<table>
<thead>
<tr>
<th><strong>Title</strong></th>
<th>STUDY OF EXTENDED-SPECTRUM β-LACTAMASE-PRODUCTING ENTEROBACTERIACEAE AMONG ASYMPTOMATIC PEOPLE</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Author(s)</strong></td>
<td>Luvsansharav, Ulzii-Orshikh</td>
</tr>
<tr>
<td><strong>Citation</strong></td>
<td></td>
</tr>
<tr>
<td><strong>Issue Date</strong></td>
<td></td>
</tr>
<tr>
<td><strong>Text Version</strong></td>
<td>ETD</td>
</tr>
<tr>
<td><strong>URL</strong></td>
<td><a href="http://hdl.handle.net/11094/54702">http://hdl.handle.net/11094/54702</a></td>
</tr>
<tr>
<td><strong>DOI</strong></td>
<td></td>
</tr>
<tr>
<td><strong>rights</strong></td>
<td></td>
</tr>
</tbody>
</table>

*Osaka University Knowledge Archive : OUKAK*  
http://ir.library.osaka-u.ac.jp/dspace/

Osaka University
STUDY OF EXTENDED-SPECTRUM
β-LACTAMASE-PRODUCING ENTEROBACTERIACEAE
AMONG ASYMPOTOMATIC PEOPLE

Osaka University
Graduate School of Medicine
Division of Health Sciences

Ulzii-Orshikh Luvsansharav

January 2013
STUDY OF EXTENDED–SPECTRUM
\(\beta\)-LACTAMASE–PRODUCING *ENTEROBACTERIACEAE*
AMONG ASYMPTOMATIC PEOPLE

Professors: NARIAKI MATSUURA, MD, PHD
(YOSHIMASA YAMAMOTO, PHD)

Osaka University
Graduate School of Medicine
Division of Health Sciences

Ulzii–Orshikh Luvsansharav

January 2013
Acknowledgements

First of all, I would like to deeply thank the people of Japan and the Japanese Ministry of Education, Culture, Science and Technology for offering me Monbukagakusho scholarship to do this doctoral research.

I was fortunate to have professor Yoshimasa Yamamoto, Ph.D., as my supervisor in the doctoral study. I express my genuine gratitude and appreciation to him for accepting me to his laboratory, exposing me to the research of molecular microbiology, and expanding my international experience by fieldwork in Thailand, Vietnam and Laos. His insightful advice and guidance were invaluable.

I would like to express my sincere gratitude to professor Nariaki Matsuura, M.D., Ph.D., for accepting me to his laboratory after my supervisor left Osaka University, and for his expert counsel. Also I would like to thank the members of my Dissertation Committee professors Eiji Miyoshi M.D., Ph.D., and Toru Tobe, Ph.D., for sharing their time and knowledge.

There have been many people in the Molecular Microbiology Laboratory, Osaka University Graduate School of Medicine, who have contributed to the completion of this project. I am indebted to associate professor Itaru Hirai, Ph.D., for his scientific guidance and support. Thanks also to past laboratory members Marie Niki, Tadahiro Sasaki, Ph.D., Arisa Nakata, Kaori Imura, Kou Yamauchi and Aya Yoshinaga for their help and friendship.
My heartfelt gratitude goes to professors Chalit Komalamisra, Dr.Med.Sci. and Teera Kusolsuk, M.D., MSc., from Mahidol University in Thailand, without whom this work would not have been possible. Thanks to professor Kiyoko Makimoto, Ph.D., and Enkhjargal Altangerel, M.Sc., for their invaluable time and assistance with statistical analyses.

Finally, I want to thank my beloved parents, sisters and brother, and friends for their inspiration and support throughout my studies. Their love and encouragement have enabled me to realize my goals.
Table of Contents

List of figures ......................................................................................................................... 1
List of tables ............................................................................................................................... 2
ABSTRACT ................................................................................................................................. 3

CHAPTER 1. INTRODUCTION ................................................................................................. 6
  1.1. Beta-lactam antibiotics and resistance to ß-lactams ...................................................... 6
  1.2. Beta-lactamases and ESBLs ............................................................................................ 6
  1.3. Definition of ESBLs ......................................................................................................... 8
  1.4. Classification of ß-lactamases and ESBLs ....................................................................... 8
  1.5. Scope of the study ........................................................................................................... 10
  Reference ................................................................................................................................. 12

CHAPTER 2. ESBL-PRODUCING ENTEROBACTERIACEAE IN THREE PROVINCES OF THAILAND ......................................................................................... 13
  2.1. Background ..................................................................................................................... 13
  2.2. Objectives ....................................................................................................................... 14
  2.3. Materials and Methods ................................................................................................. 14
  2.4. Results ............................................................................................................................ 20
  2.5. Discussions .................................................................................................................... 25
  Reference ................................................................................................................................. 28

CHAPTER 3. CTX-M-TYPE ESBL-PRODUCING ENTEROBACTERIACEAE IN KANCHANABURI PROVINCE, THAILAND .................................................. 31
  3.1. Background ..................................................................................................................... 31
  3.2. Objectives ....................................................................................................................... 32
  3.3. Materials and Methods ................................................................................................. 32
List of figures

Figure 2.1. Location of the Thai provinces selected for the study. ..........................15
Figure 2.2. Double disk synergy test .............................................................................17
Figure 2.3. Prevalence of CTX-M-type ESBL-producing Enterobacteriaceae and $bla_{CTX-M}$ gene groups. ...........................................................................................................22
Figure 2.4. Bacterial identification of CTX-M-type ESBL-producing Enterobacteriaceae ..........................................................................................................................23
Figure 2.5. Multidrug resistance pattern of CTX-M-type ESBL-producing Enterobacteriaceae ......................................................................................................................24
Figure 3.1. Location of the Kanchanaburi districts selected for the study. ........34
Figure 3.2. ESBL confirmation test .................................................................................34
Figure 3.3. Dichotomous decision tree to determine the phylogenetic group of an E. coli strain ....................................................................................................................36
List of tables

Table 1.1. β-Lactams: class and subclass designation and generic name........7
Table 1.2. Alignment of molecular and functional β-lactamase classification schemes.................................................................9
Table 2.1. Primers used for PCR amplification..................................................18
Table 2.2. PCR reaction composition..............................................................19
Table 2.3. Zone diameter breakpoints, mm .....................................................20
Table 2.4. Characteristics of the study participants.......................................21
Table 2.5. Comparison of antibiotic usage in the 3 provinces during the last year .......................................................................................25
Table 3.1. Primers used for PCR amplification................................................36
Table 3.2. Characteristics of the study participants.......................................37
Table 3.3. Detection of CTX-M-type ESBL-producing Enterobacteriaceae......39
Table 3.4. Phylogenetic groups of the CTX-M ESBL-producing Escherichia coli isolates....................................................................................40
Table 3.5. Antibiotic susceptibility of CTX-M-type ESBL-producing Enterobacteriaceae....................................................................................40
Table 4.1. Characteristics of the study participants.......................................53
Table 4.2. Detection of CTX-M-type ESBL-producing Enterobacteriaceae......54
Table 5.1. Characteristics of the study participants.......................................62
Table 5.2. Prevalence of ESBL-producing Enterobacteriaceae in nursing homes .....................................................................................62
Table 5.3. Univariate and multivariate logistic regression analyses ............64
ABSTRACT

In recent years the spread of extended-spectrum β-lactamase (ESBL)-producing microorganisms is increasing in hospital settings around the world. The prevalence of and risk factors associated with ESBL-producing microorganisms have not been well studied among asymptomatic individuals in communities. Therefore, the aim of this study was to determine this in healthy individuals in Thailand and Japan.

The study samples were collected in Thailand and Japan: 445 samples from Thailand during 2008-2009 and 417 samples in 2010; and 443 samples from Japan during 2009-2010. One stool sample and a questionnaire were obtained from each participant. Stool samples were screened for ESBLs using MacConkey agar supplemented with cefotaxime. Results were confirmed using cefotaxime and ceftazidime with and without clavulanic acid in accordance with the Clinical and Laboratory Standards Institute recommendations. The bla_{CTX-M} genes were identified and genotyped using PCR with bacterial DNA samples. Multivariate logistic regression analysis was performed to investigate risk factors associated with the faecal carriage of CTX-M producers.

In our first part of the study we identified high prevalence of CTX-M-type ESBL-producing *Enterobacteriaceae* in the three provinces of Thailand: 29.3% in Nan, 29.9% in Nakhon Si Thammarat and 50.6% in Kanchanaburi. Genotyping of *bla*_{CTX-M} revealed that most CTX-M producers harboured genes belonging to the CTX-M-9 group, followed by the CTX-M-1 group. *Escherichia coli* was the predominant member of the *Enterobacteriaceae* producing
CTX-M-type ESBLs. No statistically significant association was observed between the presence of ESBL-producing bacteria and gender, age, education, food habits or antibiotic usage. However, the provinces that had the highest prevalence of ESBL-producing Enterobacteriaceae also had the highest prevalence of use and purchase of antibiotics without a prescription.

After 2 years since the initial study we conducted another study in Kanchanaburi province with a more detailed questionnaire and increased the number of participants. The prevalence of CTX-M-type ESBL-producing Enterobacteriaceae was increased to 65.7%. Similar to previous findings, the CTX-M-9 group (60.6%) was dominant, followed by the CTX-M-1 group (38.7%). Most of the bacteria were E. coli (85.4%) and Klebsiella pneumoniae (4.7%). In a multivariate logistic regression model, better education status, history of hospitalization and the use of antibiotics within the last 3 months were independently associated with faecal carriage.

In contrast, faecal carriage of CTX-M producers among asymptomatic Japanese people was very low (6.0%). CTX-M-9 group was dominant, followed by CTX-M-2 group. E. coli was predominantly identified among CTX-M producers. Statistical analysis did not reveal any significant association between ESBL production and antibiotic usage or hospitalization experience. However, the prevalence of CTX-M-producers in nursing homes of Japan was much higher (19.6%). In multivariate logistic regression analysis, inability to turn over in bed, diabetes, and invasive procedures within the last 2 years were the only variables independently associated with faecal carriage of CTX-M-type ESBL producers among nursing home residents.
In summary, faecal carriage of CTX-M-type ESBL-producing *Enterobacteriaceae* among asymptomatic individuals in rural Thailand is alarmingly high, and previous antibiotic use and a history of hospitalization may contribute to its dissemination. In comparison to Thailand, faecal carriage of ESBL producers in healthy Japanese people is low but has an increasing trend. This may also be one of the causes of the increased spread of ESBL-producing bacteria in hospitals or vice versa, with nursing homes acting as a possible reservoir for community spread of CTX-M producers.
CHAPTER 1. INTRODUCTION

1.1. Beta-lactam antibiotics and resistance to β-lactams

β-Lactams comprise a very large family of different groups of bactericidal compounds all containing β-lactam ring and inhibit bacterial cell wall synthesis by binding to penicillin-binding proteins (PBPs). Class and subclass designation and generic names of β-lactam antibiotics are shown in Table 1.1.

Resistance to β-lactams may involve one or more of the three possible mechanisms:

1. Resistance by alteration in target site,
2. Resistance by alteration in access to the target site,

In gram-negative bacteria, β-lactamases are the major defense against multiple classes of β-lactam antibiotics.

1.2. Beta-lactamases and ESBLs

β-Lactamases are enzymes that catalyze the hydrolysis of the β-lactam ring and inactivate these antibiotics before they reach PBPs located at the cytoplasmic membrane. Genes encoding for these enzymes are found on the chromosome and on plasmids. Hundreds of different β-lactamase enzymes have been described and by late 2009, the number of unique protein sequences exceeded 890. All β-lactamases have same function but with differing amino acid-sequences their spectra of β-lactam substrates change. A group of β-lactamases specifically target the newer β-lactam antibiotics, including
Table 1.1. β-Lactams: class and subclass designation and generic name (adapted from Clinical and Laboratory Standards Institute)

<table>
<thead>
<tr>
<th>Antimicrobial Class</th>
<th>Antimicrobial Subclass</th>
<th>Agents Included; Generic Names</th>
</tr>
</thead>
<tbody>
<tr>
<td>Penicillins</td>
<td>Penicillin</td>
<td>Penicillin</td>
</tr>
<tr>
<td></td>
<td>Aminopenicillin</td>
<td>Amoxicillin, Ampicillin</td>
</tr>
<tr>
<td></td>
<td>Ureidopenicillin</td>
<td>Azlocillin, Mezlocillin, Piperacillin</td>
</tr>
<tr>
<td></td>
<td>Carboxypenicillin</td>
<td>Carbenicillin, Ticarcillin</td>
</tr>
<tr>
<td></td>
<td>Penicillinase-stable penicillins</td>
<td>Cloxacillin, Dicloxacillin, Methicillin, Nafcillin, Oxacillin</td>
</tr>
<tr>
<td></td>
<td>Amidopenicillin</td>
<td>Mecillinam</td>
</tr>
<tr>
<td>β-Lactam/β-lactamase inhibitor combinations</td>
<td></td>
<td>Amoxicillin-clavulanic acid, Ampicillin-sulbactam, Piperacillin-tazobactam, Ticarcillin-clavulanic acid</td>
</tr>
<tr>
<td>Cephems (parenteral)</td>
<td>Cephalosporin I</td>
<td>Cefazolin, Cephalothin, Cephapirin, Cephradine</td>
</tr>
<tr>
<td></td>
<td>Cephalosporin II</td>
<td>Cefamandole, Cefonicid, Cefuroxime (parenteral)</td>
</tr>
<tr>
<td></td>
<td>Cephalosporin III</td>
<td>Cefoperazone, Cefotaxime, Ceftazidime, Ceftizoxime, Ceftriaxone</td>
</tr>
<tr>
<td></td>
<td>Cephalosporin IV</td>
<td>Cefepime</td>
</tr>
<tr>
<td></td>
<td>Cephalosporins with anti-MRSA activity</td>
<td>Ceftaroline, Ceftobiprole</td>
</tr>
<tr>
<td></td>
<td>Cephemycin</td>
<td>Cefnetazole, Cefotetan, Cefoxitin</td>
</tr>
<tr>
<td></td>
<td>Oxacephem</td>
<td>Moxalactam</td>
</tr>
<tr>
<td>Cephems (oral)</td>
<td>Cephalosporin</td>
<td>Cefaclor, Cefadroxil, Cefdinir, Cefditoren, Cefetamect, Cefixime, Cefpodoxime, Cefprozil, Cefitbuten, Cefuroxime (oral), Cephalexin, Cephradine</td>
</tr>
<tr>
<td></td>
<td>Carbacephem</td>
<td>Loracarbef</td>
</tr>
<tr>
<td>Monobactams</td>
<td></td>
<td>Aztreonam</td>
</tr>
<tr>
<td>Penems</td>
<td>Carbapenem</td>
<td>Doripenem, Ertapenem, Imipenem, Meropenem, Razupenem</td>
</tr>
<tr>
<td></td>
<td>Penem</td>
<td>Faropenem, Sulopenem</td>
</tr>
</tbody>
</table>

Abbreviations: ESBL, extended-spectrum β-lactamase; MRSA, methicillin-resistant S. aureus.

Penicillinase labile; hydrolyzed by staphylococcal penicillinase.

Not hydrolyzed by staphylococcal penicillinase.

Cephalosporin I, II, III, and IV are sometimes referred to as 1st-, 2nd-, 3rd-, and 4th-generation cephalosporins, respectively. Cephalosporin III and IV are also referred to as “extended-spectrum cephalosporins”. This does not imply activity against ESBL-producing gram-negative bacteria.
extended-spectrum cephalosporins. First report of plasmid-encoded β-lactamases capable of hydrolyzing the extended-spectrum cephalosporins was published in 1983 and was soon followed by discoveries of other β-lactamases with similar abilities, and hence these new β-lactamases were named as Extended-Spectrum β-Lactamases or ESBLs.6

1.3. Definition of ESBLs

There is no consensus of the precise definition of ESBLs. A commonly used working definition is that the ESBLs are β-lactamases capable of conferring bacterial resistance to the penicillins, first-, second-, and third-generation cephalosporins, and aztreonam (but not to cephemycins or carbapenems) by hydrolysis of these antibiotics and which are inhibited by β-lactamase inhibitors such as clavulanic acid.6-8 β-Lactamase inhibitors are molecules that contain a β-lactam ring and act as “suicide inhibitors” binding to β-lactamases and preventing them from destroying β-lactams.1

1.4. Classification of β-lactamases and ESBLs

β-Lactamases are most commonly classified according to 2 general schemes: Ambler molecular classification, and Bush-Jacoby-Medieros functional classification system.6 The molecular and functional classification schemes are aligned in Table 1.2.2 ESBLs are mostly β-lactamases of Bush-Jacoby-Medieros group 2be and 2de, and those of Ambler classes A and D.

ESBLs initially arose from as a result of point mutation in the TEM and SHV β-lactamases, which did not possess extended-spectrum β-lactam substrate activity.9 For a number of years, TEM- and SHV-type ESBLs were predominant. Several types of ESBLs, other than TEM and SHV, have been
### Table 1.2: Alignment of molecular and functional β-lactamase classification schemes

<table>
<thead>
<tr>
<th>Active site</th>
<th>Molecular class</th>
<th>Functional class</th>
<th>Typical enzymes</th>
<th>Enzyme characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serine</td>
<td>A</td>
<td>2a</td>
<td>Staphylococcal penicillinases</td>
<td>Penicillins</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2b</td>
<td>TEM-1, SHV-1</td>
<td>Penicillins, narrow-spectrum cephalosporins</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2be</td>
<td>ESBLs&lt;sup&gt;6&lt;/sup&gt; (TEM, SHV, CTX-M families)</td>
<td>Penicillins, cephalosporins, monobactams (aztreonam)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2br</td>
<td>TEM-IRT enzymes, SHV-10</td>
<td>Penicillins, narrow-spectrum cephalosporins</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2c</td>
<td>PSE-1</td>
<td>Penicillins, including carbenicillin</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2e</td>
<td>Proteus and Bacteroides cephalosporinases</td>
<td>Cephalosporins</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2f</td>
<td>SME and KPC families; IMI-1</td>
<td>Penicillins, cephalosporins, carbapenems</td>
</tr>
<tr>
<td>Serine</td>
<td>C</td>
<td>1</td>
<td>AmpC, Chromosomal cephalosporinases</td>
<td>Cephalosporins</td>
</tr>
<tr>
<td>Serine</td>
<td>D</td>
<td>2d</td>
<td>OXA-1, OXA-10</td>
<td>Penicillins, including cloxacillin/oxacillin</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2de</td>
<td>OXA-ESBLs</td>
<td>Penicillins, including cloxacillin/oxacillin; cephalosporins except cephapymcins</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2df</td>
<td>OXA-24, OXA-40</td>
<td>Penicillins, including cloxacillin/oxacillin; carbapenems</td>
</tr>
<tr>
<td>Zinc</td>
<td>B</td>
<td>3</td>
<td>L1, CcrA, VIM-and IMP families</td>
<td>Penicillins, cephalosporins, carbapenems, but not aztreonam</td>
</tr>
</tbody>
</table>

<sup>a</sup>CA clavulanic acid; TZB tazobactam; EDTA ethylenediaminetetraacetic acid

<sup>b</sup>Extended-spectrum β-lactamase

<sup>c</sup>Dependent upon specific enzyme

9
described, including the CTX-M-, OXA-, PER-type and several others. Among these, the CTX-M-type ESBLs are a rapidly growing group and by far the most successful in terms of spread and their impact. The name CTX-M reflects the potent hydrolytic activity of these ESBLs against cefotaxime antibiotics.

The presence of ESBLs in various members of the Enterobacteriaceae family is of great microbiological and clinical importance and the CTX-M type of ESBLs are becoming increasingly more prevalent in E. coli and K. pneumoniae. The Infectious Diseases Society of America identified ESBL-producing Enterobacteriaceae as one of the six top priority dangerous pathogens for which there are few or no drugs in late-stage development, further making the choice of an appropriate and safe treatment for these infections limited.

1.5. Scope of the study

In this doctoral study I intended to address two basic issues regarding the rapidly spreading ESBLs, in particular CTX-M-type ESBLs. Firstly, we expanded the ESBL study beyond the traditional focus of hospital settings, and studied the prevalence of faecal carriage of ESBL-producing microorganisms among asymptomatic individuals in communities.

Secondly, we tried to identify risk factors associated with the rapid spread of the ESBL producers in communities and to test possible reservoir populations such as nursing homes that may be acting as a bridge for spreading the ESBL-producing microorganisms between the hospitals and communities.

Furthermore, since antibiotic resistance is an international public health problem, we carried out this study in two distinct countries, Thailand and Japan. This provided us with a possibility to compare the prevalence of and risk factors
associated with faecal carriage of ESBL-producing *Enterobacteriaceae* in developed and developing nations.
Reference


CHAPTER 2. ESBL-PRODUCING ENTEROBACTERIACEAE IN THREE PROVINCES OF THAILAND

2.1. Background

Multidrug-resistant Enterobacteriaceae that produce extended spectrum β-lactamases (ESBLs) have spread rapidly worldwide causing treatment failure and hindering infection control efforts. The wide spread of ESBL-producing bacteria not only affects the choice of antimicrobials but may also cause excessive morbidity and mortality, as well as increased healthcare costs.

Among the ESBLs, the CTX-M-type enzymes are more prevalent, and Escherichia coli strains that produce CTX-M-type β-lactamases are frequently true community-acquired ESBL-producing pathogens. These E. coli strains cause community-acquired urinary tract and bloodstream infections. The prevalence of and risk factors associated with the carriage of ESBL-producing microorganisms have often been studied in patients with these infections. This prevalence varies among countries, and risk factor analyses have shown conflicting and varying results. In contrast, only a few studies have been performed on the community carriage of ESBL-producing E. coli. In recent years, researchers have reported a 2.3, 3.7, 7.0 and 13.1% prevalence of ESBL-producing Enterobacteriaceae in healthy subjects in Lebanon, Spain, China and Saudi Arabia, respectively. However, in a previous study by us, we observed a surprisingly high prevalence of CTX-M-type ESBL-producing
Enterobacteriaceae (82 of 160 samples) in asymptomatic individuals in Kanchanaburi province, Thailand.12

2.2. Objectives

The aim of the present study was to analyse the factors that may have contributed to the extensive spread of ESBL-producing Enterobacteriaceae in asymptomatic individuals in Thailand. We extended our study beyond the hospital environment and performed an epidemiological analysis of the risk factors in order to predict the possible threat of the spread of ESBL-producing Enterobacteriaceae.

2.3. Materials and Methods

Location of provinces

The study was conducted from May to July 2009 in Nan and Nakhon Si Thammarat provinces in Thailand. Epidemiological data from a previous study conducted in 2008 in Kanchanaburi province, Thailand, were also used in this study.12

The three provinces were selected to represent geographically different regions of the country: Nan is in the northern region, Nakhon Si Thammarat is in the southern region and Kanchanaburi is in the central region (Figure 2.1). Written informed consent was obtained from each individual participating in the study.

Study samples

We analysed a total of 468 stool samples from the three Thai provinces: one sample was obtained from each asymptomatic volunteer. The participants were screened on the basis of age (≥20 years) and medical history.
Asymptomatic individuals with a history of antibiotic treatment 3 months prior to sample collection and with a confirmed diagnosis of digestive tract diseases were excluded from the study.

**Figure 2.1.** Location of the Thai provinces selected for the study.

**Questionnaire**

All participants were interviewed using a standardized questionnaire based on general demographic and socioeconomic circumstances. They were questioned about their food intake, drinking water resources and toilet facilities. The participants were also questioned regarding their alcohol intake and smoking habits. We questioned them about their antibiotic usage, including the purchase of antibiotics without a prescription. After excluding the questionnaires in which age and gender data had not been filled in, we used 445 questionnaires (183 men and 262 women) for statistical analyses. In Kanchanaburi, four of the excluded participants had ESBL-producing *Enterobacteriaceae*. 

15
The following scheme shows the methodology used for the study.

**Scheme 2.1.** The methodology of the research in schematic presentation.

**Screening tests for ESBLs**

Stool samples were collected from asymptomatic participants to identify ESBL-producing *Enterobacteriaceae* by phenotypic and genotypic methods. The phenotypic methods included plating the stool samples on MacConkey agar supplemented with 2 μg/ml cefotaxime ('CTX-MacConkey') within 24 h after collection. A single colony was inoculated from CTX-MacConkey plates into a nutrient broth supplemented with 2 μg/ml cefotaxime and incubated at 37°C for 24 h. The inoculated broth was used for preparation of bacterial stock and DNA extraction.
Subsequently, the ESBL production was confirmed by a double-disc synergy test as described previously. The test was performed on Mueller-Hinton agar with 30 µg disks of cefotaxime, ceftazidime, cefepime, and aztreonam and a disk of amoxicillin–clavulanate (containing 10 µg of clavulanate) positioned at a distance of 30 mm (centre to centre). The test is considered as positive when a decreased susceptibility to an antibiotic is combined with a clear-cut enhancement of the inhibition zone in front of the clavulanate-containing disk, often resulting in a characteristic shape-zone referred to as “champagne-cork” or “keyhole” (Figure 2.2).

(a) The inhibition zone is enhanced between cefotaxime (CTX) and amoxicillin–clavulanate (AMC) disks, indicating synergy between cefotaxime and clavulanate.

(b) The inhibition zones are enhanced between the disks of cefotaxime, ceftazidime, cefepime, and aztreonam and a disk of amoxicillin–clavulanate positioned at the center.

Figure 2.2. Double disk synergy test.

Identification of bacteria

Isolates were identified using conventional biochemical tests. Growth from a single colony was inoculated into biochemical test tubes with the following medium: TSI-Triple Sugar Iron; LIM-Lysine Indole Motility; SIM-Sulfide Indole Motility; MR-VP-Methyl Red-Voges Proskauer; SC-Simmons Citrate. The tests were read at 24 and 48 h. The isolates with unclear biochemical test results were identified by the API 20E system, according to the manufacturer’s instructions (Sysmex-bioMerieux, Tokyo, Japan).
DNA extraction and PCR analysis

Bacterial DNA was extracted from the isolates by boiling the bacterial suspensions. DNA samples with a concentration of 0.1 ng/μl were used as template for PCR analysis. The universal primers CTX-M-U1 and CTX-M-U2 were used to detect the bla\text{CTX-M} gene, as described previously. DNA from the reference \textit{E. coli} bla\text{CTX-M}-positive strain was used as a positive control. For genotyping the bla\text{CTX-M} genes, we used four primer sets that amplify group-specific bla\text{CTX-M} genes, as described elsewhere: CTX-M-1 group, CTX-M-1, CTX-M-3, CTX-M-10–CTX-M-12, CTX-M-15, CTX-M-22, CTX-M-23, CTX-M-28, CTX-M-29 and CTX-M-30; CTX-M-2 group, CTX-M-2, CTX-M-4–CTX-M-7, CTX-M-20 and Toho-1; CTX-M-8 group, CTX-M-8; and CTX-M-9 group, CTX-M-9, CTX-M-13, CTX-M-14, CTX-M-16–CTX-M-19, CTX-M-21, CTX-M-27 and Toho-2 (Table 2.1).

Table 2.1. Primers used for PCR amplification

<table>
<thead>
<tr>
<th>Target(s)</th>
<th>Primer</th>
<th>Sequence (5' - 3' direction)</th>
<th>Product size (bp)</th>
<th>Annealing temp (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CTX-M</td>
<td>CTX-M-U1</td>
<td>ATG TGC AGY ACC AGT AAR GTK</td>
<td>593</td>
<td>54°</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ATG GC</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>CTX-M-U2</td>
<td>TGG GTR AAR TAR GTS ACC AGA</td>
<td></td>
<td>58°</td>
</tr>
<tr>
<td></td>
<td></td>
<td>AYC AGC GG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CTX-M-1 group</td>
<td>CTXM1-F3</td>
<td>GAC GAT GTC ACT GGC TGA GC</td>
<td>499</td>
<td>55°</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GAC GAT GTC ACT GGC TGA GC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CTX-M-2 group</td>
<td>CTXM1-R2</td>
<td>AGC CG C CGA CGC TAA TAC A</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CTX-M-8 group</td>
<td>TOHO1-2F</td>
<td>GCG ACC TGG TTA ACT ACA ATC C</td>
<td>351</td>
<td>55°</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GCG ACC TGG TTA ACT ACA ATC C</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CTX-M-9 group</td>
<td>CTXM825F</td>
<td>CGC TTT GCC ATG TGC AGC ACC</td>
<td>307</td>
<td>55°</td>
</tr>
<tr>
<td>CTX-M-9 group</td>
<td>CTXM825R</td>
<td>GCT CAG TAC GAT CGA GCC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CTX-M-9 group</td>
<td>CTXM914F</td>
<td>GCT GGA GAA AAG CAG CGG AG</td>
<td>474</td>
<td>62°</td>
</tr>
<tr>
<td>CTX-M-9 group</td>
<td>CTXM914R</td>
<td>GTA AGC TGA CGC AAC GTC TG</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

°Melting temperature.
PCR reaction composition was setup as shown in Table 2.2:

Table 2.2. PCR reaction composition

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td>1.2 µl</td>
</tr>
<tr>
<td>2 x PCR buffer for KOD FX</td>
<td>5.0 µl</td>
</tr>
<tr>
<td>2.0 µM deoxynucleotide triphosphates (dNTP)</td>
<td>2.0 µl</td>
</tr>
<tr>
<td>10 µM CTX-M primer mix (5’ and 3’)</td>
<td>0.6 µl</td>
</tr>
<tr>
<td>1.0 U/µl KOD FX (DNA Polymerase)</td>
<td>0.2 µl</td>
</tr>
<tr>
<td>Template DNA</td>
<td>1.0 µl</td>
</tr>
<tr>
<td><strong>Total volume</strong></td>
<td><strong>10.0 µl</strong></td>
</tr>
</tbody>
</table>

PCR amplification conditions were as follows: initial denaturation step at 94°C for 2 min; 35 cycles of denaturation at 98°C for 10 sec, annealing at 55-62°C for 30 sec and extension at 68°C for 40 sec. The PCR products were visualized by 2% agarose gel electrophoresis followed by staining with GelRed Nucleic Acid Gel Stain (Biotium).

Antimicrobial susceptibility testing

All ESBL-producing Enterobacteriaceae isolates were tested for susceptibility to imipenem, meropenem, amikacin, gentamicin, norfloxacin, ciprofloxacin and tetracycline by the disc diffusion method using an SN Disc (Nissui Pharmaceutical Co., Ltd, Tokyo, Japan) according to the manufacturer’s instructions. Colonies from overnight growth on a nutrient agar plate were directly suspended in a sterile saline and vortexed thoroughly. Turbidity of the bacterial suspension was compared to the 0.5 McFarland standard (approximately $10^8$ CFU/ml). Then a portion of the suspension was diluted 1:100 with saline ($10^8$ CFU/ml). After adjusting the turbidity of the suspension, the Mueller-Hinton agar was inoculated with 100 µl of bacterial suspension which was spread using T-shaped spreader. Antimicrobial discs were applied to
the plates using sterile forceps. After overnight incubation at 37°C, the diameter of the zones of complete inhibition were measured. Zone diameter breakpoints are shown in Table 2.3.

Table 2.3. Zone diameter breakpoints, mm

<table>
<thead>
<tr>
<th>Antimicrobial agent</th>
<th>Susceptible</th>
<th>Intermediate</th>
<th>Resistant</th>
</tr>
</thead>
<tbody>
<tr>
<td>Imipenem</td>
<td>≥ 16</td>
<td>14–15</td>
<td>≤ 13</td>
</tr>
<tr>
<td>Meropenem</td>
<td>≥ 16</td>
<td>14–15</td>
<td>≤ 13</td>
</tr>
<tr>
<td>Amikacin</td>
<td>≥ 17</td>
<td>15–16</td>
<td>≤ 14</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>≥ 15</td>
<td>13–14</td>
<td>≤ 12</td>
</tr>
<tr>
<td>Norfloxacin</td>
<td>≥ 17</td>
<td>13–16</td>
<td>≤ 12</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>≥ 21</td>
<td>16–20</td>
<td>≤ 15</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>≥ 15</td>
<td>12–14</td>
<td>≤ 11</td>
</tr>
</tbody>
</table>

Statistical analysis

Statistical analysis was performed using SPSS v.16.0. Data from the three provinces were examined separately for each province and for all provinces together. All variables were analysed by univariate analysis using a \( \chi^2 \) test or Fisher's exact test, as appropriate. Statistical significance was set at \( P < 0.05 \).

2.4. Results

The characteristics of the 445 study participants from Nan, Nakhon Si Thammarat and Kanchanaburi provinces in Thailand are listed in Table 2.4. The median age of the study participants was 54 years (range 25–86 years). The study participants were from the rural areas of Thailand and were predominantly farmers (72%). Sixty-two percent of the participants had received primary school education, whilst 26% had not received any formal education.
Table 2.4. Characteristics of the study participants

Missing data were excluded from the analysis.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Nan</th>
<th>Nakhon Si Thammarat</th>
<th>Kanchanaburi</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(n=147)</td>
<td>(n=144)</td>
<td>(n=154)</td>
</tr>
<tr>
<td>Age (years): median (range)</td>
<td>48 (30-71)</td>
<td>59 (39-86)</td>
<td>55 (25-86)</td>
</tr>
<tr>
<td>Gender: male sex</td>
<td>70 (48%)</td>
<td>53 (37%)</td>
<td>60 (39%)</td>
</tr>
<tr>
<td>Education: no formal education</td>
<td>55 (38%)</td>
<td>10 (7%)</td>
<td>48 (32%)</td>
</tr>
<tr>
<td>Occupation: farmer</td>
<td>132 (92%)</td>
<td>89 (64%)</td>
<td>88 (61%)</td>
</tr>
<tr>
<td>Lifestyle</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Current alcohol usea</td>
<td>72 (50%)</td>
<td>23 (18%)</td>
<td>68 (45%)</td>
</tr>
<tr>
<td>Current smokingb</td>
<td>19 (13%)</td>
<td>33 (23%)</td>
<td>66 (43%)</td>
</tr>
<tr>
<td>Meat consumptionc</td>
<td>138 (94%)</td>
<td>127 (88%)</td>
<td>145 (94%)</td>
</tr>
<tr>
<td>Housing condition</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tap water at home</td>
<td>54 (37%)</td>
<td>51 (36%)</td>
<td>106 (70%)</td>
</tr>
<tr>
<td>Flush toilet at home</td>
<td>19 (13%)</td>
<td>47 (33%)</td>
<td>31 (20%)</td>
</tr>
</tbody>
</table>

*aSubjects who drink alcoholic beverages once or more times a month.

bCurrent smoking includes both daily and non-daily or occasional smoking.

cSubjects who consume meat once or more times a week.

The double-disc synergy test revealed that 32.0% (47 of 147 samples), 32.6% (47 of 144 samples) and 53.9% (83 of 154 samples) of the isolates in the Nan, Nakhon Si Thammarat and Kanchanaburi provinces, respectively, were ESBL-producing bacteria. The prevalence of CTX-M-type ESBL-producing Enterobacteriaceae, as confirmed by phenotypic and genotypic methods, was 29.3% in the Nan province (43 of 147 samples), 29.9% in the Nakhon Si Thammarat province (43 of 144 samples) and 50.6% in the Kanchanaburi province (78 of 154 samples) (Figure 2.3). The prevalence in Kanchanaburi was significantly higher than that in the other two provinces (P < 0.001). Genotyping of blaCTX-M gene-positive isolates revealed that 33 (7.4%), 1 (0.2%)
and 127 (28.5%) of the 445 samples belonged to CTX-M-1, CTX-M-8 and CTX-M-9 groups, respectively (Figure 2.3). Three of the isolates did not belong to any of the groups tested.

![Prevalence of CTX-M-type ESBL-producing Enterobacteriaceae and bla<sub>CTX-M</sub> gene groups.](image)

**Figure 2.3.** Prevalence of CTX-M-type ESBL-producing *Enterobacteriaceae* and *bla<sub>CTX-M</sub>* gene groups.

*P < 0.001; prevalence was compared between the provinces using a χ² test.

On performing bacterial identification for CTX-M-type ESBL-producing *Enterobacteriaceae*, we found that *E. coli* was the predominant CTX-M-type ESBL-producing member of the *Enterobacteriaceae* in these isolates (40 of 43 isolates in Nan, 39 of 43 isolates in Nakhon Si Thammarat and 70 of 78 isolates in Kanchanaburi) (Figure 2.4). The other CTX-M-type ESBL-producing bacterial species isolated were *Citroacter*, *Klebsiella* and *Enterobacter*. 

22
Antibiotic susceptibility tests showed that there was no CTX-M-type ESBL-producing isolate that was resistant or intermediate to imipenem, meropenem and amikacin. Tetracycline resistance was identified in 88.4% (38 of 43), 86.0% (37 of 43) and 91.0% (71 of 78) of the samples in Nan, Nakhon Si Thammarat and Kanchanaburi provinces, respectively. Furthermore, 69.8% (30 of 43), 62.8% (27 of 43) and 67.9% (53 of 78) of the isolates from Nan, Nakhon Si Thammarat and Kanchanaburi provinces, respectively, were resistant to gentamicin. Resistance to both ciprofloxacin and norfloxacin was identified in 10 samples (23.3%) in Nan province, 14 (32.6%) in Nakhon Si Thammarat and 5 samples (6.4%) in Kanchanaburi province.

Of the CTX-M-type ESBL-producing bacteria, 67.7% (111 of 164) were
resistant to two or more drugs tested and only 4.3% (7 of 164) were susceptible to all the antibiotics tested (Figure 2.5).

Figure 2.5. Multidrug resistance pattern of CTX-M-type ESBL-producing *Enterobacteriaceae*.

CPFX, Ciprofloxacin; NFLX, Norfloxacin; GM, Gentamicin; TC, Tetracycline.

No statistically significant association was observed between the presence of ESBL-producing bacteria and gender, age, education and food habits of or antibiotic usage by the participants (data not shown). However, on comparing antibiotic usage in the three provinces, we observed a significant difference between the three provinces. In particular, the pattern of antibiotic purchase without a prescription was similar to the pattern of the prevalence of ESBL-producing *Enterobacteriaceae* in these provinces (Table 2.5).
Table 2.5. Comparison of antibiotic usage in the 3 provinces during the last year

<table>
<thead>
<tr>
<th>Variable</th>
<th>Nan (n=147)</th>
<th>Nakhon Si Thammarat (n=144)</th>
<th>Kanchanaburi (n=154)</th>
<th>P value&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antibiotic usage</td>
<td></td>
<td></td>
<td></td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>yes</td>
<td>65 (44.2%)</td>
<td>94 (65.3%)</td>
<td>104 (67.5%)</td>
<td></td>
</tr>
<tr>
<td>no</td>
<td>81 (55.1%)</td>
<td>48 (33.3%)</td>
<td>44 (28.6%)</td>
<td></td>
</tr>
<tr>
<td>unknown or missing</td>
<td>1 (0.7%)</td>
<td>2 (1.4%)</td>
<td>6 (3.9%)</td>
<td></td>
</tr>
<tr>
<td>Used and purchased antibiotics</td>
<td></td>
<td></td>
<td></td>
<td>0.006</td>
</tr>
<tr>
<td>without a prescription</td>
<td>34 (23.1%)</td>
<td>34 (23.6%)</td>
<td>62 (40.3%)</td>
<td></td>
</tr>
<tr>
<td>with a prescription</td>
<td>30 (20.4%)</td>
<td>56 (38.9%)</td>
<td>40 (26.0%)</td>
<td></td>
</tr>
<tr>
<td>unknown or missing</td>
<td>1 (0.7%)</td>
<td>4 (2.8%)</td>
<td>2 (1.3%)</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup>Data for Kanchanaburi province was obtained from the previous study conducted by Sasaki T et al.<sup>12</sup>

<sup>b</sup>By χ² test. Missing or unknown data were excluded from the χ² test.

2.5. Discussions

In this study, the prevalence of CTX-M-type ESBL-producing *Enterobacteriaceae* in asymptomatic Thai individuals (29.3–50.6%) was alarmingly high compared with that observed in previous studies performed on healthy subjects; these studies reported a 2.4–13.1% prevalence of faecal carriage of ESBL-producing *Enterobacteriaceae* in different countries.<sup>8–11</sup> A recent publication by Lo et al. reported 50.3% faecal carriage of ESBL-producing bacteria in household adults of children admitted to a paediatric department with mild febrile illness.<sup>16</sup> In contrast, our study was conducted in community settings among healthy subjects.

The faecal carriage of ESBL-producing *Enterobacteriaceae* in asymptomatic Thai individuals is close to or sometimes higher than the prevalence of ESBL-producing bacteria in clinical isolates collected from
patients in Thailand (30–48% in 2005–2007).\textsuperscript{17, 18} Lactamases belonging to CTX-M-1 group (20 %) and CTX-M-9 group (77%) were predominant among the 164 CTX-M β-lactamases identified in asymptomatic individuals; this pattern is identical to that of patient isolates in Thailand, where CTX-M-14 (CTX-M-9 group) and CTX-M-15 (CTX-M-1 group) were the ESBLs mainly responsible for resistance in *E. coli*.\textsuperscript{19, 20} Our findings suggest that ESBL-producing bacterial isolates obtained from clinical specimens in hospital settings represent only a fraction of the actual prevalence, and that a much more rapid and broader community-wide spread of ESBL-producing bacteria may already be under way. Therefore, an increase in the prevalence of ESBL-producing microorganisms among patients is to be expected in the future.

Even though the presence of ESBL-producing microorganisms was not associated with any of the risk factors examined in this study, the comparison of antibiotic usage between the provinces studied suggested that unregulated and high consumption of antibiotics may be a risk factor for the high prevalence of ESBL-producing *Enterobacteriaceae*. Previous studies have reported correlations between antimicrobial resistance and antibiotic usage.\textsuperscript{10, 21} The reason that our analysis did not show a direct association between the presence of ESBL-producing bacteria and antibiotic usage could be that individuals who had recently undergone antibiotic therapy had been excluded.

Furthermore, several studies have linked the high prevalence of antibiotic-resistant bacteria in the human population to the use of antibiotics in the farming industry.\textsuperscript{22–25} Recent studies have revealed an increased
prevalence of ESBL-producing microorganisms in poultry at chicken farms.\textsuperscript{26, 27} Other studies have also reported that farmers may be more likely than the general population to acquire antibiotic-resistant bacteria of food-animal origin.\textsuperscript{28, 29} However, the current study did not reveal any direct association between the prevalence of ESBL-producing bacteria and meat consumption or the occupation of the participants. This may be because of the homogeneity in the population of farmers who participated in the study or because most of them only had backyard chickens and not big industrialized farms. Moreover, studies indicate that contamination of the environment by large amounts of antimicrobials and heavy metal pollution may also select for antibiotic resistance in nature and favour the dissemination of antibiotic resistance genes.\textsuperscript{30} Therefore, in order to clarify these issues, environmental data collection should be considered for future studies.

In summary, faecal carriage of ESBL-producing \textit{Enterobacteriaceae} among asymptomatic individuals in Thailand is very high, with some variations in the prevalence among the provinces. Epidemiological analysis suggested that the high prevalence of ESBL-producing \textit{Enterobacteriaceae} may be linked to antibiotic abuse.
Reference

11. Valverde A, Coque TM, Sanchez-Moreno MP et al. Dramatic increase in prevalence of fecal carriage of extended-spectrum beta-lactamase-producing


CHAPTER 3. CTX-M-TYPE ESBL-PRODUCING
ENTEROBACTERIACEAE IN KANCHANABURI PROVINCE, THAILAND

3.1. Background

Recent studies have noted high levels of extended-spectrum β-lactamase (ESBL)-producing gram-negative bacilli in the Asia-Pacific region, particularly in India, China and Thailand.\textsuperscript{1-3} In Thailand, high rates of the ESBL phenotype (26\% of Enterobacteriaceae clinical isolates) were reported as early as 1994–96,\textsuperscript{1} and the CTX-M-type ESBLs were first detected during 1998–99.\textsuperscript{4} The emergence of community-onset infection with ESBL-producing Escherichia coli in Thailand was reported after an analysis of specimens collected in 2003–04.\textsuperscript{5} In 2004–05, the prevalence of ESBL-producing E. coli and Klebsiella pneumoniae causing healthcare-associated infections was 13.2\% and 12.7\%, respectively, and most strains harboured the \textit{bla}_{CTX-M} gene.\textsuperscript{6} ESBL producers continue to be widely disseminated in Thailand, and recent data from 2005–07 showed that the prevalence of ESBL-producing isolates in different clinical specimens had increased to include 30.0\%–40.1\% of E. coli and 27.1\%–39.2\% of K. pneumoniae strains.\textsuperscript{7}

The problem of ESBL production is no longer limited to community-onset or hospital-acquired infections. Faecal carriage of ESBL-producing Enterobacteriaceae, particularly the CTX-M producers, by asymptomatic individuals has been noted in many parts of the world.\textsuperscript{8-12} The highest levels of ESBL were found in the people of Thailand and China.\textsuperscript{13, 14} We previously reported significantly high levels of faecal carriage of CTX-M-type
ESBL-producing *Enterobacteriaceae* in asymptomatic volunteers in three provinces in the northern, southern and central regions of Thailand in 2008–09.\(^{13}\) Among the three provinces, Kanchanaburi province had the highest number of *bla*\(_{\text{CTX-M}}\) carriers,\(^{15}\) and this high number may have been linked to antibiotic abuse.\(^{13}\) Based on our previous findings, we calculated an appropriate sample size for another prevalence study in Kanchanaburi province and developed a more comprehensive questionnaire to include different risk factors.

### 3.2. Objectives

In order to identify factors contributing to the high prevalence of ESBL producers in asymptomatic people, we investigated a possible link between CTX-M ESBL-producing *Enterobacteriaceae* and previous antibiotic use or hospital admittance.

### 3.3. Materials and Methods

**Sample size determination**

The sample size was calculated using OpenEpi open source software\(^{16}\) and Cochran's sample size calculation formula for categorical data.\(^{17}\) The population of Kanchanaburi province was 734394 people, according to the Population and Housing Census 2000 data.\(^{18}\) Our previous study indicated that the prevalence of CTX-M-type ESBL-producing *Enterobacteriaceae* was 50.6\% in the Kanchanaburi province. The data were entered to following equation to calculate the study sample size:

\[
    n = Z^2 \frac{P (1 - P)}{e^2}
\]

- \(Z\) = level of confidence at 95\% (standard value of 1.96)
- \(P\) = estimated prevalence (50.6\% in Kanchanaburi province)
- \(e\) = margin of error at 5\% (standard value of 0.05)
Using the above equation, the initial calculation of the sample size would be:

\[ n = \frac{1.96^2 \times 0.51 \times (1-0.51)}{0.05^2} = 384 \]

Finally, \( n \) is divided by 0.90 to adjust for an anticipated 10% non-response:

\[ n = 384 \div 0.90 = 427 \]

Therefore, the final sample size required was **427**.

**Specimen collection and questionnaires**

The study was conducted in two randomly selected districts of Kanchanaburi province, Thailand: Thong Pha Phum district, which borders Burma; and Tha Maka district, located close to the capital city Bangkok (Figure 3.1). The study volunteers were selected by random door-to-door sampling. A total of 450 people aged > 18 years were approached to participate in the study, of whom 33 people refused.

Single stool samples from 417 adult volunteers from both districts were collected in November 2010 and analysed. Participants were interviewed using a standardized questionnaire, and the following data were recorded for each participant: age; gender; education; employment; number of persons in the household; monthly household income; consumption of raw meat and vegetables; household water supply and toilet conditions; possession of animals; antibiotic usage in the previous year; antibiotic usage in the previous 3 months; visits to a doctor or health officer during the previous year; admission to hospital in the previous year; and invasive procedures during the previous year.

This study was approved by the Ethics Committee of the Faculty of Tropical Medicine, Mahidol University, Thailand. Written informed consent was obtained from each individual participating in the study.
Screening for and confirming the presence of ESBLs

Stool samples were collected using Cary-Blair transport media. The samples were plated on MacConkey agar supplemented with 2 μg/ml cefotaxime (‘CTX-MacConkey’) and incubated at 37°C for 24 h. ESBL expression was confirmed by the disc diffusion method on Mueller–Hinton agar using cefotaxime (30 mg) and ceftazidime (30 mg) with and without clavulanic acid (10 mg), as recommended by the Clinical and Laboratory Standards Institute (CLSI). A ≥ 5-mm increase in a zone diameter for either antimicrobial agent tested in combination with clavulanic acid vs. its zone when tested alone was considered to be a positive test (Figure 3.2). Each set of samples was tested with CLSI quality control strains *E. coli* ATCC 25922 and *K. pneumoniae* ATCC 700603.
Positive isolates were identified using conventional biochemical tests and the API 20E system (Sysmex-bioMerieux, Tokyo, Japan), as described in the previous chapter.

**Antimicrobial susceptibility testing**

All ESBL-producing *Enterobacteriaceae* isolates were tested for susceptibility to imipenem, meropenem, amikacin, gentamicin, ciprofloxacin and tetracycline by the disc diffusion method using an SN Disc (Nissui Pharmaceutical Co., Ltd, Tokyo, Japan) according to the manufacturer's instructions.

**bla$_{CTX-M}$ gene identification and genotyping**

The $bla_{CTX-M}$ genes were identified and genotyped by PCR using DNA extracted by boiling suspensions of positive isolates. DNA samples at a concentration of 0.1 ng/µl were used as PCR templates and $bla_{CTX-M}$ genes were amplified using the universal primers CTX-M-U1 and CTX-M-U2, as described previously. DNA from a reference *E. coli* $bla_{CTX-M}$-positive strain was used as a positive control. For genotyping of the $bla_{CTX-M}$ genes, four primer sets were used to amplify group-specific $bla_{CTX-M}$ genes, as described elsewhere.

Phylogenetic grouping of the identified *E. coli* isolates was determined by triplex PCR, using a combination of two genes (chuA and yjaA) and the DNA fragment TSPE4.C2, as described elsewhere (Table 3.1 and Figure 3.3). PCR products were visualized by 2% agarose gel electrophoresis and staining with GelRed nucleic acid gel stain (Biotium, Hayward, CA, USA).
**Table 3.1.** Primers used for PCR amplification

<table>
<thead>
<tr>
<th>Target(s)</th>
<th>Primer</th>
<th>Sequence (5' - 3' direction)</th>
<th>Product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>chuA</td>
<td>ChuA.1</td>
<td>GAC GAA CCA ACG GTC AGG AT</td>
<td>279</td>
</tr>
<tr>
<td></td>
<td>ChuA.2</td>
<td>TGC CGC CAG TAC CAA AGA CA</td>
<td></td>
</tr>
<tr>
<td>yjaA</td>
<td>YjaA.1</td>
<td>TGA AGT GTC AGG AGA CGC TG</td>
<td>211</td>
</tr>
<tr>
<td></td>
<td>YjaA.2</td>
<td>ATG GAG AAT GCG TTC CTC AAC</td>
<td></td>
</tr>
<tr>
<td>TSPE4.C2</td>
<td>TspE4C2.1</td>
<td>GAG TAA TGT CGG GGC ATT CA</td>
<td>152</td>
</tr>
<tr>
<td></td>
<td>TspE4C2.2</td>
<td>CGC GCC AAC AAA GTA TTAC G</td>
<td></td>
</tr>
</tbody>
</table>

*Appl Environ Microbiol* 66: 4555-8

**Figure 3.3.** Dichotomous decision tree to determine the phylogenetic group of an *E. coli* strain by using the results of PCR amplification of the *chuA* and *yjaA* genes and DNA fragment TSPE4.C2.

**Statistical analysis**

Data were analysed using SPSS version 16 software. Categorical data were compared using the $\chi^2$ test, and continuous data were compared using the Mann–Whitney *U*-test. Univariate and multivariate logistic regression analyses were used to determine risk factors associated with the faecal carriage of ESBL-producing *Enterobacteriaceae*. Factors identified as statistically significant
in the univariate analysis were included in a multivariate logistic regression model, and the results are presented as ORs with 95% CIs. Statistical significance was set at $P < 0.05$.

3.4. Results

In all, we analysed 417 stool samples, 232 of which were collected from Thong Pha Phum district and 185 of which were collected from Tha Maka district. Data on demographics, history of hospitalization, recent visits to doctors or health officers, and antibiotic use are shown in Table 3.2. There were no significant age or gender differences between participants from the two districts. However, participants from Tha Maka district had significantly better education and employment status, and more frequent use of antibiotics.

Table 3.2. Characteristics of the study participants

| Characteristic                                      | Thong Pha Phum n=232 | Tha Maka n=185 | $P$ value
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Median age, years (range)</td>
<td>48 (20–84)</td>
<td>46 (20–85)</td>
<td>0.057</td>
</tr>
<tr>
<td>Male gender</td>
<td>87 (37.5%)</td>
<td>71 (38.4%)</td>
<td>0.854</td>
</tr>
<tr>
<td>No formal education</td>
<td>60 (26.0%)</td>
<td>12 (6.5%)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Unemployed</td>
<td>67 (29.0%)</td>
<td>28 (15.1%)</td>
<td>0.001</td>
</tr>
<tr>
<td>Visited a doctor or health officer within the last year</td>
<td>155 (68.0%)</td>
<td>116 (64.4%)</td>
<td>0.452</td>
</tr>
<tr>
<td>Admitted to a hospital within the last year</td>
<td>98 (42.4%)</td>
<td>62 (34.8%)</td>
<td>0.119</td>
</tr>
<tr>
<td>Was prescribed antibiotics within the last year</td>
<td>145 (63.9%)</td>
<td>140 (76.5%)</td>
<td>0.006</td>
</tr>
<tr>
<td>Used antibiotics within the last year</td>
<td>159 (68.8%)</td>
<td>142 (78.0%)</td>
<td>0.037</td>
</tr>
<tr>
<td>Used antibiotics within the last 3 months</td>
<td>114 (50.0%)</td>
<td>122 (68.2%)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Used antibiotics without a prescription within the last year</td>
<td>120 (53.1%)</td>
<td>116 (64.1%)</td>
<td>0.026</td>
</tr>
</tbody>
</table>

*Calculated by $\chi^2$ or Mann Whitney $U$-tests, as appropriate.
Genotypes of CTX-M ESBL producers

Phenotypic screening and confirmation tests showed that 289 of 417 (69.3%) samples contained ESBL-producing bacteria (Table 3.3). All the 289 isolates were identified from different participants. After PCR analysis, 65.7% (274 of 417) of the isolates were found to be positive for the \( \text{bla}_{\text{CTX-M}} \) gene. The prevalence of CTX-M-type ESBL-producing Enterobacteriaceae was significantly higher in Tha Maka district than in Thong Pha Phum district (P<0.001). Genotyping of \( \text{bla}_{\text{CTX-M}} \) revealed that most CTX-M producers harboured genes belonging to the CTX-M-9 group (60.6%), followed by the CTX-M-1 group (38.7%). Only two (0.7%) of the \( \text{bla}_{\text{CTX-M}} \) gene-positive isolates belonged to the CTX-M-8 group, and no samples belonged to the CTX-M-2 group.

Among the CTX-M ESBL-producing Enterobacteriaceae, 85.4% (234 of 274) were identified as E. coli and 4.7% (13 of 274) were identified as K. pneumoniae. The remainder of the CTX-M-type ESBL producers were Citrobacter, Enterobacter and other bacteria. Phylogenetic group analysis of the E. coli strains showed that the highest proportion (114 of 234, 48.7%) of isolates belonged to phylogenetic group A and the next highest proportion belonged to group B1 (67 of 234, 28.6%) (Table 3.4).

Antibiotic susceptibility

All CTX-M-type ESBL-producing isolates were susceptible or intermediate to imipenem, meropenem and amikacin, except for two isolates: one was resistant to imipenem, meropenem, tetracycline and gentamicin, and the other was resistant to amikacin and tetracycline. Moreover, 88.0% (241 of
Table 3.3. Detection of CTX-M-type ESBL-producing *Enterobacteriaceae*

<table>
<thead>
<tr>
<th>District</th>
<th>Participants</th>
<th>ESBL phenotype&lt;sup&gt;a&lt;/sup&gt;</th>
<th>CTX-M gene&lt;sup&gt;b&lt;/sup&gt;</th>
<th>CTX-M-1&lt;sup&gt;d&lt;/sup&gt;</th>
<th>CTX-M-2&lt;sup&gt;e&lt;/sup&gt;</th>
<th>CTX-M-8&lt;sup&gt;f&lt;/sup&gt;</th>
<th>CTX-M-9&lt;sup&gt;g&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thong Pha Phum</td>
<td>232</td>
<td>148 63.8%</td>
<td>133 57.3%</td>
<td>48 36.1%</td>
<td>0 0.0%</td>
<td>2 1.5%</td>
<td>83 62.4%</td>
</tr>
<tr>
<td>Tha Maka</td>
<td>185</td>
<td>141 76.2%</td>
<td>141 76.2%</td>
<td>58 41.1%</td>
<td>0 0.0%</td>
<td>0 0.0%</td>
<td>83 58.9%</td>
</tr>
<tr>
<td>Total</td>
<td>417</td>
<td>289 69.3%</td>
<td>274 65.7%</td>
<td>106 38.7%</td>
<td>0 0.0%</td>
<td>2 0.7%</td>
<td>166 60.6%</td>
</tr>
</tbody>
</table>

<sup>a</sup>ESBL phenotype was determined according to the Clinical and Laboratory Standards Institute recommendations.

<sup>b</sup>CTX-M gene was determined by PCR.

<sup>c</sup>Genotype of CTX-M genes was determined by PCR.

<sup>d</sup>CTX-M-1 group includes CTX-M-1 and several other variants such as CTX-M-3 and CTX-M-15.

<sup>e</sup>CTX-M-2 group includes CTX-M-2 and several other variants.

<sup>f</sup>CTX-M-8 group includes CTX-M-8.

<sup>g</sup>CTX-M-9 group includes CTX-M-9 and several other variants such as CTX-M-14.
274) of them were resistant to tetracycline, 63.1% (173 of 274) to gentamicin and 19.7% (54 of 274) to ciprofloxacin (Table 3.5). Of CTX-M ESBL-producing bacteria, 56.4% (75 of 133) from Thong Pha Phum district and 70.2% (99 of 141) from Tha Maka district were resistant to two or more of the antibiotics tested. The prevalence of such multidrug-resistant CTX-M ESBL producers was significantly higher in Tha Maka district than in Thong Pha Phum district (P<0.018).

Table 3.4. Phylogenetic groups of the CTX-M ESBL-producing *Escherichia coli* isolates

<table>
<thead>
<tr>
<th>Phylogenetic group</th>
<th>Thong Pha Phum</th>
<th>Tha Maka</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>66 (54.5%)</td>
<td>48 (42.5%)</td>
</tr>
<tr>
<td>B1</td>
<td>34 (28.1%)</td>
<td>33 (29.2%)</td>
</tr>
<tr>
<td>B2</td>
<td>4 (3.3%)</td>
<td>12 (10.6%)</td>
</tr>
<tr>
<td>D</td>
<td>17 (14.1%)</td>
<td>20 (17.7%)</td>
</tr>
<tr>
<td>Total</td>
<td>121 (100.0%)</td>
<td>113 (100.0%)</td>
</tr>
</tbody>
</table>

Table 3.5. Antibiotic susceptibility of CTX-M-type ESBL-producing *Enterobacteriaceae*<sup>a</sup>

<table>
<thead>
<tr>
<th>Antimicrobial agent</th>
<th>Susceptible</th>
<th>Intermediate</th>
<th>Resistant</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n (%)</td>
<td>n (%)</td>
<td>n (%)</td>
</tr>
<tr>
<td>Imipenem</td>
<td>273 (99.6)</td>
<td>0 (0.0)</td>
<td>1 (0.4)</td>
</tr>
<tr>
<td>Meropenem</td>
<td>273 (99.6)</td>
<td>0 (0.0)</td>
<td>1 (0.4)</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>94 (34.3)</td>
<td>7 (2.6)</td>
<td>173 (63.1)</td>
</tr>
<tr>
<td>Amikacin</td>
<td>272 (99.3)</td>
<td>1 (0.4)</td>
<td>1 (0.4)</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>208 (75.9)</td>
<td>12 (4.4)</td>
<td>54 (19.7)</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>33 (12.0)</td>
<td>0 (0.0)</td>
<td>241 (88.0)</td>
</tr>
</tbody>
</table>

<sup>a</sup>Susceptibility results were interpreted according to SN Disc (Nissui Pharmaceutical, Co, Ltd., Tokyo, Japan) zone diameter breakpoints.
Risk factors for faecal carriage

Using univariate logistic regression models, we found that the following factors were associated with a risk of carrying CTX-M-type ESBL-producing Enterobacteriaceae: (i) enrolment in formal education (OR 2.095; 95% CI 1.251–3.508; P<0.004); (ii) a history of hospitalization within the last year (OR 1.550; 95% CI 1.009–2.381; P<0.045); (iii) use of antibiotics within the last year (OR 1.592; 95% CI 1.018–2.381; P<0.041); (iv) use of antibiotics within the last 3 months (OR 1.879; 95% CI 1.241–2.844; P<0.003); and (v) being prescribed antibiotics within the last year (OR 1.569; 95% CI 1.015–2.424; P<0.042). However, the final multivariate logistic regression model identified enrolment in formal education (OR 2.245; 95% CI 1.297–3.884; P<0.004), a history of hospitalization within the last year (OR 1.643; 95% CI 1.036–2.603; P<0.035) and use of antibiotics within the last 3 months (OR 1.883; 95% CI 1.221–2.903; P<0.004) as the only variables independently associated with faecal carriage of CTX-M-type ESBL producers. The three variables significantly predicted carriage of CTX-M ESBL producers (χ²=20.47; df=3; P<0.001).

3.5. Discussions

Genotypes of CTX-M ESBL producers

The results of this study confirmed our previous findings of a high prevalence (29.3%–50.6% faecal carriage\textsuperscript{13}) of CTX-M-type ESBL-producing Enterobacteriaceae among asymptomatic individuals in rural Thailand. The current finding of 65.7% (274 of 417 samples) carriage of CTX-M ESBL producers in Kanchanaburi province shows a rapid spread of CTX-M-type ESBLs compared with our previous finding of 51.3% (82 of 160) prevalence in
Moreover, we observed variations in the prevalence of ESBLs within the Kanchanaburi province: Tha Maka district, which had a better education and employment status and higher use of antibiotics (Table 3.2), also had a significantly higher prevalence of CTX-M ESBL producers than did Thong Pha Phum district (Table 3.3). To the best of our knowledge, these are the highest prevalences of ESBL-producing Enterobacteriaceae that have been reported among healthy subjects in the community to date.

Prevalent identification of ESBLs belonging to the CTX-M-9 (60.6%) and CTX-M-1 (38.7%) groups is consistent with previous findings of community and clinical isolates from Thailand. A recent study in healthy people of China showed 50.5% faecal carriage of E. coli producing ESBLs, mainly of the CTX-M-type, among which also the CTX-M-9 and CTX-M-1 groups were dominant (74.5% and 29.1%, respectively). Other Asian countries may soon follow the same pattern of rapidly spreading CTX-M ESBLs in the community.

In the current study, most ESBL producers were identified as E. coli belonging to phylogenetic groups A and B1. In China, phylogenetic groups D and A were found to be prevalent among ESBL-producing E. coli strains in asymptomatic carriers. A report on the population genetics of E. coli stated that commensal E. coli strains isolated from Asia mainly belonged to phylogenetic groups A and B1, whereas a recent worldwide dissemination of bla genes encoding CTX-M-14 and CTX-M-15 was considered to be driven by epidemic E. coli strains belonging to phylogroup B2 (ST131). However, of the 234 CTX-M ESBL-producing E. coli strains in this study, only 16 isolates belonged to phylogroup B2. After multilocus sequence typing, 11 of these were
identified as strain ST131, with 7 samples belonging to the CTX-M-9 group and 4 to the CTX-M-1 group (data not shown). This indicates that horizontal plasmid transfer has played a more important role than clonal expansion in the current massive community spread of CTX-M-type ESBLs in Asia.

Antibiotic susceptibility

Our findings are similar to those of hospital studies in Thailand, where all ESBL-producing \( E. \ coli \) strains isolated from clinical specimens were susceptible to carbapenems, but 57.8% were resistant to gentamicin, 81.0% were resistant to ciprofloxacin and 89.1% were resistant to tetracycline. However, quinolone resistance was lower (19.7%) in CTX-M ESBL producers isolated from asymptomatic people than in those isolated from clinical samples.

Risk factors for faecal carriage

Our risk factor analyses show that even in the community setting, previous healthcare contact and antibiotic exposure have a major contribution to the faecal carriage of resistant bacteria. A history of hospitalization within the last year and antibiotic use within the last 3 months indicated higher risks of carrying CTX-M ESBL-producing \( Enterobacteriaceae \). However, these factors may be specific to countries where antibiotic management programmes are uncommon and antibiotics can be purchased over the counter without a prescription, which is the case in Thailand.

Previous antibiotic use and a history of hospitalization have been identified as risk factors for community-onset urinary tract infections (UTIs) caused by ESBL-producing bacteria, but have rarely been found to be risk factors for the faecal carriage of ESBL producers. Only one report, to our
knowledge, has found a strong association between the use of antibiotics in the previous 3 months and the rectal carriage of ESBL-producing *E. coli* in elderly people in community settings.\(^{31}\) In contrast, most other studies showed that prior hospitalization or previous use of antimicrobial drugs was irrelevant for the faecal carriage of ESBL-producing *Enterobacteriaceae* in healthy people.\(^{32,33}\) Neither have studies on faecal carriage of patients attending health centres or at admission to hospitals found association with prior antibiotic use.\(^{11,34}\) Thus, although our results regarding risk factors for faecal carriage differ from previous findings, they are similar to factors associated with community-onset UTIs caused by ESBL producers. This similarity may be explained by the fact that faecal carriage of ESBL-producing *E. coli* contributes substantially to the development of UTIs and that almost half of these infections are caused by the same strains found in the faeces of patients before the development of UTIs.\(^{35}\)

Interestingly, we found a significant association between the faecal carriage of CTX-M producers and enrolment of the participants in formal education. Since the study was conducted in rural areas, enrolment in formal education may be a proxy for socio-economic status. Therefore, it may indicate that those who have better socio-economic status can also afford and/or have better access to medical services, including over-the-counter antibiotics.

Furthermore, different factors, such as acquisition of ESBL-producing *E. coli* from food or by person-to-person transmission from faecal carriers, dissemination of ESBL-producing *E. coli* in the environment and carriage by domestic and wild animals, have been proposed to explain the extensive spread of ESBL-producing *E. coli* in community settings.\(^{36}\) However, our
findings did not show any association between the faecal carriage of CTX-M producers and household conditions, food habits or animals possessed by the participants.

Finally, it is important to recognize that the current study was conducted only in one province of Thailand and the findings may not be representative of the whole country. In addition, the study was conducted in community settings among asymptomatic individuals and risk factor analysis for faecal carriage of ESBL producers was therefore based on the participants’ self-reporting, which may have potential for recall bias with regard to factors such as previous antibiotic use. Despite these limitations, our data indicate that implementation of antimicrobial stewardship interventions, such as limitation of over-the-counter antibiotics, prescribing the proper antibiotics for the proper duration, and proper hospital infection control, can assist in the prevention of further spread of resistant bacteria among healthy people in rural communities.

In summary, faecal carriage of CTX-M-type ESBL-producing Enterobacteriaceae among asymptomatic individuals in the Kanchanaburi province of Thailand remains alarmingly high. Public health efforts should focus on educating rural Thai communities and healthcare professionals on the proper use of antibiotics.


CHAPTER 4. ESBL-PRODUCING ENTEROBACTERIACEAE AMONG ASYMPTOMATIC JAPANESE PEOPLE

4.1 Background

*Escherichia coli* strains that produce CTX-M-type β-lactamases have become widely prevalent in the general population and are probably being imported into the hospital community.¹

In Japan, the prevalence of ESBL producers among clinical isolates has shown an increasing trend. In 2000, Komatsu et al.² reported a 0.5% prevalence of ESBL producers in the feces of diarrhea patients. Another review reported prevalences of 0.6, 7.2, 10.0, and 14% in clinical isolates of *E. coli* and *Klebsiella pneumoniae* in Japan in 1990, 1995, 2000, and 2003, respectively.³ A hospital study in southwestern Japan showed that the prevalence of ESBL-producing bacteria increased from 3.6 to 15.2% between 2003 and 2009.⁴ However, little is known about the frequency of ESBL-producing bacteria in healthy Japanese people.

4.2 Objectives

The aim of this study was to investigate the prevalence of the faecal carriage of ESBL-producing *Enterobacteriaceae* among asymptomatic Japanese adults.

4.3 Materials and Methods

Stool samples from adult volunteers in Osaka, Japan, were collected from July 2009 to June 2010. Written informed consent was obtained from each individual who volunteered to participate in the study. We analyzed a total of 218
samples, 1 from each participant.

All participants were asked to fill out a standardized questionnaire including general demographic circumstances, antibiotic usage within 3 months prior to sample collection, and lifetime history of hospitalization.

Stool samples were assessed for the presence of ESBL-producing *Enterobacteriaceae* by phenotypic and genotypic methods. The phenotypic methods included plating the stool samples on MacConkey agar supplemented with 2 µg/ml cefotaxime (CTX-MacConkey). The results were confirmed using cefotaxime and ceftazidime with and without clavulanic acid, as recommended by the Clinical and Laboratory Standards Institute.\(^5\) Isolates were identified using conventional biochemical tests. Tests for susceptibility to imipenem, meropenem, amikacin, gentamicin, ciprofloxacin, norfloxacin, and tetracycline were conducted by the disc diffusion method using an SN Disc (Nissui Pharmaceutical, Tokyo, Japan), as described in previous chapters.

Bacterial DNA was extracted from the isolates by boiling the bacterial suspensions. DNA samples with a concentration of 0.1 ng/µl were used as the templates for polymerase chain reaction (PCR) analysis. The universal primers CTX-M-U1 and CTX-M-U2 were used to detect the *bla*\(_{\text{CTX-M}}\) gene, and the primers *bla*-SHV.SE (5'-ATG CGT TAT ATT CGC CTG TG-3') and *bla*-SHV.AS (5'-TGC TTT GTT ATT CGG GCC AA-3') were used to detect the *bla*\(_{\text{SHV}}\) gene, as described previously.\(^6\) DNA from the reference *E. coli* *bla*\(_{\text{CTX-M}}\)-positive strain was used as the positive control. The PCR products were visualized by 2% agarose gel electrophoresis, followed by staining with GelRed Nucleic Acid Gel Stain (Biotium, Hayward, CA, USA).
DNA sequencing was used for genotyping the \( \text{bla}_{\text{CTX-M}} \) genes. First, the DNA fragment was extracted from agarose gel using QIAEX II Agarose Gel Extraction Kit (QIAGEN, Valencia, CA, USA). Then cycle sequencing was performed using BigDye® Terminator v3.1 Cycle Sequencing Kit and purification was done using BigDye XTerminator® Purification Kit, according to the manufacturer's instructions (Applied Biosystems, Foster, CA, USA). The nucleotide sequences were analyzed with CLUSTALW and BLAST software (http://clustalw.ddbj.nig.ac.jp/top-j.html).

Statistical analysis was performed using SPSS v.16.0. Variables were analyzed by univariate analysis using \( \chi^2 \) or Fisher's exact test, as appropriate. Statistical significance was set at \( P < 0.05 \).

4.4 Results

The characteristics of the study participants are shown in Table 4.1. The participants were aged between 20 and 70 years, with a median age of 41.9 years. Most had not used antibiotics within the 3 months prior to sample collection and most did not have a history of hospitalization.

Of the 218 samples, 65 (29.8%) showed bacterial growth on CTX-MacConkey agar and 14 (6.4%) showed ESBL production (Table 4.2). All the 14 isolates were identified from different participants. On PCR analysis, 92.9% (13 of 14) of the ESBL-positive isolates were positive for the \( \text{bla}_{\text{CTX-M}} \) gene, and 1 sample carried the \( \text{bla}_{\text{SHV}} \) gene. By genotyping of \( \text{bla}_{\text{CTX-M}} \) genes, CTX-M-14 (38.5%) and CTX-M-2 (30.8%) ESBL types were predominantly identified. The other samples harbored the CTX-M-8, CTX-M-3, and CTX-M-15 types (15.4, 7.7, and 7.7%, respectively).
Among the CTX-M ESBL-producing *Enterobacteriaceae*, 84.6% (11 of 13) were identified as *E. coli* and 15.4% (2 of 13) as *K. pneumoniae*. The SHV-type ESBL producer was identified as *E. coli*. We did not identify any participants who harbored both ESBL-producing *K. pneumoniae* and *E. coli* as well as different CTX-M genotypes. Antibiotic susceptibility tests showed that 92.9% (13 of 14) of the ESBL-positive bacteria were susceptible to all the tested antibiotics, except for tetracycline. Five of the 14 ESBL producers were resistant to only tetracycline, and 1 was resistant to gentamicin and quinolones in addition to tetracycline.

Statistical analysis did not reveal any significant association between ESBL production and antibiotic usage or hospitalization experience, although...
<table>
<thead>
<tr>
<th>Participants tested</th>
<th>ESBL phenotype (^a)</th>
<th>CTX-M gene (^b)</th>
<th>Organisms</th>
<th>Genotype of CTX-M genes (^c)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>CTX-M-1 group (^d)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td><em>Escherichia coli</em></td>
<td>11 (84.6%)</td>
</tr>
<tr>
<td></td>
<td>218</td>
<td>14 (6.4%)</td>
<td><em>Klebsiella pneumoniae</em></td>
<td>2 (15.4%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>13 (92.9%)</td>
<td>Total</td>
<td>13 (100%)</td>
</tr>
</tbody>
</table>

\(^a\) ESBL phenotype was determined according to the Clinical and Laboratory Standards Institute recommendations.

\(^b\) The CTX-M gene was determined by polymerase chain reaction (PCR).

\(^c\) The genotype of CTX-M genes was determined by DNA sequencing.

\(^d\) The CTX-M-1 group includes CTX-M-1 and several other variants, such as CTX-M-3 and CTX-M-15.

\(^e\) The CTX-M-2 group includes CTX-M-2 and several other variants.

\(^f\) The CTX-M-8 group includes CTX-M-8.

\(^g\) The CTX-M-9 group includes CTX-M-9 and several other variants, such as CTX-M-14.
these findings may be attributed to the small number of ESBL-positive cases identified in this study.

4.5 Discussions

To the best of our knowledge, this is the first published data regarding the prevalence of ESBL-producing Enterobacteriaceae among healthy Japanese adults. Other countries have reported a 2.4–13.1% prevalence of faecal carriage of ESBL-producing Enterobacteriaceae in healthy people.7-10 This value reached 58.2% in developing countries, such as Thailand, where antibiotics are used without prescription.11 However, in Japan, where antibiotic usage is strictly regulated, the 6.4% prevalence of ESBL-producing Enterobacteriaceae among healthy individuals is relatively high.

The high prevalence (92.9%) of CTX-M-type ESBLs identified in the present study is consistent with the global trend of CTX-M dominance. The prevalence of the CTX-M-14 enzyme belonging to the CTX-M-9 group and the CTX-M-2 enzyme belonging to the CTX-M-2 group was compatible with the findings of previous studies carried out in hospital settings, which have reported that the CTX-M-9, CTX-M-2, and CTX-M-1 groups are the predominant CTX-M-type ESBLs in E. coli in Japan.3, 4, 12, 13 This similarity between the CTX-M-type ESBL groups identified in community and hospital settings may indicate that people who are already carriers of ESBL producers are visiting hospitals. In previous studies of clinical isolates in Japan, ESBL-producing bacteria showed co-resistance to fluoroquinolones.4, 14 However, our data showed that most of the ESBL producers (92.9%) identified from healthy individuals were susceptible to all antibiotics, except tetracycline. Furthermore,
our study did not show any significant association between the faecal carriage of ESBL-positive Enterobacteriaceae and recent antibiotic use or hospitalization, which indicates the existence of other risk factors. Because the study enrolled only a limited number of participants, possible factors that contribute to the prevalence of ESBL-producing bacteria in healthy people in Japan should be investigated in a larger number of participants.

In summary, our results suggest that the prevalence of the faecal carriage of CTX-M-type ESBL producers, mostly E. coli, by healthy Japanese people is rapidly increasing. This may be one of the causes of the dissemination of ESBL-producing bacteria in hospitals. A broader and more detailed study of the prevalence of ESBL producers in the community is needed to prevent potential treatment failures associated with serious infections caused by these bacteria.


CHAPTER 5. ESBL-PRODUCING ENTEROBACTERIACEAE AMONG NURSING HOME RESIDENTS IN JAPAN

5.1 Background

The detection rate of extended-spectrum β-lactamases (ESBLs) in clinical isolates of Escherichia coli and Klebsiella pneumoniae in the Kinki region of midwestern Japan significantly increased from 0.2% to 7.3% and from 0.0% to 2.4%, respectively, during a 10-year period from 2000 to 2009.¹ Our recent study among healthy adults in the same region showed 6.4% prevalence of ESBL producers, most of which were CTX-M-type ESBLs.² The reasons for the increased prevalence of ESBL producers in Japan have not been adequately investigated. In the USA and Europe, nursing homes have been identified as a reservoir of antibiotic-resistant bacteria and possibly play an important role in their emergence and spread.³⁻⁵

5.2 Objectives

Therefore, we studied the prevalence of and risk factors associated with faecal carriage of CTX-M-type ESBL-producing Enterobacteriaceae among the residents of nursing homes.

5.3 Materials and Methods

The study was conducted from March to September 2010 in 3 nursing homes located in midwestern Japan. Nursing home A was a privately owned 150-bed nursing home, whereas nursing homes B and C were 100- and 50-bed institutions, respectively, run by the local government.

We analyzed 225 stool samples from the 3 nursing homes, and 1 sample
was obtained from each resident. Data on resident characteristics, including length of stay in the current nursing home, co-morbidities, and past and present antibiotic use, were collected from resident charts. Detailed information was also collected from resident charts in nursing home A, which had the highest prevalence of ESBL-producing *Enterobacteriaceae* carriers. The information included residents' physical and mental health status, history of invasive procedures within the last 2 years, past and present urinary tract infections, history of stroke, and other health conditions, such as diabetes.

ESBL production was identified by phenotypic and genotypic methods, as described elsewhere. The phenotypic methods included plating the stool samples on MacConkey agar supplemented with 2 µg/ml cefotaxime (CTX-MacConkey). ESBL expression was confirmed using cefotaxime and ceftazidime, with and without clavulanic acid, as recommended by the Clinical and Laboratory Standards Institute (CLSI), and each set of samples was tested with CLSI quality control strains *E. coli* ATCC 25922 and *K. pneumoniae* ATCC 700603. Isolates were identified using conventional biochemical tests. Drug susceptibility tests for imipenem, meropenem, amikacin, gentamicin, ciprofloxacin, norfloxacin, and tetracycline were performed with the disc diffusion method using SN Discs (Nissui Pharmaceutical Co. Ltd., Tokyo, Japan).

Bacterial DNA was extracted from the isolates by boiling the bacterial suspensions. DNA samples with a concentration of 0.1 ng/µl were used as a template for PCR analysis. The universal primers CTX-M-U1 and CTX-M-U2 were used to detect the *bla*<sub>CTX-M</sub> gene, as described previously. DNA from the
reference *E. coli* bla\(_{\text{CTX-M}}\)-positive strain was used as a positive control. For genotyping the *bla\(_{\text{CTX-M}}\)* genes, we used 4 primer sets that amplify group-specific *bla\(_{\text{CTX-M}}\)* genes, as described elsewhere.\(^8\) To identify the *qnrA*, *qnrB*, and *aac(6\(^\prime\))\-lb* genes, PCR analysis was performed on the samples collected from nursing home A, as described elsewhere.\(^9,10\) PCR products were visualized on 2% agarose gel electrophoresis followed by staining with GelRed Nucleic Acid Gel Stain (Biotium, Hayward, CA, USA).

Statistical analysis was performed using SPSS v.16.0. All qualitative variables were analyzed using the \(\chi^2\) or Fisher's exact tests, and quantitative variables were analyzed with the Mann-Whitney *U* test, as appropriate. Factors identified in univariate models as being statistically significant were included in the multivariate logistic regression model and are presented as odds ratios with 95% confidence intervals. Statistical significance was set at *P* < 0.05.

5.4 Results

The characteristics of the 225 study residents from the 3 nursing homes are listed in Table 5.1. The median age of the residents was 85 years (range, 52–100 years). The majority (74.7%) of the residents were women. The median length of stay in the 3 nursing homes varied from 87 to 300 days.

Bacterial growth on CTX-MacConkey agar was observed in 103 of 225 samples (45.8%). The prevalence of CTX-M-type ESBL-producing *Enterobacteriaceae*, as confirmed by phenotypic and genotypic methods, was 22.9% (33 of 144 samples) in nursing home A, 18.8% (9 of 48 samples) in nursing home B, and 6.1% (2 of 33 samples) in nursing home C (Table 5.2). *E. coli* was the predominant CTX-M-type ESBL-producing bacterium among
these isolates (41 of 44 isolates). Genotyping of $\text{bla}_{\text{CTX-M}}$ gene-positive isolates showed that 30 (68.2%), 13 (29.5%), and 1 (2.3%) of the 44 samples belonged to groups CTX-M-9, CTX-M-1, and CTX-M-2, respectively.

**Table 5.1.** Characteristics of the study participants

<table>
<thead>
<tr>
<th>Variable</th>
<th>A</th>
<th>B</th>
<th>C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Capacity</td>
<td>150</td>
<td>100</td>
<td>50</td>
</tr>
<tr>
<td>No. of participants</td>
<td>144</td>
<td>48</td>
<td>33</td>
</tr>
<tr>
<td>Age, years</td>
<td>Median (range)</td>
<td>85.0 (53-100)</td>
<td>86.5 (52-100)</td>
</tr>
<tr>
<td>Gender</td>
<td>Female sex</td>
<td>71.5%</td>
<td>83.3%</td>
</tr>
<tr>
<td>Length of stay, days</td>
<td>Median±SDa</td>
<td>300±345 (2-1490)</td>
<td>105±194 (2-1015)</td>
</tr>
</tbody>
</table>

*aSD, standard deviation

**Table 5.2.** Prevalence of ESBL-producing *Enterobacteriaceae* in nursing homes

<table>
<thead>
<tr>
<th>Variable</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of participants</td>
<td>144</td>
<td>48</td>
<td>33</td>
<td>225</td>
</tr>
<tr>
<td>ESBL-producing <em>Enterobacteriaceae</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(25.7%)</td>
<td>(18.8%)</td>
<td>(9.1%)</td>
<td>(21.7%)</td>
<td></td>
</tr>
<tr>
<td>33</td>
<td>9</td>
<td>2</td>
<td>44</td>
<td></td>
</tr>
<tr>
<td>CTX-M-type ESBL</td>
<td>(89.2%)</td>
<td>(100.0%)</td>
<td>(66.7%)</td>
<td>(89.8%)</td>
</tr>
<tr>
<td>Bacterial species</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>(93.9%)</td>
<td>(88.9%)</td>
<td>(100.0%)</td>
<td>(93.2%)</td>
</tr>
<tr>
<td>(6.0%)</td>
<td>(11.1%)</td>
<td>(0.0%)</td>
<td>(6.8%)</td>
<td></td>
</tr>
</tbody>
</table>

Disc diffusion test for multidrug resistance revealed high levels of co-resistance of CTX-M-type ESBL producers to quinolone antibiotics (33 of 33, 8 of 9, and 1 of 2 isolates in nursing homes A, B, and C, respectively). More than half of these isolates (25 of 42) were resistant to tetracycline in addition to...
quinolones. However, all the 44 identified CTX-M-type ESBL producers were susceptible to carbapenems and amikacin. Only 1 sample was susceptible to all the antibiotics tested, and another sample was resistant to gentamicin and tetracycline only. In nursing home A, where 100% (33 of 33 isolates) quinolone co-resistance was observed, we detected the \( aac(6')-Ib \) gene in 22 of the 33 isolates (66.7%); however, none of the samples carried the \( qnrA \) or \( qnrB \) genes.

Analysis of the data on resident characteristics showed no significant differences in terms of age, gender, or length of stay between carriers and non-carriers of CTX-M-type ESBL producers. Furthermore, carriers and non-carriers of CTX-M-producing bacteria did not differ in the current and past antibiotic use (data not shown). However, univariate analysis of the data collected from the resident charts in nursing home A revealed the following risk factors for CTX-M-type ESBL-producing Enterobacteriaceae carriage: (i) resident's condition that requires the highest level of care, (ii) inability to turn over in bed, (iii) diaper use, (iv) diabetes, (v) urine infection, (vi) history of hospitalization within the last year, and (vii) invasive procedures within the last 2 years (Table 5.3). In multivariate logistic regression analysis, inability to turn over in bed, diabetes, and invasive procedures within the last 2 years were the only variables independently associated with faecal carriage of CTX-M-type ESBL producers.

5.5 Discussions

Our findings show that nursing homes in midwestern Japan have almost 3 times higher prevalence (19.6%) of CTX-M-type ESBL producers than that reported in the previous clinical study and among healthy people in the same
Thus, as predicted, nursing homes may play the important role of a reservoir in the rapid spread of CTX-M-type ESBLs in Japan. However, the prevalence of faecal carriage of ESBL producers in Japanese nursing homes is low compared to the findings in some other countries.

Table 5.3. Univariate and multivariate logistic regression analyses of risk factors associated with CTX-M-type ESBL-producing Enterobacteriaceae carriage in nursing home A

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Univariate analysis</th>
<th>Multivariate analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>OR (95% CI)</td>
<td>P value</td>
</tr>
<tr>
<td>Required care level</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Level 5</td>
<td>3.2 (1.29–7.914)</td>
<td>0.009</td>
</tr>
<tr>
<td>Level 1–4</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Ability to turn over in bed</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Incapable</td>
<td>3.50 (1.56–7.86)</td>
<td>0.002</td>
</tr>
<tr>
<td>Capable</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Use of diapers</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>2.61 (1.14–5.99)</td>
<td>0.021</td>
</tr>
<tr>
<td>No</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Diabetes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>2.78 (1.11–6.99)</td>
<td>0.025</td>
</tr>
<tr>
<td>No</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Past and present urine infection</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>4.13 (1.61–10.56)</td>
<td>0.002</td>
</tr>
<tr>
<td>No</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>History of hospitalization within the last one year</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>3.16 (1.37–7.30)</td>
<td>0.006</td>
</tr>
<tr>
<td>No</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Invasive procedures within the last two years</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>4.73 (2.06–10.85)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>No</td>
<td>1</td>
<td></td>
</tr>
</tbody>
</table>

OR odds ratio, 95% CI 95% confidence interval

*a Required care level is assessed by the authorized professional people according to the Long Term Care system of Japan. Five levels of care are distinguished: Care Level 1 is defined as requiring partial care, whereas Care Level 5 is defined as impossible to live without care.
In Northern Ireland, United Kingdom, 40.5% (119 of 294 samples) of nursing home residents were gut carriers of ESBL-producing *E. coli*, which were also resistant to fluoroquinolones;³ in Bolzano, Italy, 41.4% of 111 residents in a long-term-care facility (LTCF) were colonized with ESBL-producing *E. coli*.¹²

The dominance of CTX-M among the ESBLs in nursing homes is consistent with the global trend. The prevalent identification of *E. coli* (93.2%; 41 of 44 isolates) among the ESBL producers and the CTX-M-9 group dominance (68.2%; 30 of 44 isolates) are similar to the previous findings in Japan, both in clinical settings and among asymptomatic individuals.²,¹³,¹⁴

Among the CTX-M-type ESBL-producing *Enterobacteriaceae* found in nursing homes, 95% (42 of 44 isolates) were co-resistant to quinolone antibiotics. A hospital study in Japan reported that most of the CTX-M-producing isolates were resistant to fluoroquinolones,¹⁴ whereas fluoroquinolone resistance was observed in only 7.1% (1 of 14 isolates) of asymptomatic ESBL carriers in Japan.² However, all the CTX-M-producing *Enterobacteriaceae* isolated at the nursing homes were susceptible to carbapenems and amikacin.

Poor functional status, diabetes, and invasive procedures have also been previously identified as risk factors for ESBL colonization or infection among residents of LTCFs and general patients.⁴,⁵ Not only residents, but also 11.6% of 69 staff members in an LTCF were colonized with ESBL-producing *E. coli*.¹² These may imply contact transmission of CTX-M-type ESBL-producing *Enterobacteriaceae*. Furthermore, among the 3 studied nursing homes, faecal carriage of CTX-M-type ESBL producers was the lowest (6.1%) in the nursing
home C. Infection control measures may be better implemented in the nursing home with fewer residents. Therefore, infection control must be closely monitored in nursing homes to prevent resident-to-resident, staff-to-resident, and vice versa transmission of ESBL producers.

In summary, nursing home residents in Japan have a high prevalence of CTX-M-type ESBL-producing Enterobacteriaceae carriage, with a high level of co-resistance to quinolones. Further monitoring and public health efforts focusing on nursing homes are needed to control the spread of ESBL-producing bacteria in Japan's aging society.
Reference


12. March A, Aschbacher R, Dhanji H et al. Colonization of residents and
staff of a long-term-care facility and adjacent acute-care hospital geriatric unit by multiresistant bacteria. *Clin Microbiol Infect* 2010; **16**: 934-44.


List of publications

Publications included in this thesis


Publications not part of this thesis


Conferences


2. Luvsansharav UO, Hirai I, Nakata A, editors. A 2-year trend of CTX-M beta-lactamase-producing Enterobacteriaceae in asymptomatic Thai
