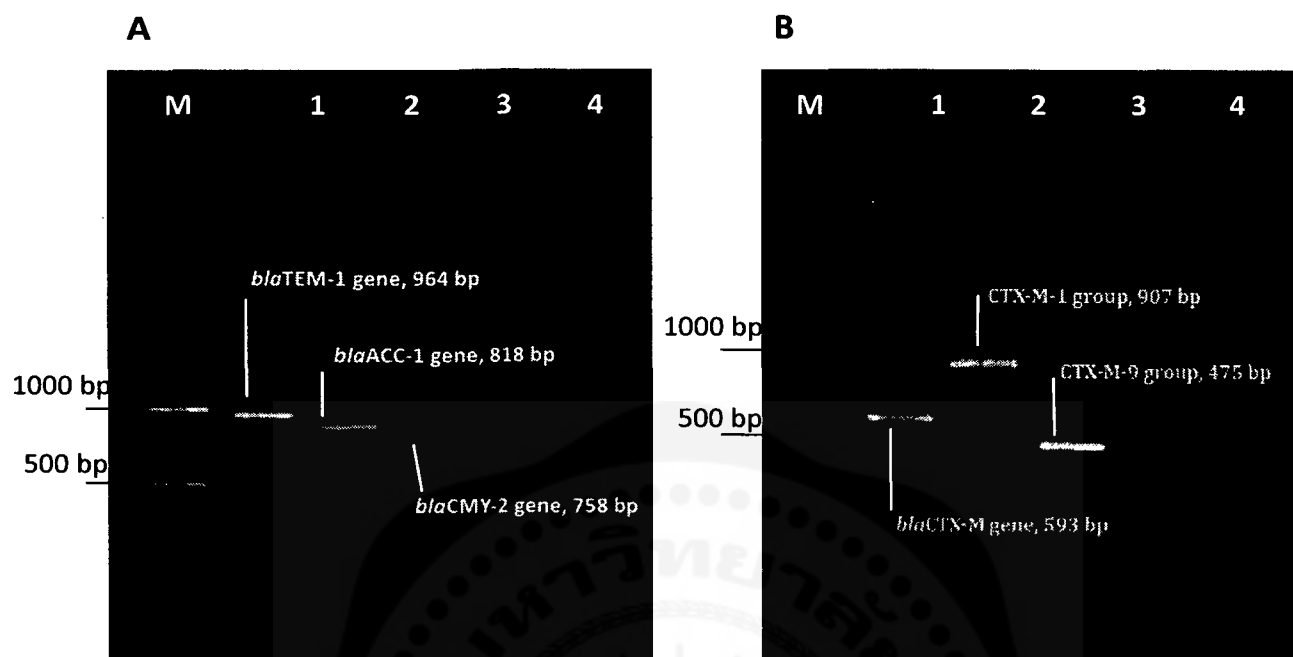


Figure 16 (A), The PCR analysis for *bla*_{TEM}, *bla*_{ACC}, and *bla*_{CMY-2} genes: Lane M, 100-bp+1.5 kb DNA ladder; Lanes 1-3 Templates, *bla*_{TEM} (964 bp), *bla*_{ACC} (818 bp), and *bla*_{CMY-2} (758 bp); Lanes 4-5, negative control (sterile DW) and (B), The PCR analysis for *bla*_{CTX-M}, CTX-M-1 group, and CTX-M-9 group genes: Lane M, 100-bp+1.5 kb DNA ladder; Lanes 1-3 Templates, *bla*_{CTX-M} (593 bp), *bla*_{CTX-M-1} group (907 bp), and *bla*_{CTX-M-9} group (475 bp); Lanes 4-5, negative control (sterile DW).

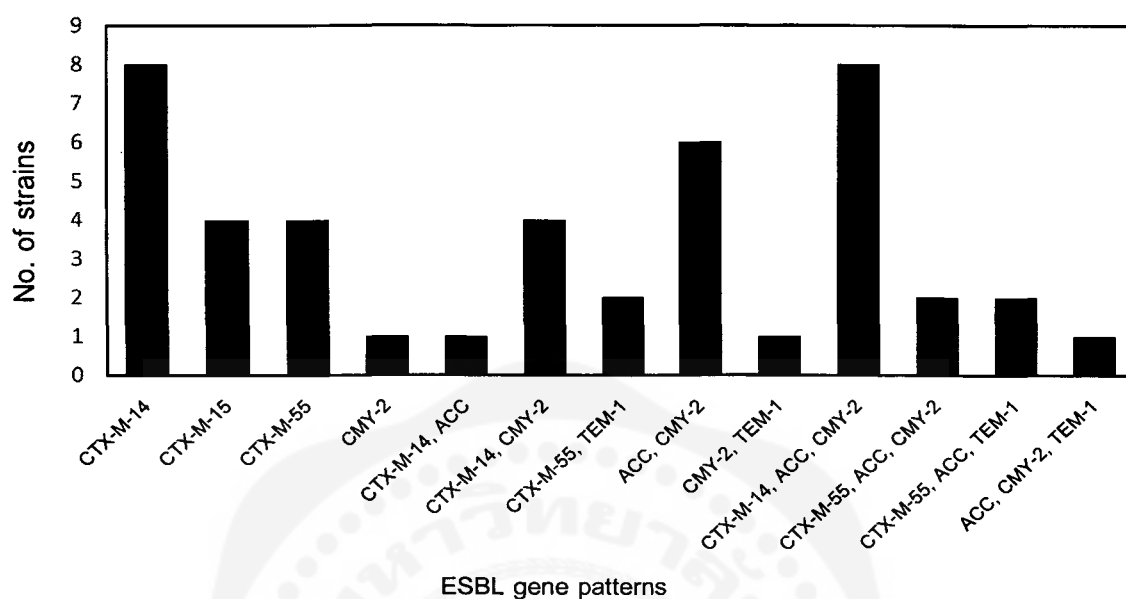


Figure 17 The 13 patterns of β -lactamase-resistant genes of 44 ESBL - producing *Salmonella Choleraesuis* strains

3.2 DNA sequencing analysis of entire bla_{CTX-M} genes of 44 ESBL-producing *S. Choleraesuis* strains

DNA sequencing was performed in 14 (CTX-M-1 groups) and 21 (CTX-M-9 groups) strains, respectively. The nucleotide sequence analyses were compared to the published sequences using an NCBI blast search available at the National Center for Biotechnology Information website (<http://www.ncbi.nlm.nih.gov>). The results of DNA sequencing are summarized in Table 18. All of 14 strains carrying $bla_{CTX-M-1}$ group showed 100% nucleotide and amino acid identity to CTX-M-15 enzyme (4 strains) and CTX-M-55 enzyme (10 strains). The results showed that CTX-M-55 shared 99% amino acid identity with CTX-M-15 (Ala77Val, Ambler numbering). Amino acid sequence alignments of CTX-M-15 (accession no.

AVD98608), and CTX-M-55 (accession no. AVD98611) compared with CTX-M-1 group from our isolates are showed in Figure 18 Forty-four isolates carried blaCTX-M-9 group showed 100% nucleotide and amino acid identity to CTX-M-14 enzyme. Alignments for amino acid sequence of CTX-M-14 (accession no. AVD98606) compared with CTX-M-9 group from our isolates are showed in Figure 19.

Table 18 Sequencing analysis of entire *bla* gene of 44 ESBL-producing *S. Choleraesuis* strains

isolate	Presence of <i>bla</i> genes					Type of <i>bla</i> gene for DNA sequencing
	<i>bla</i> _{CTX-M-1} group	<i>bla</i> _{CTX-M-9} group	<i>bla</i> _{TEM}	<i>bla</i> _{ACC}	<i>bla</i> _{CMY-2}	
SH1544/10	—	+	—	+	+	<i>bla</i> _{CTX-M-14} , <i>bla</i> _{ACC} , <i>bla</i> _{CMY-2}
SH2539/10	—	+	—	+	+	<i>bla</i> _{CTX-M-14} , <i>bla</i> _{ACC} , <i>bla</i> _{CMY-2}
SH495/10	+	—	—	+	+	<i>bla</i> _{CTX-M-55} , <i>bla</i> _{ACC} , <i>bla</i> _{CMY-2}
SH1660/10	—	+	—	—	—	<i>bla</i> _{CTX-M-14}
SH1407/10	+	—	—	—	—	<i>bla</i> _{CTX-M-55}
SH1208/10	—	+	—	+	+	<i>bla</i> _{CTX-M-14} , <i>bla</i> _{ACC} , <i>bla</i> _{CMY-2}
SH3124/10	—	+	—	—	+	<i>bla</i> _{CTX-M-14} , <i>bla</i> _{CMY-2}
SH1007/10	—	+	—	+	+	<i>bla</i> _{CTX-M-14} , <i>bla</i> _{ACC} , <i>bla</i> _{CMY-2}
SH2788/10	—	+	—	—	—	<i>bla</i> _{CTX-M-14}
SH862/10	—	+	—	+	+	<i>bla</i> _{CTX-M-14} , <i>bla</i> _{ACC} , <i>bla</i> _{CMY-2}
SH2223/10	—	+	—	—	+	<i>bla</i> _{CTX-M-14} , <i>bla</i> _{CMY-2}
SH2789/10	—	+	—	+	+	<i>bla</i> _{CTX-M-14} , <i>bla</i> _{ACC} , <i>bla</i> _{CMY-2}
SH2821/10	+	—	—	—	—	<i>bla</i> _{CTX-M-55}
SH1036/10	—	+	—	+	—	<i>bla</i> _{CTX-M-14} , <i>bla</i> _{ACC}
SH2543/10	—	—	+	+	+	<i>bla</i> _{TEM-1} , <i>bla</i> _{CMY-2} , <i>bla</i> _{ACC}
SH2609/10	+	—	—	—	—	<i>bla</i> _{CTX-M-15}
SH2347/10	+	—	—	—	—	<i>bla</i> _{CTX-M-55}
SH3141/10	—	+	—	—	—	<i>bla</i> _{CTX-M-14}
SH2736/10	—	+	—	—	+	<i>bla</i> _{CTX-M-14} , <i>bla</i> _{CMY-2}
SH787/10	—	—	—	+	+	<i>bla</i> _{ACC} , <i>bla</i> _{CMY-2}

+, positive result; —, negative result

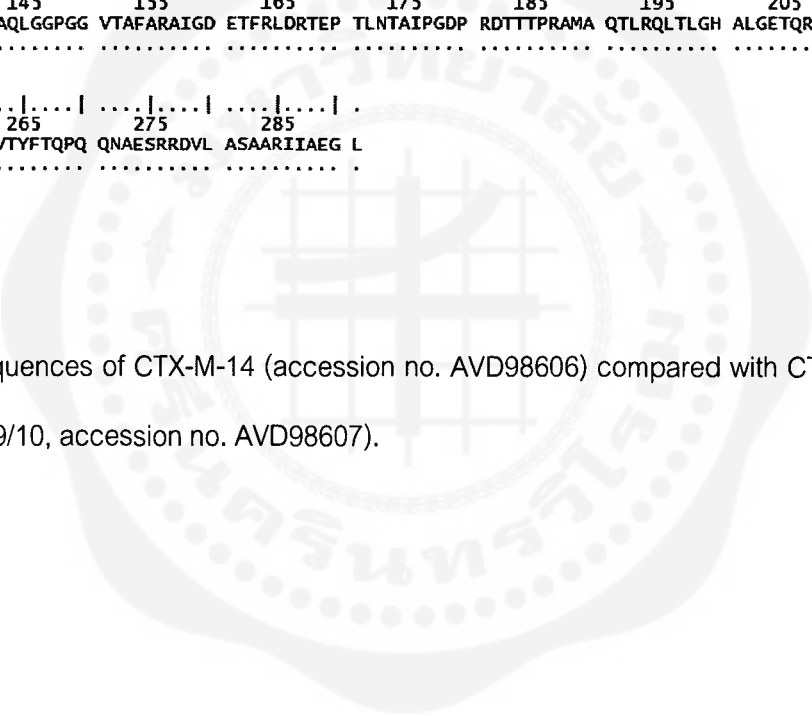
Table 18 Sequencing analysis of entire *bla* gene of 44 ESBL-producing *S. Choleraesuis* strains (cont.)

isolate	Presence of <i>bla</i> genes					Type of <i>bla</i> gene for DNA sequencing
	<i>bla</i> _{CTX-M-1} group	<i>bla</i> _{CTX-M-9} group	<i>bla</i> _{TEM}	<i>bla</i> _{ACC}	<i>bla</i> _{CMY-2}	
SH876/10	—	—	—	+	+	<i>bla</i> _{ACC} , <i>bla</i> _{CMY-2}
SH564/10	—	+	—	—	+	<i>bla</i> _{CTX-M-14} , <i>bla</i> _{CMY-2}
SH735/10	—	—	—	+	+	<i>bla</i> _{ACC} , <i>bla</i> _{CMY-2}
SH737/10	+	—	—	+	+	<i>bla</i> _{CTX-M-55} , <i>bla</i> _{ACC} , <i>bla</i> _{CMY-2}
SH598/10	+	—	—	—	—	<i>bla</i> _{CTX-M-55}
SH1364/10	—	+	—	+	+	<i>bla</i> _{CTX-M-14} , <i>bla</i> _{ACC} , <i>bla</i> _{CMY-2}
SH809/10	—	+	—	+	+	<i>bla</i> _{CTX-M-14} , <i>bla</i> _{ACC} , <i>bla</i> _{CMY-2}
SH218/10	—	+	—	—	—	<i>bla</i> _{CTX-M-14}
SH646/11	—	—	—	+	+	<i>bla</i> _{ACC} , <i>bla</i> _{CMY-2}
SH293/11	—	—	—	+	+	<i>bla</i> _{ACC} , <i>bla</i> _{CMY-2}
SH1421/11	—	—	—	—	+	<i>bla</i> _{CMY-2}
SH359/12	—	+	—	—	—	<i>bla</i> _{CTX-M-14}
SH1697/12	—	+	—	—	—	<i>bla</i> _{CTX-M-14}
SH1423/12	+	—	—	—	—	<i>bla</i> _{CTX-M-15}
SH1504/12	—	+	—	—	—	<i>bla</i> _{CTX-M-14}
SH1424/12	+	—	—	—	—	<i>bla</i> _{CTX-M-15}
SH1450/12	—	+	—	—	—	<i>bla</i> _{CTX-M-14}
SH1456/12	+	—	—	—	—	<i>bla</i> _{CTX-M-15}
SH1155/12	—	—	—	+	+	<i>bla</i> _{ACC} , <i>bla</i> _{CMY-2}
SH575/12	+	—	+	—	—	<i>bla</i> _{CTX-M-55} , <i>bla</i> _{TEM-1}
SH577/12	+	—	+	—	—	<i>bla</i> _{CTX-M-55} , <i>bla</i> _{TEM-1}
SH85/12	—	—	+	—	+	<i>bla</i> _{TEM-1} , <i>bla</i> _{CMY-2}
SH121/12	+	—	+	+	—	<i>bla</i> _{CTX-M-55} , <i>bla</i> _{ACC} , <i>bla</i> _{TEM-1}
SH120/12	+	—	+	+	—	<i>bla</i> _{CTX-M-55} , <i>bla</i> _{ACC} , <i>bla</i> _{TEM-1}

+, positive result; —, negative result

	
	5 15 25 35 45 55 65 75 85 95 105 115
ctx-m-15	MVKKSLRQFT LMATATVTLL LGSVPLYAQT ADVQQKLAEL ERQSGGRLGV ALINTADNSQ ILYRADERFA MCSTSKVMAA AAVLKKSESE PNLLNQRVEI KKSOLDVNYNP IAEKHVNGTM
ctx-m-55	V.....
SH1456	A.....
SH2609	A.....
SH1423	A.....
SH577	V.....
	
	125 135 145 155 165 175 185 195 205 215 225 235
ctx-m-15	SLAELSAAL QYSDNVAMNK LIAHVGGPAS VTAFARQLGD ETFRLDRTEP TLNTAIPGDP RDTTSPRAMA QTLRNLTGK ALGDSQRAQL VTWMKGNTTG AASIQAGLPA SWVVGDKTGS
ctx-m-55	
SH1456	
SH2609	
SH1423	
SH577	
	
	245 255 265 275 285
ctx-m-15	GGYGTNDIA VIWPKDRAPL ILVTYFTQPQ PKAESRRDVL ASAAKIVTDG L
ctx-m-55	
SH1456	
SH2609	
SH1423	
SH577	

Figure 18 Alignments of amino acid sequences of CTX-M-15 (accession no. AVD98608), CTX-M-55 (accession no. AVD98611) compared with CTX-M-1 group from ESBL- producing *S. Cholerasuis* strains (Lab ID: SH1456/12, accession no. AVD98609; Lab ID: SH2609/10, accession no. AVD98610; Lab ID: SH1423/12, accession no. AVD98613 and Lab ID: SH577/12, accession no. AVD98612).



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      .....|.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|
      5       15      25      35      45      55      65      75      85      95     105     115
CTX-m-14  MVTKRVRMM FAAAACIPLL LGSAPLYAQT SAVQQKLAAL EKSSGGRLGV ALIDTADNTQ VLYRGDERFP MCSTSKVMAA AAVLKQSETQ KQLLNQPVEI KPADLVNYPN IAEKHVNGTM
SH2789    .....|.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|
      125     135     145     155     165     175     185     195     205     215     225     235
ctx-m-14  TLAELSAAAL QYSDNTAMNK LIAQLGGPGG VTAFAARAIGD ETFRLDRTEP TLNTAIPGDP RDTTTPRAMA QTLRQLTLGH ALGETQRAQL VTWLKGNTTG AASIRAGLPT SWTVGDKTGS
SH2789    .....|.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|
      245     255     265     275     285
ctx-m-14  GDYGTNDIA VIWPQGRAPL VLVTYFTQPQ QNAESRRDVL ASAARIIEG L
SH2789    .....|.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|

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Figure 19 Alignments of amino acid sequences of CTX-M-14 (accession no. AVD98606) compared with CTX-M-9 group from ESBL - producing *S. Cholerasuis* strain (Lab ID: SH2789/10, accession no. AVD98607).

3.3 Screening for the presence of QRDR and PMQR genes in co-resistant strains

In addition, 20.5% (9/44 isolates) of the ESBL-producing strains were also co-resistant to ciprofloxacin. Screening the mutation in *gryA* (312 bp) and *parC* (261 bp) and presence of *qnrA* (579 bp), *qnrB* (263 bp), *qnrS* (427 bp), and *aac(6')-Ib-cr* (482 bp) genes are shown in Figure 20. Of the nine strains, 6 patterns of PMQR genes were identified, all of the co-resistant strains harbored at least one PMQR gene; the *qnr* genes (*qnrA* or/and *qnrB* or/and *qnrS*) and the *aac(6')-Ib-cr* gene were the most prevalent mutations detected. The most common PMQR genes patterns were *qnrA* – *qnrB* - *qnrS1* - *aac(6')-Ib-cr* gene (3 strains, 33.3%), followed by *qnrB* - *qnrS1* - *aac(6')-Ib-cr* gene (2 strains, 22.2%), *qnrA* - *qnrS1* - *aac(6')-Ib-cr* gene (1 strain, 11.1%), *qnrA* – *qnrB* - *qnrS1* gene (1 strain, 11.1%), *qnrA* - *qnrS1* gene (1 strain, 11.1%) and *qnrS1* and *aac(6')-Ib-cr* gene (1 strain, 11.1%). All co-resistant isolates displayed point mutation in QRDR including in the *gryA* (D87Y and D87G) and *parC* (T57S) genes (Figure 21 and Figure 22).

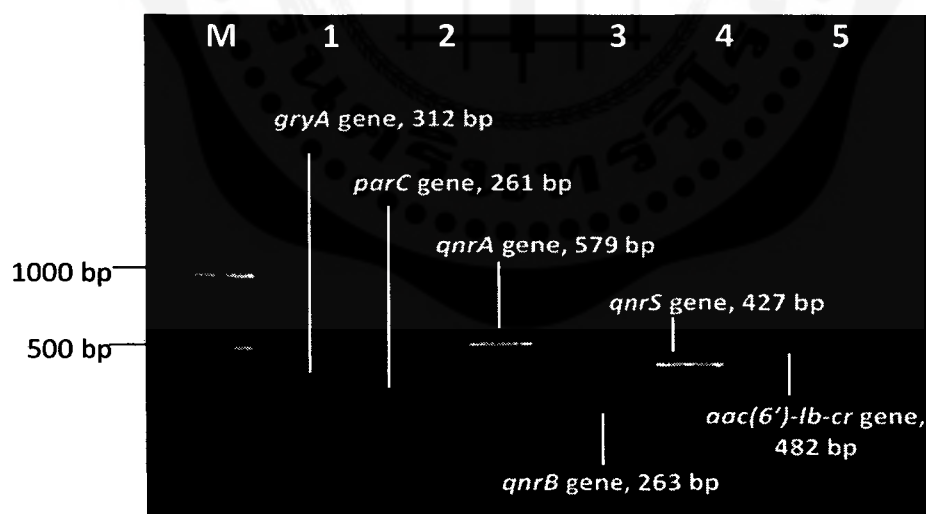


Figure 20 The PCR analysis of QRDR and PMQR genes: Lane M, 100-bp+1.5 kb DNA ladder; Lanes 1-2 Templates of QRDR genes, *gryA* (312 bp), and *parC* (261 bp); Lanes 3-6 Templates of PMQR genes, *qnrA* (579 bp), *qnrB* (263 bp), *qnrS* (427 bp), and *aac(6')-Ib-cr* (482 bp); Lane 7, negative control (sterile DW).

4. Pulse Field Gel Electrophoresis (PFGE) Analysis

After digestion by the XbaI enzyme, the genetic relatedness of ESBL-producing *S. Choleraesuis* strains was evaluated by PFGE. The dendrograms in Figure 23 demonstrates the banding patterns and the genetic relatedness of the isolates evaluated by PFGE. Multidrug antimicrobial resistance patterns and ESBL genes were also reported. Thirty-one fingerprint-patterns generated by PFGE were identified amongst the strains recovered from various locations at different times and source isolation, providing information on associations amongst the ESBL-producing strains and evidence of the diversity of ESBL genes harboured on plasmids in Thailand during 2010-2012. Forty-four strains were clustered into 6 predominant PFGE pulsotypes with ≥ 2 indistinguishable strains, which showed a close relationship (Dice correlation coefficient of 95%) based on Tenover criteria⁽¹⁶⁰⁾. However, this study represented a high diversity of PFGE fingerprint patterns of ESBL-producing *S. Choleraesuis* isolates in Thailand. The PFGE and antimicrobial resistance patterns revealed a high degree of clonal diversity amongst the 44 ESBL-producing strains. All of the analyses indicate that multiple clones and multiple resistance genes on the plasmids are responsible for the extended-spectrum cephalosporin resistance amongst the *S. Choleraesuis* isolates obtained from patients in Thailand.

Dice (Opt1.50%) (Tol 1.5%-1.5%) (P>0.0% S>0.0%) (D.0%-100.0%)
PFGE-XbaI

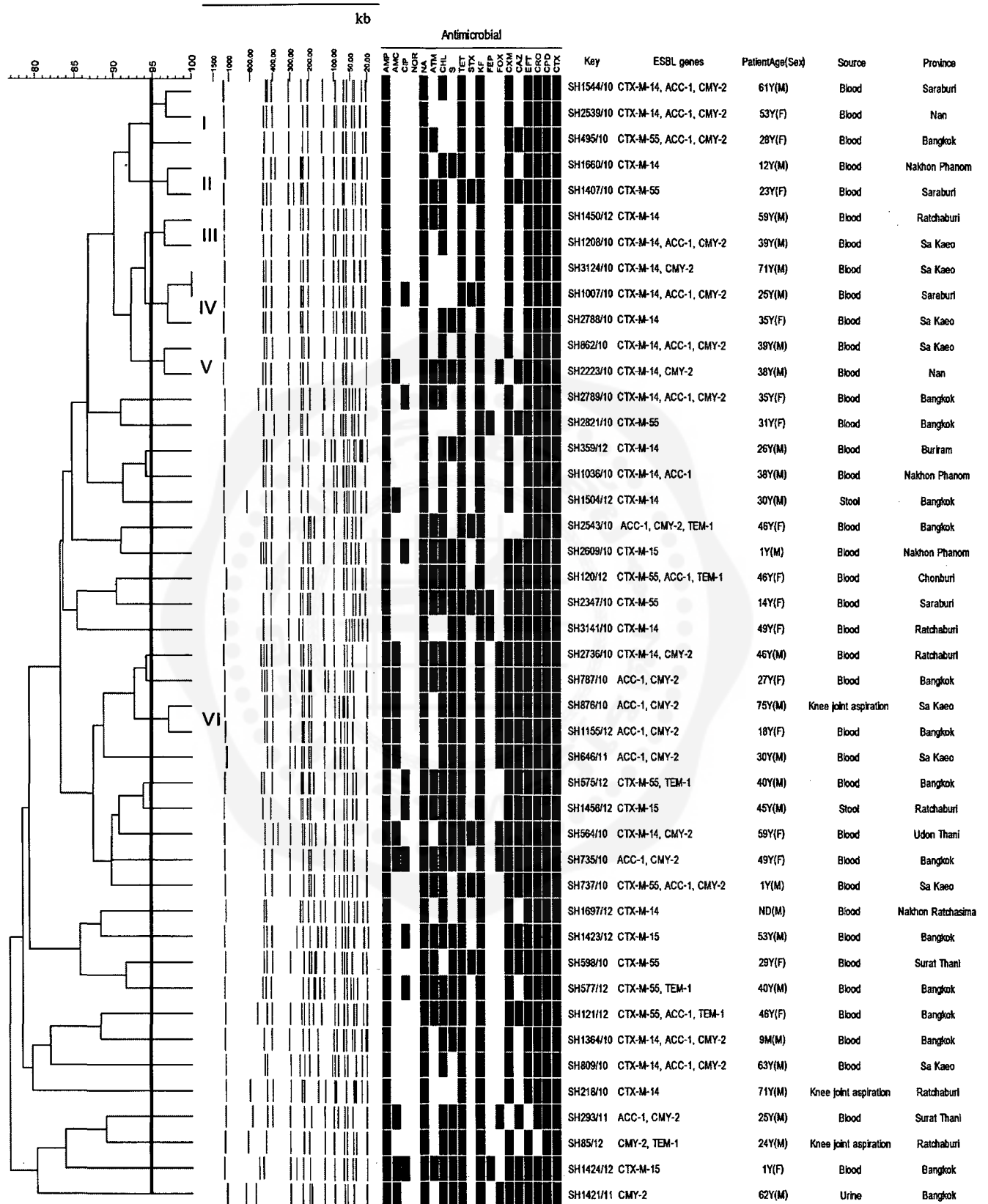


Figure 23 PFGE characteristics of 44 ESBL-producing *Salmonella Choleraesuis* strains.

CHAPTER 5

DISCUSSIONS

Multidrug-resistant *Salmonella* may pose a risk to humans, particularly individuals resistant to quinolones and third-generation cephalosporin drugs. Previous studies conducted in Thailand have described the presence of multidrug-resistant *Salmonella* from a wide range of serovars ⁽²⁵⁾. The WHO National *Salmonella* and *Shigella* Center in Thailand received all presumptive *S. Choleraesuis* isolates from all public health laboratories in Thailand. During 2010-2012, there were a total of 6,483 nontyphoidal *Salmonella* submissions, of which 174 (2.7%) were confirmed to be *S. Choleraesuis*. Of those, 113 (64.9%) were identified as *S. Choleraesuis* isolated from bacteremia patients. The overall percentage of *S. Choleraesuis* bacteremia cases versus total infection cases during the study period is shown in Figure 25. The number of *S. Choleraesuis* cases decreased from 92 in 2010 to 25 in 2012. During this same period, a high proportion of bacteremia *S. Choleraesuis* cases was observed in 2010 (90.2%). However, a decrease in the proportion of bacteremia cases and total infection cases was observed in 2011 and 2012 compared to previous years.

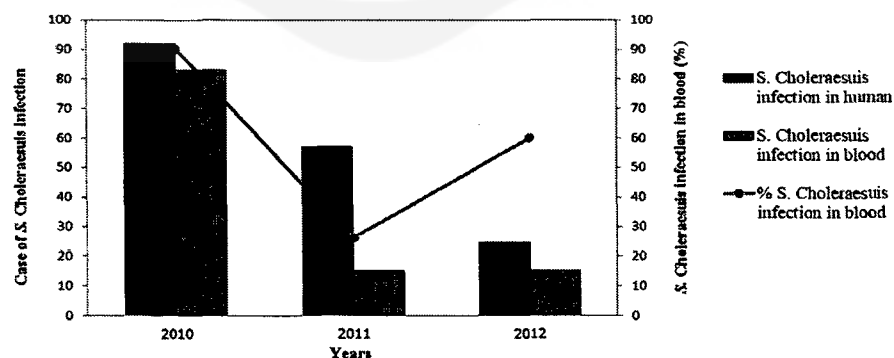


Figure 25 Proportion of *Salmonella* Choleraesuis cases reported in Thailand compared to the proportion of bacteremia cases of *S. Choleraesuis* for the entire country during 2010-2012.

According to data obtained from Thailand, *S. Weltevreden*, *S. Stanley*, *S. Anatum*, and *S. Rissen* were the most common serovars in patient stool samples. Conversely, *S. Choleraesuis*, *S. Enteritidis*, *S. Typhimurium*, and *S. Typhi* were observed in blood samples⁽²³⁾. In a previous study in Thailand during 2005, 135 nontyphoidal *Salmonella* isolated from blood in Siriraj Hospital showed that *Salmonella* group C (47%) was the most common serogroup and 17.8%, 14.1%, 17.8% and 5.8% of isolates were resistant to ceftriaxone, ceftazidime, cefotaxime and ciprofloxacin, respectively⁽¹⁴⁸⁾. Furthermore, a high rate of ceftriaxone resistance was reported in *S. Choleraesuis* isolated from bacteremia patients during 2003-2005.

In the present study, 44 (70.9%) *S. Choleraesuis* were identified as ESBL producers. The high ESBL production therefore showed that *Salmonella* compete with *E. coli* and *Klebsiella* species as ESBL producers, and care should be taken when handling such isolates. Moreover, antimicrobial treatment enhances the selection of resistant strains and results in an increase in the resistance gene pool, which ultimately increases the risk of the spreading resistance gene-swapping mechanisms⁽¹⁴⁹⁾. These mechanisms enhance the incidence of *Salmonella* and other gastrointestinal tract Gram-negative isolates as strong ESBL producers. According to Spanu et al.⁽¹⁵⁰⁾ and Zaki⁽¹⁵¹⁾ many ESBL producers carry other genes that confer resistance to other antimicrobial agents, such as aminoglycosides and fluoroquinolones.

In addition, 9 ESBL producers isolated from humans were co-resistant to third-generation cephalosporins (including cefotaxime or ceftriaxone, cefpodoxime and ceftazidime) and ciprofloxacin, the standard drug for treating *Salmonella* infections in humans. This antimicrobial finding highlights a serious epidemiological situation. This increase is particularly concerning amid recent reports of *S. Choleraesuis* strains resistant to fluoroquinolone and extended-spectrum cephalosporin⁽¹¹⁸⁾. Our data indicate that rates of antimicrobial resistance against (fluoro)quinolones and third-generation cephalosporin in *S.*

Choleraesuis are increasing when compared to previous reported. A recent study of 54 *S. Choleraesuis* isolates reported that approximately 60% of the study isolates were nalidixic acid-resistant and 15% were ceftriaxone-resistant⁽²⁴⁾. This increase is particularly concerning amid recent reports of *S. Choleraesuis* strains that are resistant to fluoroquinolone and extended-spectrum cephalosporin⁽¹¹⁸⁾. To date, only a few reports from Taiwan and Thailand have described resistance to third-generation cephalosporins and fluoroquinolones in *S. Choleraesuis*^(23,152-153). Lee et al.⁽²³⁾ found a level of resistance to ciprofloxacin and ceftriaxone among non-typhoidal *Salmonella* in seven Southeast Asian countries. They found the highest frequencies of resistance to ciprofloxacin and ceftriaxone in Taiwan and Thailand.

This study provides the first description of the diversity of β -lactamase-resistant gene patterns in *S. Choleraesuis* isolates from patients with systemic infection in Thailand (Figure 25), in which dominant ESBL isolates harboured *bla*_{CTX-M-14} with/without other ESBL resistance genes. The understanding of the various reports worldwide is that plasmid-mediated CTX-M-15 and CTX-M-14 are the most important type, posing a threat to human health⁽¹⁵⁴⁾. The occurrence of ESBL phenotypes and genotypes in this study was similar to those of other studies in Thailand and Taiwan^(25,118). The *bla*_{CTX-M-14} and *bla*_{CMY-2} genes were found in extended-spectrum cephalosporinase-producing *S. Choleraesuis* isolates recovered from Thai patients⁽²⁵⁾. The *bla*_{CMY-2} gene for ampicillin resistance was also reported in ESC-resistant *S. Choleraesuis* strains isolated in Taiwan in 2006⁽¹¹⁸⁾. To our knowledge, we report for the first time of CTX-M-15 and CTX-M-55-producing *S. Choleraesuis* isolates obtained from patients with septicaemia. Previous paper has reported that plasmids harbouring the *bla*_{CTX-M-14}, *bla*_{CTX-M-15} and *bla*_{CTX-M-55} genes were found in *S. Typhimurium* isolates from paediatric patients in China⁽¹⁴²⁾, but they did not report the *bla*_{CTX-M-15} and *bla*_{CTX-M-55} genes in clinical *S. Choleraesuis*. In the past, plasmids carrying *bla*_{CTX-M-55} and *bla*_{CTX-M-15} have been found in ESBL-producing *E. coli* and *Klebsiella* in China and Thailand⁽¹⁵⁵⁻¹⁵⁶⁾. The molecular characterization showed that *bla*_{CTX-M-55} is a derivative of *bla*_{CTX-M-15}, in which the Ala in position

77 is substituted with a Val. With regard to antibiotic resistance, the MIC ranges for cefotaxime, ceftazidime, ceftriaxone and cefpodoxime of isolates carrying the *bla*_{CTX-M-55} and *bla*_{CTX-M-15} genes were significantly higher than those of isolates carrying *bla*_{CTX-M-14} (Table 17). The *bla*_{CTX-M-15} and *bla*_{CTX-M-55} are the main genes that cause ceftazidime resistance ⁽¹⁵⁵⁾. Ceftazidime-hydrolysing CTX-M-15 and CTX-M-55 enzymes possess a Gly substitution in position 240, which may be associated with a reduced susceptibility to ceftazidime ⁽¹⁵⁷⁾. The data suggest that ESBL-producing isolates have emerged in Thailand on several plasmids and in multiple clones of *S. Choleraesuis*. The clonal spread of CTX-M-mediated resistance may be due to horizontal transfer, but this study did not perform gene or plasmid transfer experiments on our ESBL-producing isolates. However, an epidemiological study of plasmid transfer between bacteria in the *Enterobacteriaceae* family has been reported previously ⁽¹⁵⁵⁻¹⁵⁶⁾. In intensive health-care hospital settings, the development and spread of ESBL genes has most likely been caused by the overuse of antibiotic drugs ⁽¹⁴⁹⁾.

In addition, 20.5% (9/44 strains) of the ESBL-producing isolates were also co-resistant to ciprofloxacin. All co-resistant isolates displayed a QRDR mutation including in the *gyrA* (D87Y and D87G) and *parC* (T57S) genes. The most frequently observed point mutations in the *gyrA* gene in fluoroquinolone-resistant *Salmonella* are the amino acid changes at serine-83 to phenylalanine, tyrosine or alanine or aspartic acid-87 to glycine or asparagine, or/and mutations in *parC* involving changes at threonine-57 to serine and tyrosine, which have been found in fluoroquinolone-resistant *Salmonella* ⁽¹⁵⁸⁾.

In all of the ESBL producing-ciprofloxacin resistance isolates, PMQR genes were identified, and all of the co-resistant isolates harboured at least one PMQR gene; the *qnr* genes (*qnrS* or *qnrA* and *qnrB*) and/or the *aac(6')-Ib-cr* gene. To our knowledge, this study is the first report of QRDR mutations and *qnr*-positive plasmids in clinical *Salmonella* isolates in Thailand, and the results also showed an improved MIC of ciprofloxacin at 0.12-2 µg/ml. The

PMQR determinants mediate low-level quinolone resistance, and the presence of PMQR genes is of great concern because these genes can promote mutations within the QRDR⁽¹⁵⁹⁾.

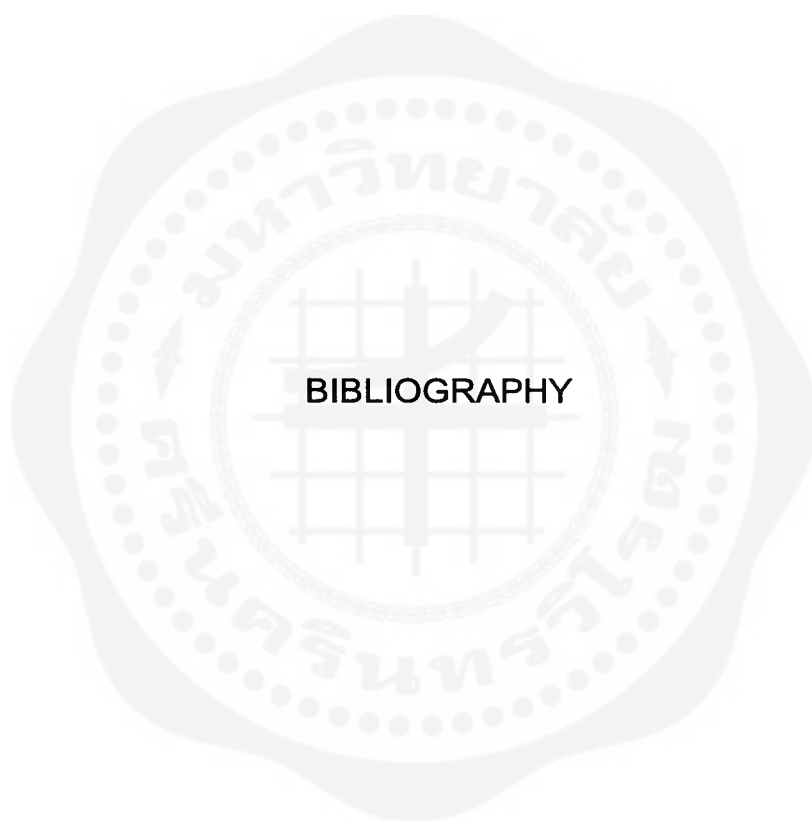
In this study, 4 strains (SH2609, SH735, SH575, and SH577) isolated from the blood samples showed high MIC levels for ciprofloxacin. Only one strain in a 40-year-old patient, SH575/12, showed high MIC levels for both ciprofloxacin (MIC 2 µg/ml) and cephalosporins (cefotaxime MIC ≥ 256 µg/ml, ceftazidime MIC 48 µg/ml, ceftriaxone MIC ≥ 256 µg/ml and cefpodoxime ≥ 256 µg/ml). This strain contained 2 types of ESBL genes (*bla*_{TEM-1} and *bla*_{CTX-M-55}), 4 types of PMQR genes (*qnrA*, *qnrB*, *qnrS* and *aac(6')-Ib-cr*) and presented a mutation in both *gyrA* and *parC*. In addition, 4 co-resistant *S. Choleraesuis* strains obtained from 3 patients with a systemic infection showed a higher MIC for ciprofloxacin (2 µg/ml) than previously reported, which reduced susceptibility to ciprofloxacin to ≥ 0.125 µg/mL⁽²⁴⁾.

This study represented a high diversity of PFGE fingerprint patterns of ESBL-producing *S. Choleraesuis* isolates in Thailand. The PFGE and antimicrobial resistance patterns revealed a high degree of clonal diversity amongst the 44 ESBL-producing isolates. All of the analyses indicate that multiple clones and multiple resistance genes on the plasmids are responsible for the extended-spectrum cephalosporin resistance amongst the *S. Choleraesuis* isolates obtained from patients in Thailand. Similarly, Sirichote *et al.* reported multiple clones and multiple ESBL resistance genes of *S. Choleraesuis* isolates obtained from patients in Thailand and one patient in Denmark⁽²⁵⁾. In addition, 16 unique *Xba*I PFGE patterns of 22 *S. Choleraesuis* isolates were obtained.

In conclusion, the multidrug resistance of *S. Choleraesuis* isolated from patients in Thailand showed a high diversity of ESBLs and fluoroquinolone-resistant genes. This is the first report of plasmids carrying *bla*_{CTX-M-15} or *bla*_{CTX-M-55} with and without other ESBL resistance genes in *S. Choleraesuis* obtained from patients with bacteremia. In addition, according to these findings, the PFGE typing suggests that ESBL-producing *S. Choleraesuis* strains

isolated from patients in different regions in Thailand are multiple clones, which may indicate that isolates of this serovar have recently spread and that resistance has evolved locally among the isolates in Thailand.





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APPENDICES



APPENDIX A



APPENDIX A

REAGENT AND MEDIA PREPARATION

1. Media and Biochemical for *Salmonella*

1.1 Trypticase Soy Agar (TSA) is used to culture *Salmonella* for biochemical and antimicrobial susceptibility tests.

Trypticase agar base	40	g/L
Distilled water	1000	ml

The medium was sterilized by autoclaving at 121°C, 15 pounds/inch² pressures, for 15 minutes. The sterile medium was cooled to 50°C. Dispense 20 ml per Petri dish. Cool and store at 4°C until used.

1.2 Muller Hinton agar (MHA) was used for antimicrobial susceptibility test.

Muller Hinton agar base	38	g/L
Distilled water	1000	ml

The medium was sterilized by autoclaving at 121°C, 15 pounds/inch² pressures, for 15 minutes. The sterile medium was cooled to 50°C. Dispense 25 ml per Petri dish. Cool and store at 4°C until used.

1.3 Xylose-Lysine Deoxycholate (XLD) agar was a selective medium used for isolating *Salmonella* spp.

XLD agar base	58	g/L
Distilled water	1000	ml

Heat with frequent agitation until the medium boils. DO NOT AUTOCLAVE. Dispense 20 ml per Petri dish. Cool and store at 4°C until used.

1.4 Triple sugar iron (TSI) slant is used for screening test.

Interpret result: A/A mean bacteria can utilize glucose and other saccharide.

K/A mean bacteria can utilize only glucose.

K/K, K/N, N/N mean bacteria cannot utilize any sugar.

Gas⁺ mean the bubble in medium: end product from the saccharide fermentation producing acidity and gas.

Gas ⁻	mean end product from the saccharide fermentation producing only acidity without gas.
H ₂ S ⁺	mean utilization of ferrous compound resulted in forming black in the medium.
H ₂ S ⁻	mean unable to utilize ferrous compound, black color is not observed at the butt area.

Ingredient and preparation: Mix the ingredients, heat to boiling, dispense into tubes, and sterilized by autoclaving at 121°C, 15 pounds/inch² pressures, for 15 minutes, and allow tubes to cool in a slanted position.

TSI agar base	65	g/L
Distilled water	1000	ml

1.5 Lysine Indole Motility (LIM) medium is used for screening test.

Interpret result:	indole ⁺ mean red ring occur after dropping Kovac' reagent
	indole ⁻ mean yellow color occur after dropping Kovac' reagent
	lysine ⁺ mean the color still purple
	lysine ⁻ mean the color of medium changing into yellow
	motile ⁺ mean turbidity
	motile ⁻ mean no turbidity

Ingredient and preparation: Mix the ingredients, heat to boiling, dispense into tubes, and sterilized by autoclaving at 121°C, 15 pounds/inch² pressures, for 15 minutes, cool and store at 4°C until used.

LIM agar base	36.5	g/L
Distilled water	1000	ml

2. Reagent of Biochemical Testing

2.1 Kovac's reagent is prepared by the following:

Paradimethylamino-benzaldehyde	5 g
Conc. HCL	25 ml
Amyl alcohol	75 ml

HCL and amyl alcohol is mixed together, paradimethylamino-benzaldehyde is then added into solution. The reagent is kept at 4°C and avoided to expose with the light.

3. Medium and reagents for gDNA Extraction

3.1 Luria Bertani (LB) broth is used for culturing the bacterial cells.

Tryptone	10 g
Yeast extract	5 g
NaCl	10 g

All of compositions are dissolved in 1000 ml of distilled water and sterilized by autoclaving at 121°C, 15 pounds/inch² pressures, for 15 minutes.

3.2 20% Sodium dodecyl sulfate (SDS)

Sodium dodecyl sulfate (SDS)	20 g
Deionized water	100 ml

The solution is heated at 65°C to completely dissolve and adjusted pH to 7.2 with concentrate HCl. This reagent is sterilized by filtration and kept at room temperature.

3.3 Stock solution

3.3.1 1M Tris-HCL pH 8.0

Tris	12.11 g
Distilled water	100 ml

The solution is heated at 65°C to completely dissolve and adjusted pH to 8.0 with 0.1N HCl. This reagent is sterilized by autoclaving at 121°C, 15 pounds/inch² pressures, for 15 minutes and kept at room temperature.

3.3.2 0.5M EDTA pH 8.0

EDTA	18.6 g
Distilled water	100 ml

The solution is heated at 65°C to completely dissolve and adjusted pH to 8.0 with 0.1N HCl. This reagent is sterilized by autoclaving at 121°C, 15 pounds/inch² pressures, for 15 minutes and kept at room temperature.

3.3.3 1M sucrose

Sucrose	34.23 g
Distilled water	100 ml

The solution is heated at 65°C to completely dissolve. This reagent is sterilized by filtration and kept at 4°C.

3.4 TES buffer pH 8.0 (10 mM Tris-HCl, 25 mM EDTA, 0.6M Sucrose)

1M Tris-HCl pH 8.0	1 ml
0.5M EDTA pH 8.0	5 ml
1M Sucrose	60 ml
Distilled water	100 ml

This buffer is sterilized by autoclaving at 121°C, 15 pounds/inch² pressures, for 15 minutes. After cooling at room temperature, this buffer is kept at 4°C.

3.5 THE buffer pH 8.0

1M Tris-HCl pH 8.0	5 ml
0.5M EDTA pH 8.0	4 ml
Distilled water	100 ml

This buffer is sterilized by autoclaving at 121°C, 15 pounds/inch² pressures, for 15 minutes. After cooling at room temperature, this buffer is kept at 4°C.

3.6 1M MgCl₂

MgCl ₂	9.52 g
Distilled water	100 ml

This buffer is sterilized by autoclaving at 121°C, 15 pounds/inch² pressures, for 15 minutes. After cooling at room temperature, this buffer is kept at 4°C.

3.7 Solution III

CH ₃ COOH	11.5 ml
CH ₃ COOK	29.4 g
Distilled water	100 ml

This buffer is sterilized by autoclaving at 121°C, 15 pounds/inch² pressures, for 15 minutes. After cooling at room temperature, this buffer is kept at 4°C.

4. The Reagents Used for PFGE

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

1M Tris, pH 8.0	10 ml
0.5 mM EDTA, pH 8.0	20 ml
Sterile Type 1 water	100 ml

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

1M Tris, pH 8.0	10 ml
0.5 mM EDTA, pH 8.0	20 ml
Sterile Type 1 water	1000 ml

4.3 1% SeaKEM gold: 1% Sodium Dodecyl Sulfate Agarose (1% SKG: 1% SDS) in TE buffer

- Wight 0.50 (or 0.25) gram SKG into 250 ml screw-cap flask.
- Add 47.0 (or 13.5) ml TE buffer; swirl gently to disperse agarose.
- Remove cap, cover loosely with clear film, and microwave for 30-sec; mix gently and repeat for 10 sec interval until agarose is completely dissolved.
- Place flask in 60°C water bath for at least 5 min.
- Add 2.5 (or 1.25) ml of 20% SDS that has equilibrated to 56°C and mix well.
- Recap flask and return to 56°C water bath until ready to use.

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

1M Tris, pH 8.0	10 ml
0.5 mM EDTA, pH 8.0	20 ml
10% Sarcosyl (N-Lauryl-Sarcosine, Sodium salt)	50 ml
Sterile Type 1 water	500 ml

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

4.5 1% Sea Kem Gold Agarose (1% SKG) in 0.5X TBE buffer

- Wight 1.0 gram SKG into 500 ml screw-cap bottle.
- Add 100 ml 0.5X TBE buffer; swirl to disperse agarose.
- Remove cap, cover loosely with agarose clear film, and microwave for 60 sec; mix gently and repeat for 15 sec interval until agarose is completely dissolve



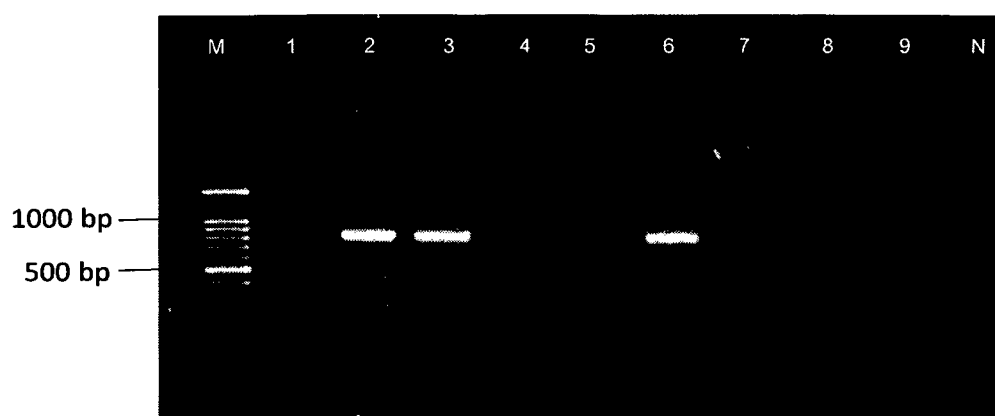
APPENDIX B

APPENDIX B

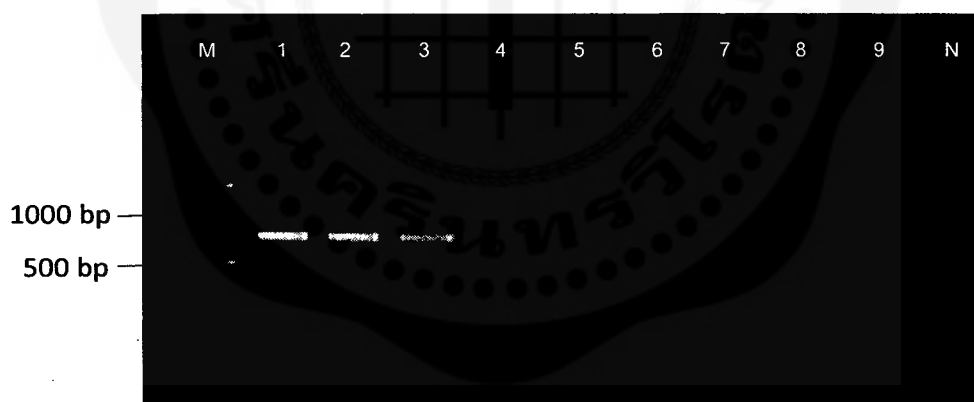
PCR AND GEL ELECTROPHORESIS



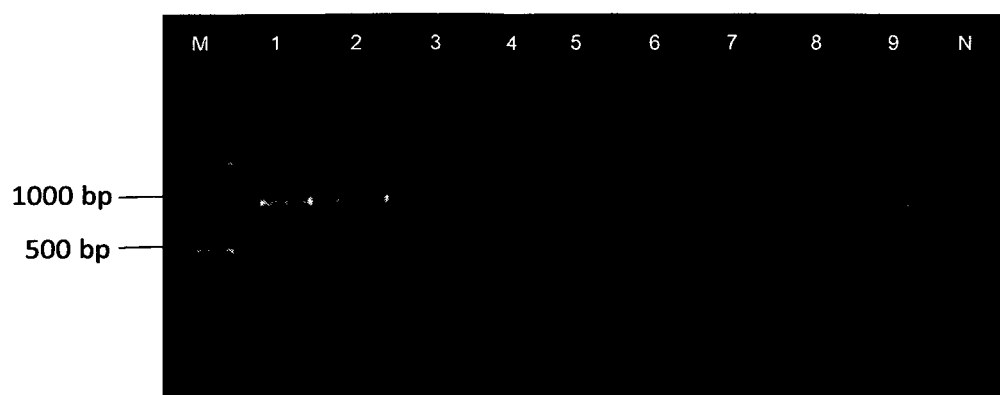
The PCR analysis for *bla*_{CTX-M-14}, *bla*_{CTX-M-15}, and *bla*_{CTX-M-55} genes in 9 co-resistance *S. choleraesuis* strains: Lane M: 100-bp+1.5 kb DNA ladder; Lanes 1-2 Templates: *bla*_{CTX-M-14} (475 bp), strain SH1007/10 and SH2789/10; Lanes 3-6 Templates: *bla*_{CTX-M-15} (907 bp), strain SH2609/10, SH1423/12, SH1424/12, and SH1456/12; Lanes 7-8 Templates: *bla*_{CTX-M-55} (907 bp), strain SH575/12 and SH577/12; Lane 9 Template: strain SH735/10; Lane N: negative control (sterile DW).



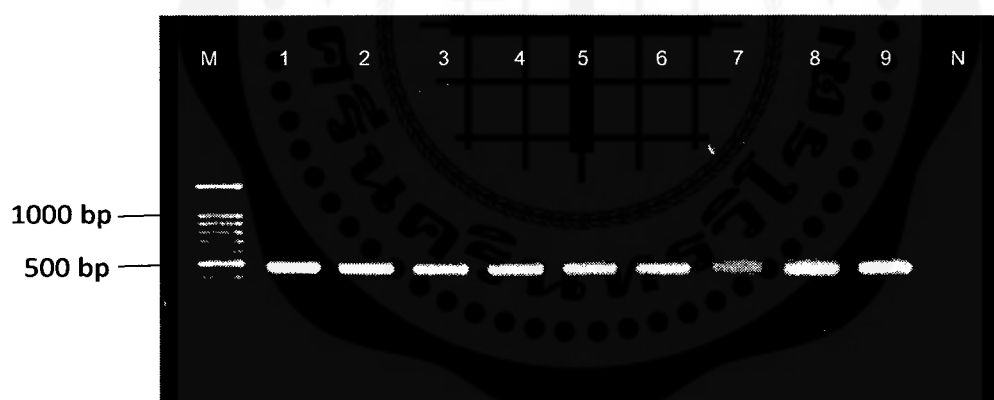
The PCR analysis for *bla*_{ACC-1} (818 bp) gene in 9 co-resistance *S. choleraesuis* strains: Lane M: 100-bp+1.5 kb DNA ladder; Lane 1: Strain SH2609/10; Lanes 2-3: Strain SH1007/10 and SH2789/10; Lanes 4-5: Strain SH575/12 and SH577/12; Lane 6: Strain SH735/10; Lanes 7-9: Strain SH1423/12, SH1424/12, and SH1456/12; Lane N: negative control (sterile DW).



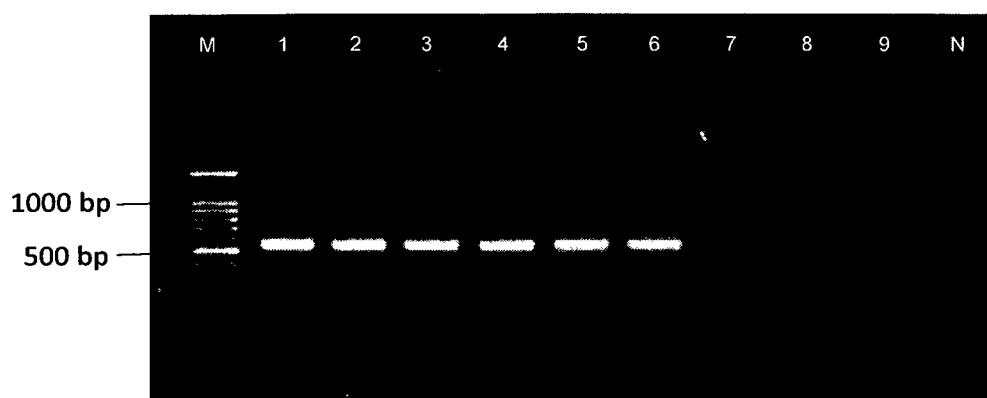
The PCR analysis for *bla*_{CMY-2} (758 bp) gene in 9 co-resistance *S. choleraesuis* strains: Lane M: 100-bp+1.5 kb DNA ladder; Lanes 1-3: Strain SH1007/10, SH2789/10, and SH735/10; Lanes 4-9: Strain SH2609/10, SH575/12, SH577/12, SH1423/12, SH1424/12, and SH1456/12; Lane N: negative control (sterile DW).



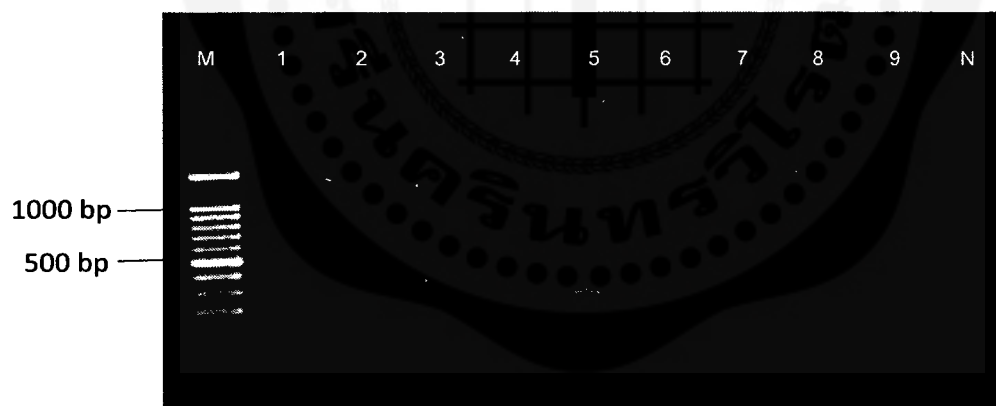
The PCR analysis for *bla*_{TEM-1} (964 bp) gene in 9 co-resistance *S. choleraesuis* strains:
 Lane M: 100-bp+1.5 kb DNA ladder; Lanes 1-2: Strain SH575/12 and SH577/12; Lanes 3-9: Strain SH1007/10, SH2789/10, SH735/10, SH2609/10, SH1423/12, SH1424/12, and SH1456/12; Lane N: negative control (sterile DW).



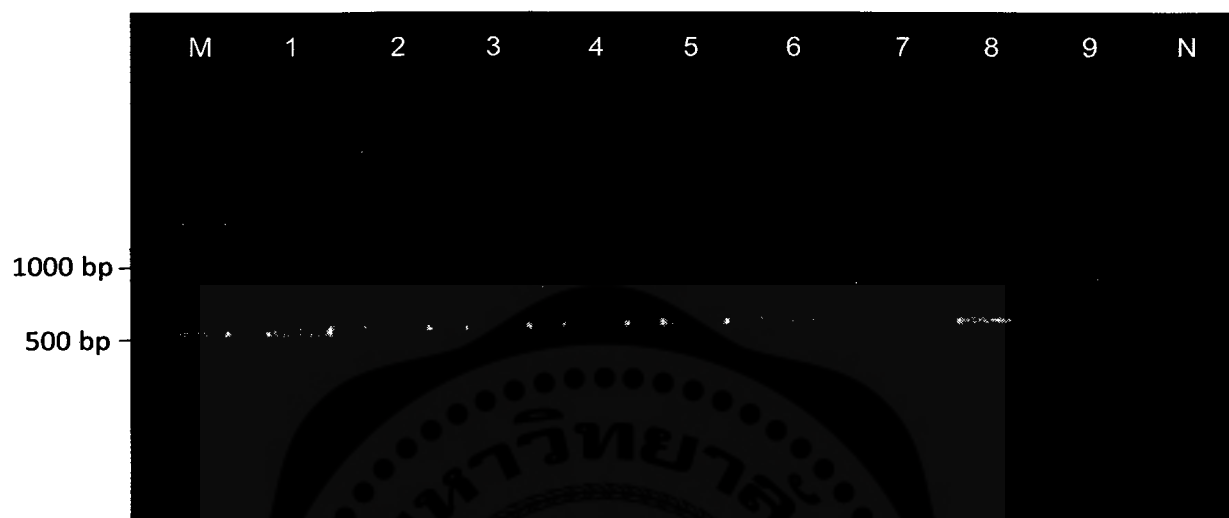
The PCR analysis for *qnrS1* (427 bp) gene in 9 co-resistance *S. choleraesuis* strains:
 Lane M: 100-bp+1.5 kb DNA ladder; Lanes 1-9: Strain SH1007/10, SH2789/10, SH735/10, SH2609/10, SH575/12, SH577/12, SH1423/12, SH1424/12, and SH1456/12; Lane N: negative control (sterile DW).



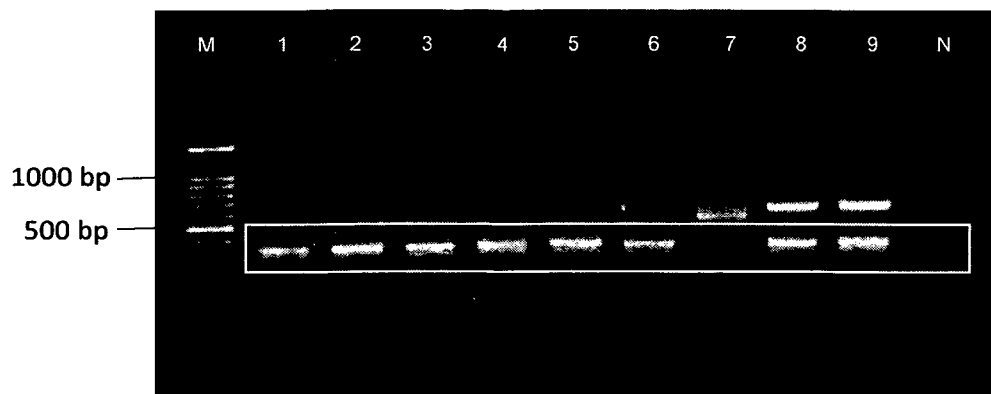
The PCR analysis for *qnrA* (579 bp) gene in 9 co-resistance *S. choleraesuis* strains: Lane M: 100-bp+1.5 kb DNA ladder; Lanes 1-6: Strain SH1007/10, SH2789/10, SH575/12, SH577/12, SH1424/12, and SH1456/12; Lanes 7-9: SH735/10, SH2609/10, and SH1423/12; Lane N: negative control (sterile DW).



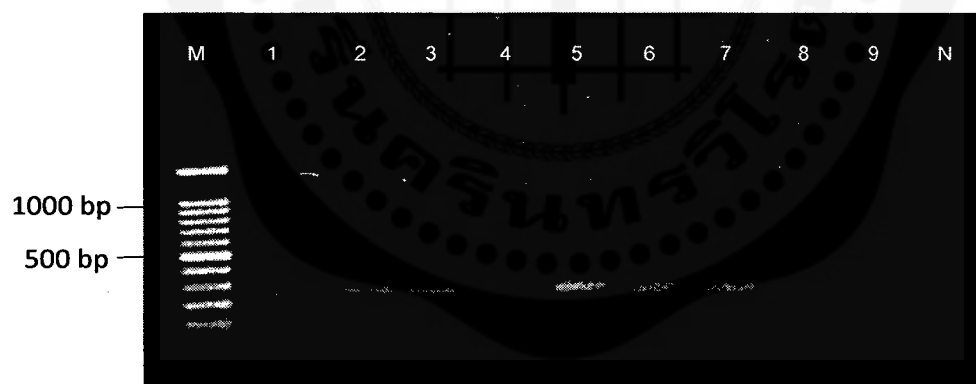
The PCR analysis for *qnrB* (263 bp) gene in 9 co-resistance *S. choleraesuis* strains: Lane M: 100-bp+1.5 kb DNA ladder; Lane 1: Strain SH2609/10; Lanes 2-3: Strain SH1007/10 and SH735/10; Lanes 4-7: Strain SH2789/10, SH575/12, SH577/12, and SH1423/12; Lanes 8-9: Strain SH1424/12 and SH1456/12; Lane N: negative control (sterile DW).



The PCR analysis for *aac(6')-Ib-cr* (482 bp) gene in 9 co-resistance *S. choleraesuis* strains:
Lane M: 100-bp+1.5 kb DNA ladder; Lanes 1-6: Strain SH2609/10, SH2789/10, SH735/10, SH575/12, SH1423/12, and SH1424/12; Lanes 7: Strain SH1007/10; Lanes 8: Strain SH1456/12; Lanes 9: Strain SH577/12; Lane N: negative control (sterile DW).



The PCR analysis for *gryA* (312 bp) gene in 9 co-resistance *S. choleraesuis* strains: Lane M: 100-bp+1.5 kb DNA ladder; Lanes 1-9: Strain SH1007/10, SH2789/10, SH735/10, SH2609/10, SH575/12, SH577/12, SH1423/12, SH1424/12, and SH1456/12; Lane N: negative control (sterile DW).



The PCR analysis for *parC* (261 bp) gene in 9 co-resistance *S. choleraesuis* strains: Lane M: 100-bp+1.5 kb DNA ladder; Lanes 1-9: Strain SH1007/10, SH2789/10, SH735/10, SH2609/10, SH575/12, SH577/12, SH1423/12, SH1424/12, and SH1456/12; Lane N: negative control (sterile DW).



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