Analysis of risk factors for a high prevalence of extended-spectrum β -lactamase-producing *Enterobacteriaceae* in asymptomatic individuals in rural Thailand

Ulzii-Orshikh Luvsansharav,¹ Itaru Hirai,^{1,2} Marie Niki,¹ Tadahiro Sasaki,¹ Kiyoko Makimoto,³ Chalit Komalamisra,⁴ Wanna Maipanich,⁴ Teera Kusolsuk,⁴ Surapol Sa-Nguankiat,⁴ Somchit Pubampen⁴ and Yoshimasa Yamamoto^{1,2}

Correspondence

Yoshimasa Yamamoto
yyamamot@sahs.med.osaka-u.ac.jp

¹Department of Bioinformatics, Osaka University Graduate School of Medicine, Osaka, Japan

²International Center for Medical Research and Treatment, Kobe University Graduate School of Medicine, Kobe, Japan

³Department of Nursing, Osaka University Graduate School of Medicine, Osaka, Japan

⁴Department of Helminthology, Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand

The prevalence of and risk factors associated with extended-spectrum β -lactamase (ESBL)-producing micro-organisms have not been well studied in healthy individuals. The aim of this study was to determine this in healthy individuals in Thailand. Stool samples and questionnaires obtained from 445 participants from three provinces in Thailand were analysed. The antimicrobial susceptibility of the isolates was assessed using phenotypic and genotypic methods. PCR analysis was performed to detect and group the *bla*_{CTX-M} genes. The prevalence of CTX-M-type ESBL-producing *Enterobacteriaceae* in the three provinces was as follows: 29.3 % in Nan (43/147), 29.9 % in Nakhon Si Thammarat (43/144) and 50.6 % in Kanchanaburi (78/154) ($P < 0.001$). Of the 445 samples, 33 (7.4 %), 1 (0.2 %) and 127 (28.5 %) isolates belonged to the *bla*_{CTX-M} gene groups I, III and IV, respectively. *Escherichia coli* was the predominant member of the *Enterobacteriaceae* producing CTX-M-type ESBLs (40/43, 39/43 and 70/78 isolates in Nan, Nakhon Si Thammarat and Kanchanaburi, respectively). 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. Thus, this study revealed that faecal carriage of ESBL-producing *Enterobacteriaceae* is very high in asymptomatic individuals in Thailand, with some variations among the provinces. This high prevalence may be linked to antibiotic abuse.

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INTRODUCTION

Multidrug-resistant *Enterobacteriaceae* that produce extended-spectrum β -lactamases (ESBLs) have spread rapidly worldwide causing treatment failure and hindering infection control efforts (Paterson & Bonomo, 2005; Pitout & Laupland, 2008). ESBLs are β -lactamases capable of inducing resistance to penicillins and to first-, second- and third-generation cephalosporins and monobactams but not to cephamycins and carbapenems (Paterson & Bonomo, 2005; Pitout & Laupland, 2008; Rodríguez-Baño & Pascual, 2008). Furthermore, 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 (Roberts *et al.*, 2009).

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 (Pitout & Laupland, 2008). These *E. coli* strains cause community-acquired urinary tract and bloodstream infections (Ben-Ami *et al.*, 2009; Rodríguez-Baño *et al.*, 2006). The prevalence of and risk factors associated with the carriage of ESBL-producing micro-organisms have often been studied in patients with these infections. This prevalence varies among countries, and risk

Abbreviation: ESBL, extended-spectrum β -lactamase.

factor analyses have shown conflicting and varying results (Ben-Ami *et al.*, 2009; Coque *et al.*, 2008; Lautenbach *et al.*, 2001; Paterson & Bonomo, 2005). 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 (Kader *et al.*, 2007; Moubareck *et al.*, 2005; Tian *et al.*, 2008; Valverde *et al.*, 2004). However, in a previous study by us, we observed a surprisingly high prevalence of CTX-M-type ESBL-producing *Enterobacteriaceae* (82/160 samples) in asymptomatic individuals in Kanchanaburi province, Thailand (Sasaki *et al.*, 2010). Therefore, 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*.

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 in Kanchanaburi province, Thailand, were also used in this study (Sasaki *et al.*, 2010). 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 (Fig. 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.

Questionnaire. All participants were interviewed using a standardized questionnaire based on general demographic and socio-economic 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*.

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 cefotaxime ml^{-1} within 24 h after collection. Subsequently, the results were confirmed by a double-disc synergy test using amoxicillin–clavulanate and cefotaxime, ceftazidime, cefepime and aztreonam, as described previously (Drieux *et al.*, 2008; Sasaki *et al.*, 2010). Isolates were identified using conventional biochemical tests.

Fig. 1. Location of the Thai provinces selected for the study.

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 $\text{ng } \mu\text{l}^{-1}$ were used as template for PCR analysis. The universal primers CTX-M-U1 (5'-ATGTGCAGYACCA-GTAARGTKATGGC-3') and CTX-M-U2 (5'-TGGGTRAARTARGTS-ACCAGAAYCAGCGG-3') were used to detect the *bla*_{CTX-M} gene, as described previously (Monstein *et al.*, 2007). DNA from the reference *E. coli* *bla*_{CTX-M}-positive strain was used as a positive control. For genotyping the *bla*_{CTX-M} genes, we used four primer sets that amplify group-specific *bla*_{CTX-M} genes, as described elsewhere (Pitout *et al.*, 2004): group I, 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; group II, CTX-M-2, CTX-M-4–CTX-M-7, CTX-M-20 and Toho-1; group III, CTX-M-8; and group IV, CTX-M-9, CTX-M-13, CTX-M-14, CTX-M-16–CTX-M-19, CTX-M-21, CTX-M-27 and Toho-2. The PCR products were visualized by 2% agarose gel electrophoresis followed by staining with GelRed Nucleic Acid Gel Stain (Biotium).

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 χ^2 test or Fisher's exact test, as appropriate. Statistical significance was set at $P < 0.05$.

RESULTS

The characteristics of the 445 study participants from Nan, Nakhon Si Thammarat and Kanchanaburi provinces in Thailand are listed in Table 1. The median age of the study

Table 1. Characteristics of the study participants

Missing data were excluded from the analysis.

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

*Subjects who drink alcoholic beverages one or more times a month.

†Current smoking includes both daily and non-daily or occasional smoking.

‡Subjects who consume meat one or more times a week.

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 per cent of the participants had received primary school education, whilst 26 % had not received any formal education.

The double-disc synergy test revealed that 32.0 % (47/147 samples), 32.6 % (47/144 samples) and 53.9 % (83/154 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

(43/147 samples), 29.9 % in the Nakhon Si Thammarat province (43/144 samples) and 50.6 % in the Kanchanaburi province (78/154 samples) (Fig. 2). The prevalence in Kanchanaburi was significantly higher than that in the other two provinces ($P < 0.001$). Genotyping of *bla*_{CTX-M} gene-positive isolates revealed that 33 (7.4 %), 1 (0.2 %) and 127 (28.5 %) of the 445 samples belonged to groups I, III and IV, respectively (Fig. 2). Three of the isolates did not belong to any of the groups tested. 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 (40/43 isolates in Nan,

Fig. 2. Prevalence of CTX-M-type ESBL-producing *Enterobacteriaceae* and *bla*_{CTX-M} gene groups. * $P < 0.001$; prevalence was compared between the provinces using a χ^2 test.

Fig. 3. Bacterial identification of CTX-M-type ESBL-producing *Enterobacteriaceae*.

39/43 isolates in Nakhon Si Thammarat and 70/78 isolates in Kanchanaburi) (Fig. 3). The other CTX-M-type ESBL-producing bacterial species isolated were *Citrobacter*, *Klebsiella* and *Enterobacter*.

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).

DISCUSSION

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 (Kader *et al.*, 2007; Moubareck *et al.*, 2005; Tian *et al.*, 2008; Valverde *et al.*, 2004). A recent publication by Lo *et al.* (2010) reported 50.3 % faecal carriage of ESBL-producing bacteria in household adults of children admitted to a paediatric department with mild febrile illness. 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; Hawser *et al.*, 2009; Kiratisin *et al.*, 2008b). Lactamases belonging to CTX-M group I (20 %) and IV (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 (group IV) and CTX-M-15 (group I) were the ESBLs mainly responsible for resistance in *E. coli* (Kiratisin *et al.*, 2008a; Niumsup *et al.*, 2008). 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

Table 2. Comparison of antibiotic usage in the three provinces during the last year

Variable	Province			P value*
	Nan (n=147)	Nakhon Si Thammarat (n=144)	Kanchanaburi (n=154)†	
Antibiotic usage				<0.001
Yes	65 (44.2 %)	94 (65.3 %)	104 (67.5 %)	
No	81 (55.1 %)	48 (33.3 %)	44 (28.6 %)	
Unknown or missing	1 (0.7 %)	2 (1.4 %)	6 (3.9 %)	
Used and purchased antibiotics				0.006
Without a prescription	34 (23.1 %)	34 (23.6 %)	62 (40.3 %)	
With a prescription	30 (20.4 %)	56 (38.9 %)	40 (26.0 %)	
Unknown or missing	1 (0.7 %)	4 (2.8 %)	2 (1.3 %)	

*Calculated by χ^2 test. Missing or unknown data were excluded from the χ^2 test.

†Data for Kanchanaburi province were obtained from a previous study conducted by Sasaki *et al.* (2010).

and high consumption of antibiotics may be a risk factor for the high prevalence of ESBL-producing *Enterobacteriaceae*. In this regard, previous studies have reported correlations between antimicrobial resistance and antibiotic usage (Goossens *et al.*, 2005; Tian *et al.*, 2008). 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 (Bailar & Travers, 2002; Dutil *et al.*, 2010; Hawkey & Jones, 2009; Larson, 2007). Recent studies have revealed an increased prevalence of ESBL-producing micro-organisms in poultry at chicken farms (Briñas *et al.*, 2005; Yuan *et al.*, 2009). Other studies have also reported that farmers may be more likely than the general population to acquire antibiotic-resistant bacteria of food-animal origin (Aubry-Damon *et al.*, 2004; Swartz, 2002). 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 (Martínez, 2008). Therefore, in order to clarify these issues, environmental data collection should be considered for future studies.

In summary, faecal carriage of ESBL-producing *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 *Enterobacteriaceae* may be linked to antibiotic abuse.

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