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Widespread dissemination of extended-spectrum β-lactamase-producing, multidrug-resistant *Escherichia coli* in livestock and fishery products in Vietnam

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Abstract

Background: Widespread dissemination of extended-spectrum β-lactamase (ESBL)-producing bacteria is a threat to public health. Since livestock products are possible reservoirs of ESBL-producing bacteria, food-borne dissemination of ESBL-producing bacteria and the characteristics of such organisms in food products should be assessed to evaluate potential sources of contamination.

Results: A total of 253 food samples from poultry, pork, shrimp, and fish were collected from local markets in a rural area of Vietnam from 2013 to 2014. ESBL-producing *Escherichia coli* were isolated from these samples, and their ESBL genotypes, phylogenetic groups, and antibiotic resistance profiles were assessed. Overall, a high percentage (68.4 %) of the food samples was contaminated with ESBL-producing *E. coli*, and samples from livestock and fishery products showed similar levels of contamination. The ESBL-producing *E. coli* isolated from the food samples harbored genes of the CTX-M-1, CTX-M-9, and TEM groups. Analysis of the antibiotic resistance profiles of the ESBL-producing *E. coli* isolates from the food samples showed a high degree of multidrug resistance. The prevalence of extensively multidrug-resistant ESBL-producing *E. coli* that were resistant to at least five antibiotic drug classes in poultry, pork, shrimp, and fish samples, was 92.1, 69, 56.5, and 62.5 %, respectively.

Conclusions: The results of this study confirmed the widespread dissemination of ESBL-producing *E. coli* in both livestock and fishery products from a rural area in Vietnam. The high prevalence of extensively multidrug-resistant ESBL-producing *E. coli* in food products highlights the importance of continuous monitoring of food products for the presence of these bacteria, particularly in underdeveloped countries.

Keywords: Antibiotic resistance, Extended-spectrum β-lactamase (ESBL), *Escherichia coli*, Livestock products, Vietnam

Background

Antimicrobial drug resistance is a growing public health concern worldwide (Hawkey and Jones 2009), and in particular, the increasing prevalence of extended-spectrum β-lactamase (ESBL)-producing bacteria is an emerging threat to public health (Pitout and Laupland 2008; Woerther et al. 2013). In the 1980s and 1990s, ESBL-producing bacteria, mainly SHV and TEM types, were almost exclusively detected in nosocomial settings (Woerther et al. 2013). However, in recent years, prevalence of the CTX-M-type ESBL-producing bacteria has increased in both nosocomial and community settings (Bush and Jacoby 2010). Furthermore, ESBL-producing bacteria often exhibit co-resistance to multiple classes of antibiotics (Coque et al. 2008), which may increase the risk of poor clinical outcomes because of the lack of effective treatment options.

Recent studies indicate that the prevalence of ESBL-producing bacteria in developing countries, especially Asian countries, has increased to more than 70 % of

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community residents (Tian et al. 2008; Coque et al. 2008; Sasaki et al. 2010; Luvsansharav et al. 2012; Nakayama et al. 2015). Food and food-producing animals are considered a potential reservoir of these bacteria (Geser et al. 2012; Liebana et al. 2013). Although limited studies on the prevalence of ESBL-producing bacteria have been conducted in Asian countries, the results show widespread dissemination of these bacteria in the community (Sasaki et al. 2010; Nakayama et al. 2015).

The present study determined the prevalence, genetic and phylogenetic groups, and antibiotic resistance profiles of ESBL-producing *Escherichia coli* (ESBL-*E. coli*) in retail livestock and fishery products in Vietnam.

Methods

Sample collection and bacterial isolation

A total of 253 food samples of poultry (chops; $n = 60$), pork (chops; $n = 69$), shrimp (freshwater shrimp; $n = 60$), and fish (ray-finned fish, Southeast Asian tropical freshwater fish, Asian carp, snakehead fish, and tilapia; $n = 64$) were purchased from three local markets in a rural area of Thai Binh, Vietnam from June 2013 to December 2014. For bacterial isolation, 25 g of each food sample was inoculated into 225 mL of buffered peptone water and incubated at 37 °C for 18 h. The cultures were then inoculated on CHROMagar ECC medium (CHROMagar, Paris, France) containing 1 μg/mL cefotaxime and cultured at 37 °C for 24 h. One to three colonies that showed the characteristics of *E. coli* were collected from each sample plate.

Screening and identification of ESBL-producing bacteria

ESBL production by the bacterial isolates was evaluated by the disc diffusion method on Mueller-Hinton agar plates (Oxoid, Cheshire, UK) using ceftazidime and cefotaxime with and without clavulanic acid as recommended by the Clinical and Laboratory Standards Institute (Clinical and Laboratory Standards Institute 2013). ESBL-producing isolates were identified as *E. coli* by the IMViC test (Thampuran et al. 2005).

Genotyping of ESBL-encoding genes

Polymerase chain reaction (PCR) was used to detect genes encoding ESBLs. Bacterial DNA was extracted by boiling the bacterial suspension in tris(hydroxymethyl)aminomethane-EDTA buffer (pH 8.0). The extracted DNA was used as the template for amplification by multiplex PCR using primers specific for genes encoding enzymes belonging to the TEM, SHV, CTX-M-1, CTX-M-2, CTX-M-9, and CTX-M-8/CTX-M-25 groups (Le et al. 2015). The PCR conditions used were as follows: initial denaturation at 95 °C for 5 min, followed by 25 cycles of denaturation at 95 °C for 30 s, annealing at 60 °C for 90 s, and extension at 72 °C for 90 s, with a final extension at 68 °C for 10 min. The PCR products were visualized by electrophoresis on a 2 % agarose gel followed by ethidium bromide staining.

Determination of phylogenetic groups

The phylogenetic groups of ESBL-*E. coli* were identified by multiplex PCR amplification of a combination of two genes, *chuA* and *yjaA*, and a DNA fragment, TspE4C2, as described previously (Chen et al. 2014).

Antimicrobial susceptibility testing

Antibiotics from seven groups, namely, β-lactams (ampicillin [AMP], cefoxitin [FOX], cefotaxime [CTX], ceftazidime [CAZ], and meropenem [MEM]), quinolones (nalidixic acid [NAL] and ciprofloxacin [CIP]), aminoglycosides (kanamycin [KAN], streptomycin [STR], and gentamicin [GEN]), tetracycline (TET), fosfomycin (FOF), phenicols (chloramphenicol [CHL]), and folic acid inhibitors (trimethoprim-sulfamethoxazole [SXT]), were used to test the antimicrobial susceptibility of the isolated ESBL-*E. coli* strains. Antibiotic susceptibility was determined by the disc diffusion method using antibiotic disks (Becton Dickinson) according to the standard CLSI protocol. The results of the susceptibility tests were interpreted using CLSI document M100-S23 (Clinical and Laboratory Standards Institute 2013).

Statistical analysis

Statistical analysis was performed using the chi-square test, and the significance level was set at $p < 0.05$.

Results

Prevalence of ESBL-*E. coli* in food samples

A high percentage (68.4 %) of the tested poultry, pork, shrimp, and fish samples was contaminated with ESBL-*E. coli* (Table 1). The prevalence of ESBL-*E. coli* in the tested samples varied significantly from 50.7 % in pork to 88.3 % in poultry ($p < 0.001$). Samples from livestock and fishery products showed similar prevalence of ESBL-*E. coli* (69.5 % in livestock products and 68.8 % in fishery products).

Genotypes of ESBL-*E. coli*

Genotype analysis showed that the majority of the ESBL-*E. coli* isolates belonged to the CTX-M group (359/372 [96.5 %]). Approximately 40 % of the ESBL-*E. coli* isolates (149/372) belonged exclusively to the CTX-M group, whereas only 3.5 % (13/372) belonged only to the TEM group.

The CTX-M ESBL-*E. coli* isolates from the food samples belonged to the CTX-M-1 and CTX-M-9 groups (Table 2). The majority of the ESBL-*E. coli* isolates (57.3 %) encoded genes for multiple ESBLs, including those of the CTX-M-9, CTX-M-1, and TEM groups.

Table 1 Prevalence of antibiotic-resistant ESBL-*E. coli* in the food samples

		Food type			
		Animal products		Fishery products	
	Total	Poultry	Pork	Shrimp	Fish
No. of samples tested	253	60	69	60	64
No. of ESBL-*E. coli*-positive samples	173 (68.4 %)	53 (88.3 %)*	35 (50.7 %)	45 (75.0 %)	40 (62.5 %)
ESBL-*E. coli* isolates[a]	372	126	81	85	80

*$p < 0.001$, significantly different from the other food type
[a]One to three representative colonies were isolated from each sample

The ESBL-*E. coli* isolates from poultry samples possessed a higher number of genes encoding multiple ESBLs than the isolates from other food samples (75.4 %, $p < 0.001$). Overall, 32.3 % of the ESBL-*E. coli* isolates obtained from the food samples encoded genes in the CTX-M-9 and TEM groups, whereas 23.9 % of the ESBL-*E. coli* isolates encoded genes of the CTX-M-1 and TEM groups. In contrast, only 0.8 % of the ESBL-*E. coli* isolates only encoded genes of the CTX-M group, which is significantly lower than the 56.5 % of the ESBL-*E. coli* isolates that encoded genes in both the CTX-M and TEM groups.

Phylogenetic groups of ESBL-*E. coli*

Phylogenetic analysis showed some similarities among all the ESBL-*E. coli* isolates from the food samples (Table 3). The prevalence of ESBL-*E. coli* isolates belonging to group B2, which includes virulent clonal groups of human extraintestinal pathogenic *E. coli* that cause a variety of infections outside of the gastric intestinal tract (Jakobsen et al. 2010), was low in the food samples tested in this study.

Multidrug resistance in ESBL-*E. coli*

The antibiotic resistance profiles of the ESBL-*E. coli* isolates from the food samples tested in this study are shown in Table 4. It should be noted that one ESBL-*E. coli* isolate from pork sample was resistant to MEM. In addition, 5.4 % of the isolates were resistant to FOX. FOF resistance was very limited, except in isolates from poultry (37.3 %). In addition, the ESBL-*E. coli* isolates from poultry samples showed significantly higher resistance to KAN, GEN, CIP, NAL, CHL, and FOF than those isolated from the other food samples ($p < 0.05$). Furthermore, 80–81 % of the ESBL-*E. coli* isolates from poultry, pork, and shrimp were resistant to SXT, compared to only 19 % of the ESBL-*E. coli* isolates from fish ($p < 0.01$).

A high level of multidrug resistance (MDR), which is defined as resistance to at least one antibiotic drug in three or more antibiotic classes (Cantón and Ruiz-Garbajosa 2011), was observed in all food sources (Fig. 1). Notably, extensive MDR, which is defined as resistance to at least five antibiotic drug classes, was observed in 92.1 % (116/126), 69 % (56/81), 56.5 % (48/85), and 62.5 % (50/80) of the ESBL-*E. coli* isolates from

Table 2 Prevalence of multiple ESBL-encoding genes in ESBL-*E. coli* isolated from the food samples tested in this study

	ESBL genotype	Total	Animal Products				Fishery Products			
			Poultry		Pork		Shrimp		Fish	
Multi- ESBL genes	CTX-M-9/CTX-M-1/TEM[a]	1 (0.3%)	95[b] (75.4%)[c]	0	43 (53.1%)	0	35 (41.2%)	0	40 (50%)	1 (1.3%)
	CTX-M-9/CTX-M-1	3 (0.8%)		1 (0.8%)		1 (1.2%)		0		1 (1.3%)
	CTX-M-9/TEM	120 (32.3%)		55 (43.7%)		17 (21%)		22 (25.9%)		26 (32.5%)
	CTX-M-1/TEM	89 (23.9%)		39 (31.0%)		25 (31%)		13 (15.3%)		12 (15.0%)
Single -ESBL genes	CTX-M-9	102 (27.4%)	31 (24.6%)	18 (14.3%)	38 (46.9%)	26 (32.1%)	50 (58.8%)	35 (41.2%)	40 (50%)	23 (28.8%)
	CTX-M-1	44 (11.8%)		10 (7.9%)		9 (11.1%)		9 (10.6%)		16 (20.0%)
	TEM	13 (3.5%)		3 (2.4%)		3 (3.7%)		6 (7.1%)		1 (1.3%)
Total		372	126		81		85		80	

[a] TEM amplicons were not sequences and it was not certain if they correspond to ESBL variants
[b] Number of isolates
[c] $p < 0.001$, significantly different from the other food type

Table 3 Phylogenetic groups of ESBL-*E. coli*

		Phylogenetic group			
Food type	Total	A	B1	B2	D
Poultry	126	42 (33.3 %)	37 (29.3 %)	9 (7.1 %)	38 (30.2 %)
Pork	81	31 (38.3 %)	25 (30.9 %)	6 (7.4 %)	19 (23.5 %)
Shrimp	85	28 (32.9 %)	28 (32.9 %)	10 (11.8 %)	19 (22.4 %)
Fish	80	20 (25 %)	34 (42.5 %)	7 (8.8 %)	19 (23.8 %)

poultry, pork, shrimp, and fish, respectively. In addition, the ESBL-*E. coli* isolates from livestock products showed significantly higher MDR than isolates from fishery products ($p < 0.05$; Fig. 1).

Discussion

A previous study conducted in Nha Trang, a medium-sized coastal city in southern Vietnam, showed that the prevalence of ESBL-*E. coli* in poultry, pork, and shrimp products was 58.7, 32, and 18.3 %, respectively (Le et al. 2015). The results of both the present and previous studies showed a higher prevalence of ESBL-*E. coli* in poultry products than in other food products in both areas of Vietnam. In addition, the results also showed that the overall prevalence of ESBL-*E. coli* is higher in Thai Binh than in Nha Trang. A study conducted in Denmark reported that 36 % of samples from imported broiler chicken were contaminated with extended-spectrum cephalosporinase-producing *E. coli* (Agersø et al. 2012), while a similar study conducted in the Netherlands showed that 76.8–94 % of poultry products were contaminated with ESBL-*E. coli* (Leverstein-van Hall et al. 2011; Overdevest et al. 2011). Thus, the prevalence of ESBL-producing bacteria in food products varies in different geographical areas.

Southeast Asian countries commonly use an integrated recycling farm system called VAC (vegetable, aquaculture, and caged animal) for food production. This system is effective for small-scale food animal breeding and agricultural production. Stool and waste from humans, animals, and plants are mixed into the soil or put into ponds as food for fish. In this system, antibiotic-resistant bacteria from improperly treated waste could easily enter the ecological cycle, especially in aquatic environments either directly or via sewage ditches. Unhygienic food-handling practices at retail markets may also contribute to the prevalence of antibiotic-resistant bacteria in livestock products. Notably, residual antibiotics are commonly detected in aquatic environments in Vietnam (Hoa et al. 2011). The heavy use of antibiotics in this area can lead to antibiotic contamination of the environment, which likely promotes the development of antibiotic-resistant bacteria (Suzuki and Hoa 2012). In Thai Binh, a typical rural area in northern Vietnam, retail poultry products from different chicken farms are brought to retailers' houses where they are processed before being sold in markets. In addition, most retail pork products originate from households in the community. Nearly 100 % of the freshwater shrimp products are from small domestic rivers near fields or from private pools next to sewage ditches. Almost all fish products are from household fish pools, domestic rivers, or rice fields. Therefore, differences among the sources of the food products and the environments in which they are processed may be the main reasons for the variations in the prevalence of ESBL-*E. coli* in food samples tested from different geographical areas.

Our results showed that 96.5 % of the ESBL-*E. coli* isolates encoded genes of the CTX-M group with or without TEM-encoding genes, suggesting the emergence of the CTX-M gene group as the main contributor to the antibiotic resistance of ESBL-*E. coli* in food, food-producing animals, healthy people, and patients not only in developed countries but also in underdeveloped countries (Sasaki et al. 2010; Overdevest et al. 2011; Börjesson et al. 2013; Chen et al. 2014; Nakayama et al. 2015).

CTX-M-1 and CTX-M-9 (with or without TEM) were the major subgroups of CTX-M genotypes detected in the ESBL-*E. coli* isolates from the food samples tested in this study. The results of this study are similar to those of previous studies conducted in other regions of Vietnam (Cortés et al. 2010; Leverstein-van Hall et al. 2011; Le et al. 2015). There was a significant difference in the multi-ESBL genes

Table 4 Percentage of ESBL-*E. coli* isolated from poultry, pork, shrimp, and fish samples exhibited resistance to antimicrobial agents

Food type	% resistance to													
	β- lactam					Aminoglycosides			Quinolones		Tetracycline	Phenicols	Phosphomycins	Folic acid inhibitors
	AMP	FOX	CTX	CAZ	MEM	STR	KAN	GEN	CIP	NAL	TET	CHL	FOF	SXT
Poultry (126)	100.0	5.6	99.2	21.4	0.0	94.4	63.5	54.0	58.7	90.0	99.2	90.0	37.3	81.0
Pork (81)	100.0	6.2	100.0	29.6	1.2	79.0	39.5	42.0	51.9	65.0	85.2	69.0	8.6	79.0
Shrimp (85)	100.0	5.9	100.0	22.4	0.0	71.8	27.1	31.8	28.2	46.0	83.5	56.0	4.7	80.0
Fish (80)	100.0	3.8	98.8	17.5	0.0	70.0	32.5	30.0	28.8	56.0	87.5	56.0	10.0	19..0
Total (327)	100.0	5.4	99.5	22.6	0.3	80.6	43.3	41.1	43.8	67.0	90.1	71.0	17.7	67.0
p							<0.01	<0.05	<0.01	<0.01		<0.01	<0.01	<0.01

Number in parentheses indicates the number of isolates

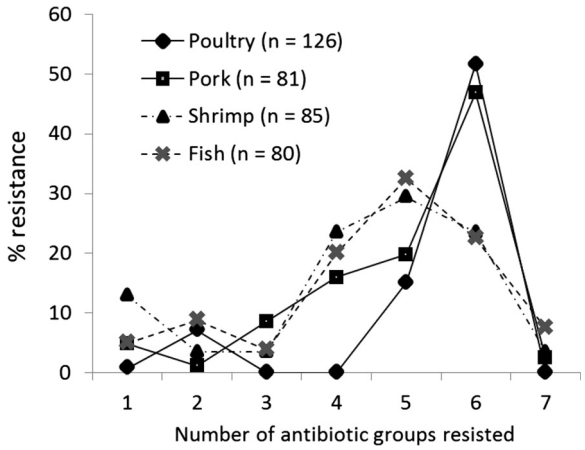

Fig. 1 Degree of MDR ESBL-*E. coli* isolated from each food sample

encoded by the isolates obtained from poultry compared to those encoded by isolates from pork, shrimp, and fish samples ($p < 0.001$). Antibiotic usage at poultry farms, particularly the long-term antibiotic treatment from hatching to grow-out, may contribute to the acquisition of multi-ESBL genes, leading to a high prevalence of ESBL-*E. coli* in poultry products.

ESBL-*E. coli* belonging to phylogenetic groups A, B1, B2, and D were present in the tested food samples, and there were no significant differences among the food sources. ESBL-*E. coli* belonging to groups B1 and A accounted for approximately two-thirds of all ESBL-*E. coli* isolates from the food samples, followed by those belonging to group D. In contrast, ESBL-*E. coli* belonging to group B2 accounted for only 8.6 % of all ESBL-*E. coli* isolates from the food samples. This order of the most to least prevalent phylogenetic groups observed in the present study was similar to that observed in a recent study conducted in Nha Trang, Vietnam (Le et al. 2015). In addition, a similar phylogenetic profile was observed for ESBL-*E. coli* isolates from chicken samples in the Netherlands, with the highest percentage (44 %) of isolates belonging to group B1, followed by groups A (28 %), D (23 %), and B2 (2 %) (Kluytmans et al. 2013). Therefore, studies performed in the Netherlands and other regions of Vietnam showed that the prevalence of ESBL-*E. coli* isolates belonging to group B2 was significantly lower than the prevalence of ESBL-*E. coli* isolates belonging to other phylogenetic groups. However, the results of the present study showed a much higher prevalence of ESBL-*E. coli* isolates belonging to group B2 than reported in previous studies performed in other areas of Vietnam or in other countries. Since group B2 usually includes bacteria isolated from humans (Jakobsen et al. 2010), our findings imply a close relationship between ESBL-*E. coli* isolates from food samples and isolates from humans.

A high percentage (89.2 %) of the ESBL-*E. coli* isolates from the food samples tested in this study showed MDR. The prevalence of extensively MDR ESBL-*E. coli* was especially high in fishery products (56–62 % isolates). Quantitative and qualitative analysis of antibiotic-resistant bacteria in fishery products (Van et al. 2007a, 2007b; Le et al. 2015) showed that up to 61 % of *E. coli* isolates exhibit MDR. The prevalence of aquaculture as well as the unique distribution method for fishery products in rural areas of Vietnam (described above) could lead to cross contamination with antibiotic-resistant bacteria, particularly in markets, resulting in a high prevalence of these bacteria.

Since the spread of carbapenem-resistant bacteria in the community is a threat to public health due to the increased risk of intractable infection, the isolation of MEM-resistant ESBL-*E. coli* from pork samples is notable. Even though only one strain was isolated from the food samples tested in this study, possible dissemination of these bacteria in food should be monitored. The high rate of quinolone resistance in ESBL-*E. coli* isolates from food is also important. The heavy use of quinolone antibiotics in agriculture may be responsible for this high prevalence.

Food contaminated with ESBL-producing bacteria is a potential source for widespread dissemination of these bacteria in humans (Lazarus et al. 2015). The results of our study, combined with data from previous studies in different regions of Vietnam and other countries, suggests that rural areas are major contributors to the dissemination of not only ESBL-*E. coli* but also extensively MDR bacteria. Rural areas with livestock and aqua-agriculture activities are one of the largest reservoirs of MDR bacteria and are therefore a major threat to public health. Thus, the development of stringent monitoring strategies and the promotion of hygienic food distribution practices are needed to control the spread of these antibiotic-resistant bacteria.

Conclusions

This study provided evidence of widespread dissemination of ESBL-*E. coli* in both livestock and fishery products obtained from a rural area in Vietnam. The high percentage of extensively MDR ESBL-*E. coli* in these food products highlights the importance of continuous monitoring for these bacteria in food distribution, particularly in underdeveloped countries.

Abbreviations

ESBL: extended-spectrum β-lactamase; MDR: multi-drug resistance; PCR: polymerase chain reaction; AMP: ampicillin; FOX: cefoxitin; CTX: cefotaxime; CAZ: ceftazidime; MEM: meropenem; NAL: nalidixic acid; CIP: ciprofloxacin; KAN: kanamycin; STR: streptomycin; GEN: gentamycin; TET: tetracycline; FOF: fosfomycin; CHL: chloramphenicol; SXT: trimethoprim-sulfamethoxazole; IMViC tests: indole, methyl red, Voges Proskauer and citrate utilization tests.

Competing interests
The authors declare that they have no competing interests.

Authors' contributions
HVL: acquisition of data, analysis and interpretation of data, drafting the manuscript; RK: study concept and design, acquisition of data; DTK: acquisition of data; HTT: acquisition of data; TNN: acquisition of data; KNP: acquisition of data; MJ: acquisition of data; YK: study concept and design; TN: study concept and design; SU: acquisition of data; YY: study concept and design, analysis and interpretation of data, drafting manuscript and critical evaluation of manuscript to ensure accuracy of content. All authors read and approved the final manuscript.

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