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Prevalence and risk factors for intestinal carriage of CTX-M-type ESBLs in Enterobacteriaceae from a Thai community

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ABSTRACT

The incidence of infections caused by antimicrobial-resistant Enterobacteriaceae in Thailand is increasing and human intestinal flora is an important reservoir for these organisms. This study was carried out to determine the intestinal carriage of bla CTX-M extended spectrum β -lactamase-positive Enterobacteriaceae (ESBL + E) and AmpC-positive Enterobacteriaceae in a community setting in Northern Thailand, and to identify potential risk factors for carriage. A total of 307 fecal samples were collected from healthy volunteers in Phitsanulok province, and cefotaxime-resistant Enterobacteriaceae (CtxRE) were isolated using selective media. Polymerase chain reaction (PCR) was used to detect ESBL and AmpC genes. Risk factors were analyzed using multiple logistic regression. Genotyping was performed by multilocus sequence typing (MLST) analysis. Two hundred ninety-one CtxRE isolates were obtained and *Escherichia coli* was the predominant organism (66.3%). The intestinal carriage rates of blaCTX-M ESBL + E and AmpC-positive Enterobacteriaceae were 52.1% and 6.2%, respectively. Comparative levels of bla CTX-M group 1 and bla CTX-M group 9 were found while bla CMY-2 was the predominant genotype among AmpC genes. Co-existence of two β -lactamase genes in a single isolate was found in 6.5% of isolates. Consumption of under-cooked meat was strongly associated with intestinal carriage of bla CTX-M ESBL + E ($p = 0.003$, OR = 2.133, 95% CI = 1.289–3.530). Phylogenetic grouping and MLST analysis of *E. coli* isolates revealed the presence of *E. coli* B2-ST131 ($n = 8$). Of these, seven carried bla CTX-M-group 9 and 1 carried blaCMY-2. Our results suggest that residents in Thailand are at high risk for developing endogenous infections caused by antibiotic-resistant Enterobacteriaceae.

INTRODUCTION

The incidence of multidrug-resistant Enterobacteriaceae (MDRE) is increasing rapidly and has now become a global threat [1]. Treatment of diseases caused by MDRE is far more challenging where treatment options are becoming limited. In particular, resistance to expanded-spectrum cephalosporins, monobactam and carbapenems among Enterobacteriaceae is increasingly reported and is usually mediated by the production of extended-spectrum β -lactamase (ESBL), AmpC and carbapenemase [1].

Due to the fact that most ESBL-, AmpC- and carbapenemase-encoding genes reside on mobile genetic elements (MGEs), i.e. plasmids and transposons, they are readily transferrable

among Enterobacteriaceae. Furthermore, MGEs invariably carry genes encoding for resistance to other classes of antibiotics, such as aminoglycosides and fluoroquinolones, hence facilitating the emergence and spread of MDRE^[1]. MDRE are now widespread in hospital settings, communities and the environment^[2]. The human intestine is increasingly recognized as an important reservoir for MDRE and thus the human-to-human transmission and dissemination of MDRE in communities can occur where living conditions have poor sanitation^[3]. Furthermore, the acquisition of MDRE in healthy individuals traveling to endemic regions have been documented, further enhancing the international spread of MDR organisms^[3].

Previous studies have shown that intestinal carriage by ESBL-positive Enterobacteriaceae (ESBL + E) has been associated with developing infection due to ESBL producers^[4, 5]. Many studies have reported the fecal carriage of ESBL + E among healthy populations^[6, 7, 8, 9]. Moreover, the high carriage rates of ESBL + E (53.9–75.5%) have been reported among healthy residents in central Thailand^[10, 11, 12, 13]. However, these rates were slightly lower (32%) in Northern and Southern parts of Thailand^[11].

Since the incidence of infections due to ESBL + E and AmpC-positive Enterobacteriaceae in Thailand has continuously increased^[14], this study was carried out to gain a better understanding of the intestinal carriage of ESBL + E and identify risk factors for ESBL + E carriage in a community setting in Northern Thailand. Furthermore, this study was extended to include the carriage rate of AmpC-positive Enterobacteriaceae. In addition, characterization of resistant genes and molecular analysis of resistant isolates were investigated.

MATERIAL AND METHODS

Study setting and ethical approval

This prospective cohort study was conducted on healthy volunteers in Wang Thong District which is located in the rural area of Phitsanulok province, Northern Thailand. All subjects were at least 15 years of age. Participants who had diarrheal diseases within the previous 1 month were excluded from the study. Participant recruitment and sample collection were assisted by local public health personnel. This study was approved by the Naresuan University Institutional Review Board (COA no. 49/2013). Written informed consent was obtained from each subject participating in the study. For the subjects who were under 18 years old, consent was provided by their parent or guardian.

Sample collection and questionnaires

Fresh fecal sampling was performed between October and December 2013. A total of 307 healthy volunteers participated in this study. Fecal samples were collected by subjects using sterile stool containers with a sterile plastic spoon. One sample was obtained from each participant. Microbiological analysis was carried out within 24 h.

Volunteers were asked to fill in a comprehensive questionnaire to collect information regarding demographic data (age, gender, education, occupation, etc.), consumption of undercooked meat and fresh vegetables, type of drinking water, previous antibiotic use, and history of hospitalization.

Isolation and identification of cefotaxime-resistant Enterobacteriaceae

The fecal samples were directly cultured on Chrome UTI agar (Oxoid Ltd., Basingstoke, Hampshire, UK) supplemented with 25 mg/L vancomycin and 1 mg/L cefotaxime (Sigma

Aldrich, St. Louis, MO, USA) and incubated under aerobic condition for 24 h at 37 °C. Colonies of different morphology were selected and subcultured onto EMB agar for further studies. Species identification was performed by using RapID™ ONE System (REMEL Inc., Lenexa, KS, USA) according to the manufacturer's instruction. Isolates showing ambiguous results were further identified by sequencing of the 16S rRNA gene.

Antimicrobial susceptibility testing and ESBL production

All isolates were subjected to susceptibility testing against 12 antimicrobial agents (amoxicillin/clavulanic acid, ceftazidime, ceftazidime, cefepime, aztreonam, imipenem, meropenem, gentamicin, amikacin, ciprofloxacin, doxycycline and trimethoprim-sulfamethoxazole) by disk diffusion method according to Clinical Laboratory Standards Institute (CLSI) protocols and the results were interpreted according to CLSI criteria [15]. An isolate was defined as MDR if it was resistant to at least three or more antimicrobial classes [16].

Screening for ESBL production was performed by combination disk method according to CLSI guidelines using cefotaxime and ceftazidime alone or in combination with clavulanic acid [15].

Determination of genes encoding for CTX-M, AmpC and carbapenemase by PCR and sequencing

The presence of genes encoding for CTX-M, AmpC and carbapenemase was performed by multiplex PCR using previously published primers and conditions [17, 18, 19]. An individual colony was suspended in 1 mL of water and used as template in PCR. Amplification was performed in a total volume of 20 µL containing 1 µL of template DNA, 1X PCR buffer, 1.5 mM MgCl₂, 0.2 mM dNTPs and 0.5 µM of each primer and 1 U of *Taq* polymerase (Vivantis Technologies, Selangor, Malaysia). DNA amplification was performed in a Veriti® Thermal Cycler (Applied Biosystems, CA, USA). PCR products were analyzed by agarose gel electrophoresis.

Selected PCR products were purified using a DNA purification kit (GF-1 Nucleic Acid Extraction Kit; Vivantis Inc., Chino, CA, USA) and were sent to a commercial facility for sequencing (First BASE Laboratories Sdn Bhd, Selangor, Malaysia). Sequences were compared with those available in the GenBank database using the BLAST algorithm available on the National Center for Biotechnology Information (NCBI) website.

Phylogenetic grouping and multilocus sequence typing (MLST) analysis

Phylogenetic grouping (A, B1, B2 and D) of *Escherichia coli* was performed by a multiplex PCR assay for *chuA*, *yjaA* and DNA fragment TspE4C2 as previously described [20]. MLST was performed by amplification and sequencing of seven housekeeping genes (*adhA*, *fumC*, *gyrB*, *icd*, *mdh*, *purA* and *recA*) according to the protocols from the *E. coli* MLST website

Repetitive palindromic polymerase chain reaction (rep-PCR)

Selected *E. coli* isolates were typed by rep-PCR as described previously [21]. The PCR reaction mixture consisted of 1 µL template DNA, 1X PCR buffer, 0.2 mM dNTPs, 0.5 µM of each primer and 1 U of *Taq* polymerase, in a total volume of 25 µL. DNA banding patterns were assessed by visual inspection.

Statistical analysis

Data were collected and analyzed using SPSS version 17.0 (SPSS, Chicago, IL, USA). Univariate analysis was performed using chi-square for categorical variables. Factors that were statistically significant in univariate analysis were included in multivariate logistic

regression analysis to determine risk factors associated with intestinal carriage of *bla*_{CTX-M} ESBL + E. The results were presented as odds ratios (OR) with 95% confidence intervals. A *p* value <0.05 was considered a significant difference.

RESULTS

Characteristics of participants

A total of 307 healthy volunteers were enrolled in this study. Volunteer demographics are shown in Table 1. The median age of participants was 51 years (range, 15–88). The majority of them were female, farmers and educated from primary school.

Isolation of cefotaxime-resistant Enterobacteriaceae from volunteers, antimicrobial susceptibility testing and ESBL production

From 307 fecal samples, cefotaxime-resistant Enterobacteriaceae (CtxRE) isolates were detected in 227 samples. One hundred sixty-three subjects carried one species while 64 subjects carried two species of CtxRE resulting in a total of 291 CtxRE isolates. Species identification showed that the majority of isolates were *Escherichia coli* (66.3%, 193/291) followed by *Enterobacter cloacae* (14.8%, 43/291) and *Klebsiella pneumoniae* (10.3%, 30/291). We also recovered *Citrobacter* spp. (3.4%), *Enterobacter* spp. (2.1%), *Klebsiella* spp. (1.4%), *Pantoea* spp. (0.7%), *Kluyvera* spp. (0.7%) and *Shigella* spp. (0.3%).

The 291 CtxRE isolates showed additional resistance to cefepime (61.9%), aztreonam (51.2%), trimethoprim/sulfamethoxazole (48.5%), ceftazidime (43.3%) and gentamicin (42.6%) (Fig. 1). A total of 10.7% of isolates were resistant to imipenem. In addition, 138 isolates (47.4%, 138/291) were considered as MDR [16]. Phenotypic analysis showed that 58.6% (180/307, 95% CI = 52.9%–64.2%) of volunteers carried ESBL + E in their intestinal tract.

Detection of genes encoding for CTX-M, AmpC and carbapenemase

Of the 291 CtxRE, 169 isolates from 160 persons were *bla*_{CTX-M} ESBL + E by phenotypic (combination disk method) and genotypic characterization (PCR and sequencing). Hence the prevalence of *bla*_{CTX-M} ESBL + E in healthy volunteers was 52.1% (160/307, 95% CI = 46.7–58.1%).

Genotyping of the *bla*_{CTX-M}-positive isolates revealed that *bla*_{CTX-M group 1} and *bla*_{CTX-M group 9} were found in 51.5% (87/169) and 57.4% (97/169) of isolates, respectively. Among these, 10.1% (17/169) of isolates carried both *bla*_{CTX-M group 1} and *bla*_{CTX-M group 9}. In addition, two *Klebsiella* spp. isolates carried *bla*_{CTX-M group 8} (Table 2).

The presence of AmpC- and carbapenemase-encoding genes was investigated. Twenty-one isolates (mostly *E. coli*) from 19 subjects carried AmpC genes: 17, 3 and 1 carried *bla*_{CMY-2}, *bla*_{DHA-1} and *bla*_{MIR}, respectively (Table 2). Therefore, the prevalence of AmpC-positive Enterobacteriaceae in healthy volunteers was 6.2% (19/307). The genes encoding for AmpC were found either alone or in combination with *bla*_{CTX-M}. No carbapenemase genes (*bla*_{IMP}, *bla*_{VIM}, *bla*_{NDM}, *bla*_{KPC} and *bla*_{OXA-48}) were detected.

Risk factors for intestinal carriage of bla CTX-M ESBL + E

Using univariate analysis, no significant differences in terms of age, gender, education, occupation, drinking tap water, consumption of fresh vegetables and history of hospitalization were associated with the carriage of *bla*_{CTX-M} ESBL + E (Table 1). In contrast, consumption of undercooked meat and previous antibiotic use were significantly

associated with *bla* CTX-MESBL + E carriage ($p < 0.05$). Accordingly, these two variables were included in multivariate logistic regression analysis. The result showed that consumption of undercooked meat was the only independent risk factor associated with *bla* CTX-M ESBL + E colonization ($p = 0.003$, OR = 2.133, 95% CI = 1.289–3.530) (Table 3).

Phylogenetic grouping, MLST analysis and rep-PCR

Phylogenetic grouping was investigated for all 193 *E. coli* isolates. The results showed that the predominant groups were A (106 isolates, 54.9%), followed by groups B1 and D (each group comprised 39 isolates, 20.2%) and B2 (9 isolates, 4.7%). Nine B2 isolates were identified as ST131 ($n = 8$) and ST95 ($n = 1$). The carriage rate of *E. coli* B2-ST131 among healthy volunteers was 2.6% (8/307). We also observed that five isolates of *E. coli* ST131 showed identical rep-PCR pattern.

DISCUSSION

In this study, the intestinal carriage of antibiotic-resistant Enterobacteriaceae in a community setting in Phitsanulok province, Northern Thailand was investigated. Our results showed a large number of CtxRE isolates in asymptomatic carriers. Several species were identified; however, the most common species was *E. coli* followed by *E. cloacae* and *K. pneumoniae*. In addition, some of these isolates showed resistance to other classes of broad-spectrum antibiotics, including cefepime, ceftazidime, aztreonam, gentamicin, and trimethoprim/sulfamethoxazole. Furthermore, nearly half (47.4%) of isolates were classified as MDR [16].

Several reports on the intestinal carriage of ESBL + E in community settings have been documented. Studies in the European countries such as the Netherlands and Norway revealed a small number (<10%) of ESBL + E in healthy subjects [7, 9]. The relatively low carriage rates of ESBL + E among healthy populations were reported from Asia, including Japan [22] and Korea [23]. Our results showed the high prevalence of intestinal carriage of *bla* CTX-M ESBL + E (52.1%) in the community. These results were consistent with those reported from China [8], Southeast Asian countries, e.g. Laos and Vietnam [6], as well as central Thailand [10, 11, 12], although there are some differences in methodologies or number of subjects who participated in each study. Our results confirmed previous observations that Thailand is a highly endemic area for *bla* CTX-M ESBL + E carriage within the community [3]. Previous reports from Thailand showed that *bla* CTX-M group 9 was the predominant ESBL-gene found in ESBL + E among healthy volunteers [10, 11, 12]. In this study we found the comparative level of *bla* CTX-M group 1 (51.5%) and *bla* CTX-M group 9 (57.4%) in ESBL + E, suggesting a shift in the prevalence of *bla* CTX-M genotypes. The *bla* CTX-M group 8, which is uncommon in Thailand, was also found at a low frequency.

Several factors have been shown to be associated with intestinal carriage of *bla* CTX-M ESBL + E in healthy volunteers, including education status, previous antibiotic usage, history of hospitalization and foreign travel [3, 8, 12]. In our study, consumption of undercooked meat was strongly identified as a risk factor associated with *bla* CTX-M ESBL + E colonization ($p = 0.003$) suggesting that carriage of *bla* CTX-M ESBL + E is driven from meat or farming sectors. A possible explanation could be due to fact that *bla* CTX-M ESBL + E is frequently found in food-producing animals and their role as major reservoirs for ESBL + E has been well documented [24]. In addition, several studies have shown the presence of ESBL + E, including those containing *bla* CTX-M, in several types of meat and food-producing animals in Thailand [13, 24, 25]. Therefore the possibility exists that transfer of ESBL + E to human intestinal flora could occur by consumption of undercooked meat.

In contrast to ESBL, data on the prevalence of AmpC-positive Enterobacteriaceae, to date, in community settings are limited although their presence in clinical isolates, environments and animals have been reported [26]. Our study is the first investigating AmpC-positive Enterobacteriaceae carriage in Thailand, the prevalence of which was 6.2%. This value is higher than that previously reported from European countries such as Spain and the Netherlands (<1%) [7, 27]. Our molecular studies of AmpC-encoding genes showed that *bla*_{CMY-2} is predominant, consistent with the study conducted in a Thai hospital [28]. Risk factors associated with the carriage of AmpC-positive Enterobacteriaceae could not be identified, probably due to the small number of carriage rate. However, our results suggested that the AmpC-producers have spread beyond hospital settings in Thailand, as seen in other studies elsewhere [7, 9]. In addition, the co-existence of two β -lactamase genes in a single isolate were found in 6.5% of isolates (19/291) (Table 2). This may enhance the level of resistance or render an organism resistant to more β -lactams. Furthermore, *bla*_{CTX-M} and *bla*_{AmpC} were not detected in some CtxRE isolates. Resistance in these isolates may be due to the presence of other enzymes such as inhibitor-resistant β -lactamases or porin alteration [1].

No carbapenemase-encoding genes were found in our study despite the fact that 10.7% of isolates showed resistance to imipenem (Figure 1). These results were similar to previous findings in the Netherlands and Switzerland [7, 29]. Although the mechanisms of carbapenem resistance remain unclear, the findings of carbapenem-resistant isolates among healthy volunteers are of concern since carbapenems are antibiotic of choice for treatment of infections caused by ESBL + E. Further experiments are underway to investigate the mechanism of carbapenem resistance in these isolates.

Our study showed that eight isolates of *E. coli* belonged to the highly pathogenic group B2-ST131. Of the eight *E. coli* ST131 isolates, seven were ESBL-producers, carried *bla*_{CTX-M-group 9} and showed ciprofloxacin resistance. The other isolate was a non-ESBL-producer, possessed *bla*_{CMY-2} and was ciprofloxacin susceptible. Recently, *E. coli* ST131 has been found in different origins (animals, environments and humans) and has been reported to cause community and hospital infections in several countries including Thailand [30, 31]. Transmission of *E. coli* ST131 among members living in the same household has been reported [32]; nevertheless, little is known about the intestinal carriage of *E. coli* ST131 among healthy subjects. Previous studies in Australia, China and France revealed that 4–7% of healthy subjects carried *E. coli* ST131 in their digestive tracts [33, 34]. In this study, we found the low carriage rate (2.6%) of *E. coli* ST131 among healthy volunteers. Moreover, we observed that five *bla*_{CTX-M-group 9}-positive ST131 isolates, which were recovered from different participants from different families, showed identical rep-PCR pattern suggesting successful clonal spread of ST131 within the community.

In conclusion, our data indicate a high rate of intestinal carriage of *bla*_{CTX-M} ESBL + E in a Thai community, and that carriage is strongly associated with the consumption of under-cooked meat. The key concern from these findings is the *E. coli* B2-ST131 carrying either *bla*_{CTX-M} or *bla*_{CMY-2}. Our results suggest that residents in Thailand are at high risk for developing infections caused by antibiotic-resistant Enterobacteriaceae. The data from this study have profound ramifications for public health and biosecurity containment in the Thai environment. Accordingly, practices such as sanitation, domestic hygiene, and infection control policies need to be enforced to prevent the spread of antibiotic-resistant human pathogens within communities.

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Table 1Demographic indices related to the intestinal colonization by *bla*_{CTX-M} ESBL-positive Enterobacteriaceae among healthy volunteers

<u>Variables</u>	<u>Number of cases, n (%)</u> <u>Total N = 307</u>	<u><i>Bla</i>_{CTX-M} ESBL + E carrier</u> <u>Number of cases, n (%) N = 161</u>	<u>non <i>bla</i>_{CTX-M} ESBL + E carrier</u> <u>Number of cases, n (%) N = 146</u>	<u>Univariate analysis</u>	
				<u>pvalue</u>	<u>Odds ratio (95% CI)</u>
Age (>50 years)	151 (49.2)	82 (50.9)	69 (47.3)	0.394	1.224 (0.769–1.947)
Female gender	188 (61.2)	102 (63.4)	86 (58.9)	0.426	0.829 (0.522–1.316)
Primary school education	195 (63.5)	105 (65.2)	90 (61.6)	0.313	0.783 (0.491–1.257)
Farmers	186 (60.6)	102 (63.4)	84 (57.5)	0.299	0.768 (0.466–1.264)
Drinking tap water ^b	142 (46.3)	72 (44.7)	70 (47.9)	0.524	0.863 (0.549–1.357)
Consumption of undercooked meat ^c	141 (45.9)	87 (54.0)	54 (37.0)	0.004 ^a	1.961 (1.231–3.124)
Consumption of fresh vegetables ^d	288 (93.8)	154 (95.7)	134 (91.8)	0.549	1.724 (0.284–10.472)
History of hospitalization within previous 6 months	31 (10.1)	14 (8.7)	17 (11.6)	0.354	0.702 (0.332–1.486)
Antibiotic usage within previous 3 months	100 (32.6)	62 (38.5)	38 (26.0)	0.046 ^a	1.672 (1.007–2.775)

^aStatistically significant ($p < 0.05$)^bVolunteers who drink tap water daily^cVolunteers who occasionally or regularly consume undercooked meat^dVolunteers who occasionally or regularly consume fresh vegetables

Table 2Distribution of β -lactamase genes in cefotaxime-resistant Enterobacteriaceae among healthy volunteers

<u>β-lactamase genes</u>	<u>Enterobacteriaceae (n)</u>
<i>bla</i> CTX-M group 1 (n = 70)	<i>E. coli</i> (61), <i>E. cloacae</i> (5), <i>K. pneumoniae</i> (4)
<i>bla</i> CTX-M group 8 (n = 2)	<i>K. pneumoniae</i> (1), <i>Klebsiella</i> spp. (1)
<i>bla</i> CTX-M group 9 (n = 78)	<i>E. coli</i> (73), <i>K. pneumoniae</i> (4), <i>Klebsiella</i> spp.(1)
<i>bla</i> CMY-2 (n = 15)	<i>E. coli</i> (14), <i>K. pneumoniae</i> (1)
<i>bla</i> DHA-1 (n = 3)	<i>E. coli</i> (1), <i>Klebsiella</i> spp. (1), <i>Citrobacter</i> spp. (1)
<i>bla</i> MIR (n = 1)	<i>E. cloacae</i> (1)
<i>bla</i> CTX-M group 1 + <i>bla</i> CTX-M group 9 (n = 17)	<i>E. coli</i> (17)
<i>bla</i> CTX-M group 9 + <i>bla</i> CMY-2 (n = 2)	<i>E. coli</i> (2)

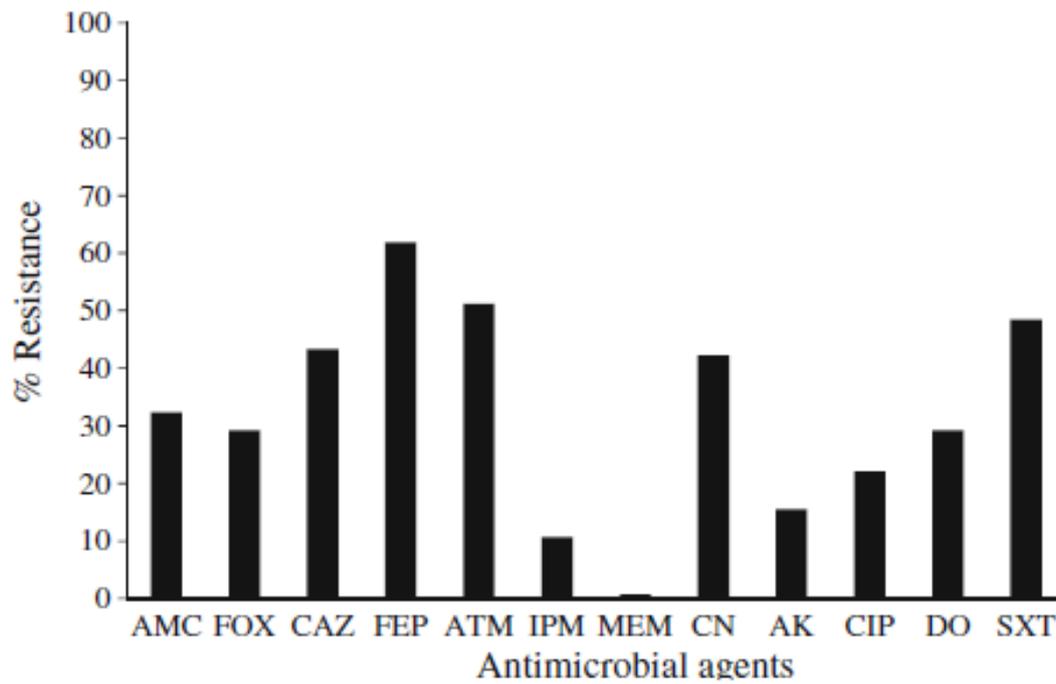
Table 3
Multivariate logistic regression analysis of risk factors associated with intestinal colonization of *bla*_{CTX-M} ESBL-positive Enterobacteriaceae among healthy volunteers

Variables	<i>p</i> value^a	Odds ratio	95% CI
Antibiotic use within previous three months	0.089	1.573	0.934–2.651
Consumption of undercooked meat ^b	0.003	2.133	1.289–3.530

^aStatistically significant ($p < 0.05$)

^bVolunteers who occasionally or regularly consume undercooked meat

Figure 1



Resistance rates of cefotaxime-resistant Enterobacteriaceae isolated from healthy volunteers ($n = 291$). *AMC* amoxicillin/clavulanate, *FOX* ceftazidime, *CAZ* ceftazidime, *FEP* cefepime, *ATM* aztreonam, *IPM* imipenem, *MEM* meropenem, *CN* gentamicin, *AK* amikacin, *CIP* ciprofloxacin, *DO* doxycycline, *SXT* trimethoprim/sulfamethoxazole