Japanese Journal of Veterinary Research 66(1): 1–12, 2018

JJVR

REGULAR PAPER Experimental Research

Isolation and molecular characterization of extended-spectrum β-lactamase producing *Escherichia coli* from industrial food animals in Mekong Delta, Vietnam

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Received for publication, July 19, 2016; accepted, October 18, 2017

Abstract

The aim of this study was to investigate if extended-spectrum β-lactamase producing *Escherichia coli* (ESBL-EC) is present in intestinal tracts of apparently healthy broiler chickens at large-scale chicken farm and pigs, and their environments in Vietnam. ESBL-EC was isolated from 86.7% cloacal swabs of chickens (13 out of 15), 55.0% rectal swabs of pigs (11 out of 20) and 100% from their surroundings (2 beddings in a chicken farm and 2 drainages in pig farms). All the isolates from chicken and pig farms were multidrug-resistant. Interestingly, 94.7% (36/38) isolates from chicken were resistant to ciprofloxacin and *mcr-1* gene-positive (related to colistin resistance), respectively, while ciprofloxacin resistance and *mcr-1* gene was found in only 12.8% (5/39) and none (0/39) from pig, respectively. CTX-M type in most of the chicken isolates belonged to group-1 whereas that in the pig isolates belonged to group-9. Virulence gene profiling revealed that some of these isolates indeed carry *eae* or *astA* pathogenic genes. Plasmid profiling and PFGE analysis indicated that most of them showed various genotypes although some isolates showed nearly identical genotype, suggesting that a number of ESBL-EC with various genotypes were distributed in chickens and pigs in Mekong Delta. To the best of our knowledge, this is the first report regarding isolation of ESBL-EC from broiler chickens in large scale-farms and pigs in Vietnam. Taken together, these results suggest that chickens and pigs in Mekong Delta, Vietnam used for food industry could also serve as reservoirs of ESBL-EC isolates carrying virulence genes.

Key Words: antimicrobials, *Escherichia coli*, pig, chicken, Vietnam

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doi: 10.14943/jjvr.66.1.1

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Introduction

The emergence of multidrug-resistant bacteria is an increasing concern in human and veterinary medicines across the world. Extended-spectrum β-lactamases (ESBLs) constitute the most important cephalosporin resistance mechanisms in *Enterobacteriaceae*, particularly *Escherichia coli*. ESBLs, which are encoded by multiple *bla* genes including TEM, SHV and CTX-M types, can hydrolyze modern extended-spectrum cephalosporins and monobactams but not cephamycins and carbapenems[22]. CTX-M typed ESBLs can be classified into 5 groups including CTX-M-1, CTX-M-2, CTX-M-8, CTX-M-9 and CTX-M-25[11]. Recently, CTX-M typed ESBLs have been emerging rapidly worldwide, principally in community-acquired human infections[8].

Recent studies have revealed that food animals, such as chickens, pigs and cattle, may carry ESBL-producing *E. coli* (ESBL-EC) in their intestinal tracts[2,26]. An association of ESBL-EC in food animals, retail meats, and humans has been suggested[10,14] and ESBL-EC in food animals and retail meats could be the source of human infections through handling or consumption of contaminated foods.

In Vietnam, isolation and characterization of ESBL-EC from community-acquired or nosocomial human infection have been reported over the past few years[1,9,19]. Recently, asymptomatic human carriers of ESBL-EC were also reported in Vietnam[6,18,28]. Moreover, domestic food demands especially in meats are rapidly growing with economic development in Vietnam. Some of harvested meats have been exported to the neighboring countries including China and Malaysia. To enhance the production efficiency, a wide range of antimicrobials has been increasingly used against food animals[3]. This might cause increasing risks for emergence, selection and spread of antimicrobial resistant bacteria worldwide. Several studies have shown that antimicrobial-resistant bacteria including ESBL-EC are present in food animals or their meat products in Vietnam[12,22,23,27,29]. However, the studies targeted chickens in backyards and in small-scale (~200 chickens) chicken farms[12,22]. And ESBL-EC was not detected in pigs probably since the study did not apply culture method selective for ESBL-EC[22], suggesting that distribution of ESBL-EC in industrial food animals is still not clear in Vietnam. The present study investigated the occurrence and characteristics of ESBL-EC in broiler chickens of a large-scale chicken farm and pigs in Mekong Delta, Vietnam by using ESBL-EC-selective culture method.

Materials and Methods

Collection of fecal and environmental samples: A total of 15 broiler chickens were used for collecting cloacal swabs in chicken farm A, which was a subcontractor of a large company, in Vinh Long province, Vietnam in 2013. Unit size of the farm was 38,000 chickens (2 houses, 19,000 chickens per house). The farm was managed by all-in, all-out scheme for individual chicken houses. Five chickens per house were sampled at 30–40 days of age (house 1 in June, and houses 1 and 2 in August 2013). Simultaneously, bedding (n = 2) was also collected from house 1 in June 2013 as well as August. Fattening period of a flock in the farm was 40–45 days. The flocks in June and August 2013 were different from each other. All the chickens of the flock in June 2013 received amoxicillin (β-lactam) at arrival to the farm, and norfloxacin before one week of harvesting, and those in August 2013 received tylosin (macrolide) and chloramphenicol from 6th to 8th days and 16th to 17th days, respectively, after installation.

Rectal swabs of 20 pigs (10 pigs per farm) were collected from 2 pig farms (farms B and C) in June and August 2013, respectively, in Vietnam. The farm B was located in Can Tho province and its unit size was ~200 pigs. The pigs received amoxicillin (β-lactam) and sulfadimidine (sulfonamide) through feeds. The farm C was located in Hau Giang province and its unit size

was ~100 pigs. The pigs received colistin (polymyxin E), kitasamycin (macrolide), and lincomycin (lincosamide) through feeds. Fattening period of the pigs in both farms was 6 months. Water was also collected (n = 2) from the ponds beside farms B and C into which sewages of the farms were drained.

All the samplings were done with approval of farms and their local governments. The samples were transported to the laboratory of Can Tho University at ambient temperature and processed within 24 h of collection.

Isolation of ESBL-EC: ESBL-EC was isolated by following the protocol described previously[15]. Briefly, fecal and water samples were directly inoculated on MacConkey agar (Eiken Chemical Co., Ltd., Tokyo, Japan) containing 2 μg/ml cefotaxime (CTX-MacConkey agar plates) (Sigma-Aldrich, St. Louis, MO, U.S.A.). Bedding (25 g) was homogenized with 225 ml of Buffered Peptone Water (Becton, Dickinson and Company, Franklin Lakes, NJ, U.S.A.) and incubated at 37°C for 24 h. One loopful enrichment culture was streaked on CTX-MacConkey agar plate and incubated at 37°C overnight. Five suspected colonies were streaked on fresh CTX-MacConkey agar and incubated at 37°C overnight for single colony isolation. The isolates were identified to be *E. coli* by conventional biochemical tests using LIM medium, SIM medium, Simmons citrate agar, Triple Sugar Iron agar and VP medium, and examined for ESBL production by antimicrobial susceptibility tests by disk diffusion method using cefotaxime and ceftazidime with and without clavulanic acid, according to the Clinical Laboratory Standards Institute (CLSI) standards (M100-S25). Among phenotypically ESBL-positive *E. coli* isolates, three colonies were randomly chosen and they were further analyzed for the presence of ESBL genes[17] and for subsequent CTX-M grouping[25] by PCRs.

Antimicrobial susceptibility: The ESBL-EC isolates were further analyzed for antimicrobial susceptibility by disk diffusion method targeting 12 antimicrobial agents including cefoxitin (FOX), meropenem (MEM), imipenem (IPM), ampicillin (AMP), fosfomycin (FOF), streptomycin (STR), kanamycin (KAN), gentamicin (GEN), chloramphenicol (CHL), tetracycline (TET), ciprofloxacin (CIP), nalidixic acid (NAL), and sulfamethoxazole-trimethoprim (SXT), based on the criteria of the CLSI.

Genetic characterization: Phylogenetic grouping was carried out by PCR as described by Clermont *et al.*[4]. Presence of virulence genes for diarrheagenic *E. coli* (*bfpA*, *eae*, EAF, *stx1*, *stx2*, *elt*, *est*, *invE*, *aatA*, *astA*, *cdt*) and *mcr-1* gene in the isolates were also checked by colony hybridization assay using ^{32}P-labeled specific gene-probes as described previously[5]. To prepare *mcr-1* gene probe, the partial sequence was amplified by PCR using a primer set of CLR5-F and CLR5-R[13] from *E. coli* strain IPN.088[12]. To analyze the clonal relationship of *astA* gene-positive ESBL-EC, pulsed-field gel electrophoresis (PFGE) was performed as described previously[28]. Location of $bla_{\text{CTX-M}}$ genes in the $bla_{\text{CTX-M}}$ gene-positive isolates was analyzed by S1 nuclease-PFGE and subsequent Southern hybridization using ^{32}P-labeled $bla_{\text{CTX-M-1}}$ and $bla_{\text{CTX-M-9}}$ gene-probes as described previously[6].

Results

Isolation of ESBL-EC from chicken farm. The mean prevalence of ESBL-EC in chickens (5 chickens per flock) collected from 3 flocks in farm A (house 1 in June, and houses 1 and 2 in August 2013) was 86.7% (13 out of 15 chicken samples). As shown in Table 1, both of the flocks in house 1 in June and August 2013 were 100% positive for ESBL-EC irrespective of antimicrobials given to chickens, whereas that in house 2 showed 60% positive. Beddings (n = 2) in house 1 collected in June and August 2013 were also positive for ESBL-EC. A total of 50 ESBL-EC

Table 1. Prevalence of ESBL-producing *E. coli* in apparently heathy chickens and pigs in Mekong Delta, Vietnam

Farm	Source	Sampling date	Antimicrobial agents used	No. of ESBL positive /No. of samples (%)	No. of isolates characterized /No. of ESBL obtained
A-house 1	Chicken	June, 2013	Amoxicillin, Norfloxacin	5/5 (100%)	14/20
	Bedding	June, 2013	–	1/1 (100%)	3/5
	Chicken	August, 2013	Tylosin, Chloramphenicol	5/5 (100%)	14/18
	Bedding	August, 2013	–	1/1 (100%)	3/3
A-house 2	Chicken	August, 2013	Tylosin, Chloramphenicol	3/5 (60%)	4/4
B	Pig	June, 2013	Amoxicillin, Sulfadimedine	10/10 (100%)	30/50
	Pond water	June, 2013	–	1/1 (100%)	3/5
C	Pig	August, 2013	Colistin, Kitasamycin, Lincomycin	1/10 (10%)	3/5
	Pond water	August, 2013	–	1/1 (100%)	3/3

isolates (20 and 18 isolates from house 1 in June and August 2013, respectively, 4 from house 2, 8 from beddings in house 1) were obtained. Among them, 32 and 6 of ESBL-EC isolates (maximum 3 isolates per sample) from chickens and beddings, respectively, were subjected for phenotypic and genotypic characterizations.

Isolation of ESBL-EC from pig farms. ESBL-EC was isolated from 100% (10 out of 10 pigs) and 10% (1/10) pigs in farms B and C, respectively (Table 1). The occurrence of ESBL-EC in farm B was significantly higher than that of farm C ($P < 0.01$, chi-squared test). ESBL-EC was also detected in the pond water samples (n = 2), where sewages were drained. Fifty and five of ESBL-EC isolates from pigs and water, respectively, were obtained in farm B whereas 5 and 3 of the isolates were from pigs and water, respectively, in farm C. Among them, 33 and 6 of the isolates (maximum 3 isolates per sample) from pigs and pond water, respectively, were subjected to the following analyses.

Antimicrobial susceptibility of ESBL-EC isolates. A total of 38 and 39 isolates from chicken and pig farms, respectively, were analyzed for antimicrobial susceptibility. As shown in Table 2, resistance of the isolates from chicken farm was 100% for CTX, AMP, and TET, followed by 97.4% in CHL, NAL and SXT, CIP and KAN (94.6%), STR (78.9%), CAZ (73.7%), FOF (47.4%), GEM and FOX (23.7%). However, none of them was resistant to MEM and IPM. All isolates were resistant to 6 to 12 antimicrobials among the 14 tested agents suggesting their multidrug-resistant phenotypes. All the isolates from pig farms were also multidrug-resistant (resistance to 5 to 10 antimicrobials) as shown in Table 3: 100% for CTX and AMP, followed by CHL (97.4%), TET (92.3%), SXT (89.7%), FOF (64.1%), STR (48.7%), KAN (35.9%), CAZ (25.6%), GEN (23.1%), NAL (23.1%), CIP (12.8%), FOX (5.1%). Similarly, none of them was resistant to MEM and IPM.

Genotypic characterization of ESBL-EC isolates. Determination of ESBL genotypes revealed that most of the chicken farm isolates (n = 26; 68.4%) were positive for both $bla_{\text{CTX-M-1}}$ and bla_{TEM} whereas only 7, 4 and 1 of the isolates were positive for bla_{TEM}, $bla_{\text{CTX-M-9}}$ and $bla_{\text{CTX-M-1}}$, respectively (Table 2). On the other hand, in the majority of ESBL genotypes in the pig farm isolates (n = 29; 74.3%) we observed the co-existence of $bla_{\text{CTX-M-9}}$ and bla_{TEM}, followed by 4 isolates having $bla_{\text{CTX-M-9}}$, 3 $bla_{\text{CTX-M-1}}$, 2 bla_{TEM} and 1 of both $bla_{\text{CTX-M-1}}$ and bla_{TEM} (Table 3).

By phylogenetic grouping analysis, the

Table 2. ESBL-genotypes, phylogenetic groups, virulence genes and antimicrobial susceptibility of ESBL-producing *E. coli* isolates from chickens

Origin	House	Month collected	ESBL genotype[*1]	PG[*5]	*n*	Strain	VG[*6]	No. of resistance	Antimicrobial susceptibility												*mcr-1*
									CTX[*7]	CAZ[*8]	FOX[*9]	AMP[*10]	FOF[*11]	STR[*12]	KAN[*13]	GEN[*14]	CHL[*15]	TET[*16]	CIP[*17]	NAL[*18]	SXT[*19]	
Chicken	1	June	1[*2]/TEM[*3]	A	1	VCF5-4[*]		11	R[*20]	R	S	R	R	R	R	S	R	R	R	R	R	+
			1/TEM	A	1	VCF2-4[*]	*astA*	11	R	R	S	R	R	R	R	S	R	R	R	R	R	+
			1/TEM	A	1	VCF5-1		10	R	I[*21]	S	R	R	R	R	S	R	R	R	R	R	+
			1/TEM	A	1[#]	VCF5-2[*]		9	R	I	S	R	R	R	R	S	S	R	R	R	R	+
			1/TEM	D	5	VCF1-1, 1-2, 1-3, 2-3, 2-5		10	R	R	S	R	R	I	R	S	R	R	R	R	R	+
			1/TEM	D	2	VCF3-4, 3-5		9	R	I	S	R	R	I	R	S	R	R	R	R	R	+
			TEM	A	1	VCF4-7		10	R	I	R	R	S	R	R	S	R	R	R	R	R	+
			TEM	A	1	VCF4-10		10	R	S[*22]	R	R	S	R	R	S	R	R	R	R	R	+
			TEM	D	1	VCF4-9	*astA*	11	R	S	R	R	R	R	R	S	R	R	R	R	R	+
		August	1/TEM	A	1	VCF10-7	*astA*	10	R	R	S	R	S	R	R	S	R	R	R	R	R	+
			1/TEM	D	5	VCF6-6, 7-1, 8-1, 9-5, 9-9		11	R	R	S	R	S	R	R	R	R	R	R	R	R	+
			1/TEM	D	2	VCF6-7, 6-10		11	R	R	S	R	S	R	R	R	R	R	R	R	R	−
			1/TEM	D	1	VCF10-1		12	R	R	R	R	S	R	R	R	R	R	R	R	R	+
			1/TEM	D	1	VCF10-4		11	R	R	S	R	S	R	R	R	R	R	R	R	R	+
			1/TEM	D	1	VCF7-8		10	R	R	S	R	S	R	R	I	R	R	R	R	R	+
			9[*4]	D	3	VCF7-7[*], VCF8-5[*], VCF8-8[*]		10	R	R	S	R	S	R	R	S	R	R	R	R	R	+
	2	August	1	B1	1	VCF11-10		6	R	S	S	R	S	R	S	S	R	R	S	S	R	+
			1/TEM	A	1	VCF12-3[*]		9	R	R	S	R	S	R	R	S	R	R	S	R	R	+
			TEM	A	1	VCF12-6		11	R	I	R	R	R	R	R	S	R	R	R	R	R	+
			TEM	D	1	VCF15-1		8	R	S	R	R	R	I	S	S	R	R	R	R	S	+
Bedding	1	June	1/TEM	A	2	VCFb1-1[*], VCFb1-3[*]		11	R	R	S	R	R	R	R	S	R	R	R	R	R	+
			1/TEM	D	1	VCFb1-2[*]	*astA*	11	R	R	R	R	S	R	R	I	R	R	R	R	R	+
		August	9	D	1	VCFb2-3[*]		10	R	R	S	R	S	R	R	S	R	R	R	R	R	+
			TEM	A	2	VCFb2-1, 2-2		12	R	R	R	R	R	R	R	S	R	R	R	R	R	+
No. of resistant strain (%)									38 (100%)	28 (73.7%)	9 (23.7%)	38 (100%)	18 (47.4%)	30 (78.9%)	36 (94.7%)	9 (23.7%)	37 (97.4%)	38 (100%)	36 (94.7%)	37 (97.4%)	37 (97.4%)	36 (94.7%)	

[*1]responsible genes for ESBL [*2]$bla_{\text{CTX-M-1}}$ [*3]bla_{TEM} [*4]$bla_{\text{CTX-M-9}}$ [*5]phylogenetic group [*6]virulence gene profile [*7]cefotaxime [*8]ceftazidime [*9]cefoxitin [*10]ampicillin [*11]fosfomycin [*12]streptomycin [*13]kanamycin [*14]gentamicin [*15]chloramphenicol [*16]tetracycline [*17]ciprofloxacin [*18]nalidixic acid [*19]sulfamethoxazole-trimethoprim [*20]resistant [*21]intermediate [*22]susceptible [#]One isolate from each category was subjected to S1 nuclease-PFGE analysis.

Table 3. ESBL-genotypes, phylogenetic groups, virulence genes and antimicrobial susceptibility of ESBL-producing *E. coli* isolates from pigs

Origin	ESBL genotype[*5]	PG[*9]	*n*	Strain	Pulsotype	VG[*10]	No. of resistance	Antimicrobial susceptibility													*mcr-1*
								CTX[*11]	CAZ[*12]	FOX[*13]	AMP[*14]	FOF[*15]	STR[*16]	KAN[*17]	GEN[*18]	CHL[*19]	TET[*20]	CIP[*21]	NAL[*22]	SXT[*23]	
PfB-Pig[*1]	1[*6]	B1	1[#]	VPF3-2			6	R[*24]	R	S	R	S	S	I	I	R	R	S	I	R	–
	1	B1	1	VPF3-3			6	R	R	S	R	S	S	S	S	R	R	S	I	R	–
	1	B1	1	VPF3-7			6	R	R	S	R	S	I	S	S	R	R	S	I	R	–
	1/TEM[*7]	A	1	VPF5-6			8	R	R	S	R	S	R	R	S	R	R	S	I	R	–
	9[*8]	A	1[#]	VPF9-3			9	R	I[*25]	S	R	R	R	R	R	R	R	S	S	R	–
	9	A	1	VPF9-2			7	R	I	S	R	S	R	R	R	S	R	S	R	I	–
	9/TEM	A	3	VPF2-1, VPF2-2, VPF2-3	I	*astA*	6	R	S[*26]	S	R	R	I	S	S	R	R	S	S	R	–
	9/TEM	A	2	VPF4-1, VPF4-2			5	R	S	S	R	S	S	S	S	R	R	S	S	R	–
	9/TEM	A	1	VPF9-1	IIIb	*astA*	6	R	S	S	R	S	S	S	S	R	S	R	R	R	–
	9/TEM	A	1	VPF7-2	IIIb	*astA*	6	R	S	S	R	S	S	S	S	R	R	S	R	R	–
	9/TEM	A	1	VPF10-1	IIIa	*astA*	6	R	S	S	R	S	I	S	S	R	S	R	R	R	–
	9/TEM	A	1	VPF10-3	IIIa	*astA*	5	R	I	S	R	S	I	S	S	R	S	S	R	R	–
	9/TEM	A	1	VPF10-2	II	*astA*	5	R	S	S	R	S	I	S	S	R	R	S	R	I	–
	9/TEM	B1	3	VPF1-1, VPF1-2, VPF1-3			9	R	S	S	R	R	R	R	R	R	R	S	S	R	–
	9/TEM	B1	3	VPF7-3, VPF8-1, VPF8-2	IVb	*astA*	6	R	I	S	R	R	I	S	S	R	R	S	I	R	–
	9/TEM	B1	2	VPF6-1, VPF6-2			9	R	I	S	R	R	R	R	R	R	R	S	S	R	–
	9/TEM	B1	2	VPF5-2, VPF5-3			8	R	I	S	R	R	R	R	I	R	R	S	S	R	–
	9/TEM	B1	1[#]	VPF7-1	IVa	*astA*	9	R	I	S	R	R	R	R	R	R	R	S	S	R	–
	9/TEM	B1	1	VPF4-3			6	R	S	S	R	S	R	S	S	R	R	S	I	R	–
	9/TEM	B1	1	VPF6-3			6	R	I	S	R	R	I	S	S	R	R	S	I	R	–
	9/TEM	D	1	VPF8-3			6	R	I	S	R	R	I	S	S	R	R	S	I	R	–
PfB-PW[*2]	9	A	1[#]	VPFp1-2	VII	*astA*	8	R	I	S	R	R	R	R	S	R	R	S	I	R	–
	9/TEM	A	2	VPFp1-1, VPFp1-3			7	R	I	S	R	R	R	S	S	R	R	S	I	R	–
PfC-Pig[*3]	9	D	1[#]	VPF11-1		*eae*	10	R	R	S	R	S	R	R	S	R	R	R	R	R	–
	TEM	D	2	VPF11-2, VPF11-3		*eae*	10	R	R	R	R	R	R	S	S	R	R	R	R	S	–
PfC-PW[*4]	9/TEM	A	1[#]	VPFp2-2	VI	*astA*	7	R	R	S	R	R	I	S	S	R	R	S	S	R	–
	9/TEM	B2	1[#]	VPFp2-4	V	*astA*	7	R	R	S	R	R	S	S	S	R	R	S	S	R	–
	9/TEM	B1	1[#]	VPFp2-8			10	R	R	S	R	R	R	R	R	R	R	S	S	R	–
No. of resistant strains (%)								39 (100%)	10 (25.6%)	2 (5.1%)	39 (100%)	25 (64.1%)	19 (48.7%)	14 (35.9%)	9 (23.1%)	38 (97.4%)	36 (92.3%)	5 (12.8%)	9 (23.1%)	35 (89.7%)	0 (0%)

[*1]pig in pig farm B [*2]pond water in pig farm B [*3]pig in pig farm C [*4]pond water in pig farm C [*5]responsible genes for ESBL [*6]$bla_{\text{CTX-M-1}}$ [*7]bla_{TEM} [*8]$bla_{\text{CTX-M-9}}$ [*9]phylogenetic group [*10]virulence gene profile [*11]cefotaxime [*12]ceftazidime [*13]cefoxitin [*14]ampicillin [*15]fosfomycin [*16]streptomycin [*17]kanamycin [*18]gentamicin [*19]chloramphenicol [*20]tetracycline [*21]ciprofloxacin [*22]nalidixic acid [*23]sulfamethoxazole-trimethoprim [*24]resistant [*25]intermediate [*26]susceptible [#]subjected to S1 nuclease-PFGE analysis.

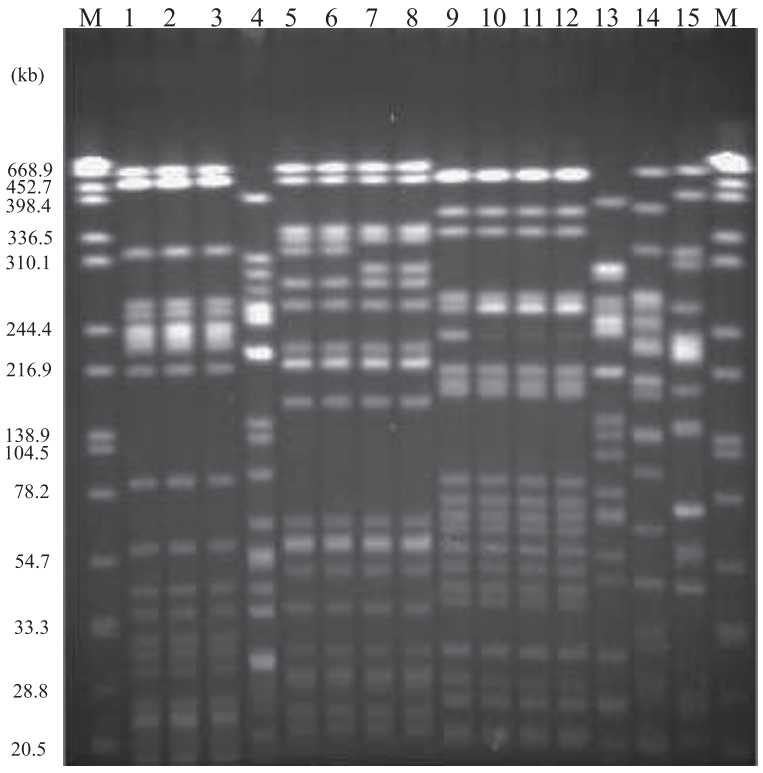

Fig. 1. *Xba*I-digested pulsed gel electrophoresis patterns of genomic DNA isolated from *astA* gene-positive ESBL-producing *E. coli* isolates from pig farms. Lane 1, VPF2-1 (phylogenetic group [PG] A, $bla_{\text{CTX-M-9}}$ and bla_{TEM} positive, pulosotype I); 2, VPF2-2 (PG A, $bla_{\text{CTX-M-9}}$/bla_{TEM}, I); 3, VPF2-3 (PG A, $bla_{\text{CTX-M-9}}$/bla_{TEM}, I); 4, VPF10-2 (PG A, $bla_{\text{CTX-M-9}}$/bla_{TEM}, II); 5, VPF10-1 (PG A, $bla_{\text{CTX-M-9}}$/bla_{TEM}, IIIa); 6, VPF10-3 (PG A, $bla_{\text{CTX-M-9}}$/bla_{TEM}, IIIa); 7, VPF9-1 (PG A, $bla_{\text{CTX-M-9}}$/bla_{TEM}, IIIb); 8, VPF7-2 (PG A, $bla_{\text{CTX-M-9}}$/bla_{TEM}, IIIb); 9, VPF7-1 (PG B1, $bla_{\text{CTX-M-9}}$/bla_{TEM}, IVa); 10, VPF7-3 (PG B1, $bla_{\text{CTX-M-9}}$/bla_{TEM}, IVb); 11, VPF8-1 (PG B1, $bla_{\text{CTX-M-9}}$/bla_{TEM}, IVb); 12, VPF8-2 (PG B1, $bla_{\text{CTX-M-9}}$/bla_{TEM}, IVb); 13, VPFp2-4 (PG B2, $bla_{\text{CTX-M-9}}$/bla_{TEM}, V); 14, VPFp2-2 (PG A, $bla_{\text{CTX-M-9}}$/bla_{TEM}, VI); 15, VPFp1-2 (PG A, $bla_{\text{CTX-M-9}}$, VII). M, *Xba*I-digested genomic DNA from *S. enterica* serotype Braenderup strain H9812 used as molecular size markers. VPFp2-2 and VPFp2-4 were isolated from farm C; other isolates were from farm B.

chicken isolates were determined to belong to A (n = 13; 34.2%), B1 (n = 1; 2.6%) and D (n = 24; 63.2%) of phylogenetic groups, irrespective of their ESBL genotypes (Table 2). The pig isolates belonged to A (n = 17; 43.6%), B1 (n = 17; 43.6%), B2 (n = 1; 2.6%), and D (n = 4; 10.3%), respectively, (Table 3). Virulence gene analysis revealed that 4 chicken and 15 pig farm isolates were positive for *astA* gene, and 3 pig farm isolates were positive for *eae* gene, indicating that they were potentially virulent (Tables 2 and 3). *mcr-1* whose gene-product is related to colistin resistance was detected in 36 (94.7%) ESBL-EC isolates from chicken farm whereas none of the ESBL-EC isolates from the pig farm was positive.

To examine the clonal relationship, *astA* gene-positive isolates from pig farms were subjected to PFGE. The 15 *astA* gene-positive isolates from pig farms showed 9 pulsotypes (I, II, IIIa, IIIb, IVa, IVb, V, VI, VII, VIII), in which IIIa and IIIb, and IVa and IVb were considered to be clonal, respectively, due to only single band difference (Fig. 1), suggesting that some clonally related ESBL-EC were distributed among pigs.

Eight strains each of $bla_{\text{CTX-M}}$ gene-positive isolates from chicken and pig farms were randomly selected (Tables 2 and 3), and were analyzed for plasmid profile by S1-nuclease PFGE. Furthermore, the location of the $bla_{\text{CTX-M}}$ genes was investigated by Southern hybridization (Fig. 2). The size of the plasmids were ranged from 60 to 120 kb in chicken isolates while that

in pig isolates were from 60 to 110 kb. The $bla_{\text{CTX-M}}$ gene was located on plasmids in all the tested strains except for a strain VPF3-2 from the pig farm B, where the gene was located on the chromosome.

Discussion

Multidrug resistance in pathogenic bacteria has been increasingly recognized as a global health concern in human and animals. Inappropriate use of antimicrobials is considered as the major factor to promote emergence and selection of resistant bacteria and dissemination of resistance genes. Abuse of antimicrobials in food animals may contribute significantly to this problem. In industrial food animals, antimicrobials are used as growth promoters, and prophylactic treatment to prevent infectious diseases as well as therapeutic purposes. ESBL is one of the most important antimicrobial resistance mechanisms in *Enterobacteriaceae*, particularly *E. coli* and is usually encoded on plasmids[24], which can be transferred among intra- and inter-bacterial species. Indeed, several reports regarding isolation of ESBL-EC from industrial food animals[2,26] have revealed that the high number of industrial food animals such as chicken and pig carry ESBL-EC in their intestines with gradual increase of the prevalence of CTX-M types[26]. Analysis of plasmids harboring ESBL genes have also suggested that they could be shared among *E. coli* in the pyramid of broiler chicken productions[30], leading to emergence of ESBL-EC. To meet the increasing food demands as well as sustain economic developments, Vietnam has intensified agriculture (i.e. chickens and pigs) and aquaculture (i.e. fishes and shrimps), especially in Mekong Delta, where the industrial food animals, fishes and shrimps are cultivated on a large-scale and a wide range of antimicrobials have been increasingly used to prevent them from infectious diseases to enhance the production efficiency. Currently Vietnam is exporting the animal products to other countries in addition to their domestic consumption, indicating that there could be increasing risk of spreading of ESBL-EC not only inside but also outside the country. ESBL-EC has been isolated from chickens cultivated in backyards of households[27] and in small scale-farms[23], and fishes[20]. There are also some reports from large-scale chicken farms and pigs targeting for a variety of antimicrobial resistant-bacteria in chickens[22,23,29], but they did not target or could not detect ESBL-EC. Since the studies applied non-selective culture method for ESBL-EC, the occurrence of ESBL-EC might have been underestimated. Thus, there is no report regarding isolation of ESBL-EC from broiler chickens in large scale-farms and pigs in Vietnam. The present study for the first time clearly demonstrated the presence of ESBL-EC in chickens in a large-scale farm and pigs in Vietnam.

Interestingly, ESBL-EC was detected in only 10% pigs in the farm C where β-lactam antimicrobial was not given to the animals, while it was detected in 100% pigs in the farm B where β-lactam antimicrobial (amoxicillin) was commonly used ($P < 0.01$, chi-squared test). This result may indicate that use of β-lactam antimicrobials have promoted emergence of ESBL-EC and their colonization in the intestines of pigs. There is another possibility that non-β-lactam antimicrobials given to pigs through food, such as colistin, could inhibit the colonization of ESBL-EC in their intestine. Although susceptibility of the ESBL-EC isolates to the antimicrobials included in the feeds given was not analyzed in the present study, none of them were positive for *mcr-1* gene encoding for a member of the phosphoethanolamine transferase enzyme family which is responsible for colistin resistance of bacterial hosts. On the other hand, in addition to the chickens to which β-lactam antimicrobial was given in June 2013 and to those not given in August 2013 both showed high contamination with ESBL-EC (100% in house 1 and 60% in house 2). This might be due to

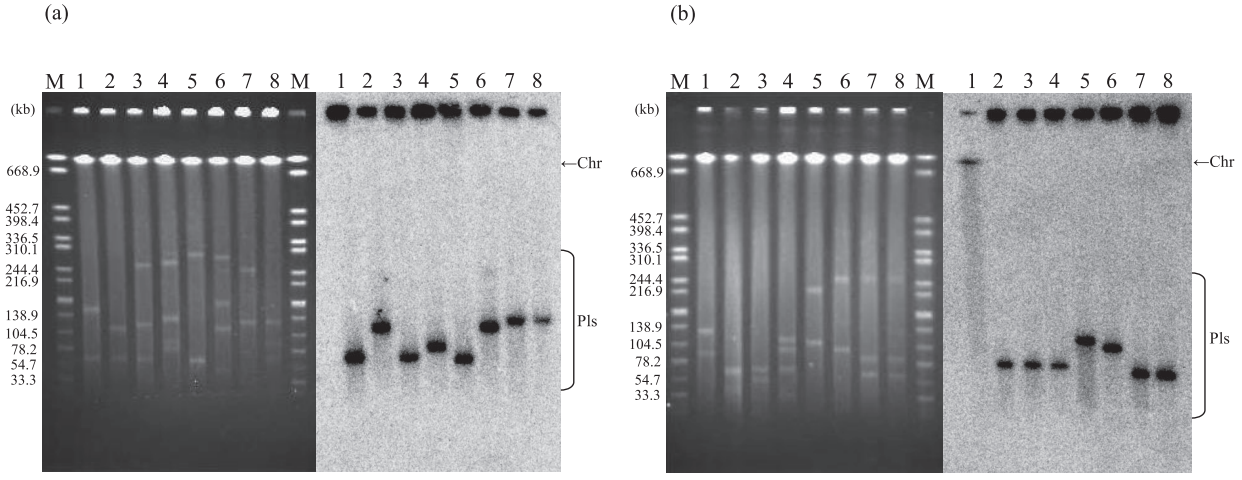

Fig. 2. Location of the *bla*$_{\text{CTX-M}}$ genes in ESBL-producing *E. coli* isolates from chicken (a) and pig farms (b). Left panel, Separation of S1 nuclease-digested DNAs by PFGE; Right panel, Southern hybridization with ^{32}P-labeled *bla*$_{\text{CTX-M-1}}$ and *bla*$_{\text{CTX-M-9}}$ gene probes. (a) Lane 1, VCF5-4 (phylogenetic group [PG] A, *bla*$_{\text{CTX-M-1}}$ and *bla*$_{\text{TEM}}$ positive); 2, VCF2-4 (PG A, *bla*$_{\text{CTX-M-1}}$/*bla*$_{\text{TEM}}$); 3, VCF2-5 (PG A, *bla*$_{\text{CTX-M-1}}$/*bla*$_{\text{TEM}}$); 4, VCF7-7 (PG D, *bla*$_{\text{CTX-M-9}}$); 5, VCF12-3 (PG A, *bla*$_{\text{CTX-M-1}}$/*bla*$_{\text{TEM}}$); 6, VCFb1-1 (PG A, *bla*$_{\text{CTX-M-1}}$/*bla*$_{\text{TEM}}$); 7, VCFb1-2 (PG D, *bla*$_{\text{CTX-M-1}}$/*bla*$_{\text{TEM}}$); 8, VCFb2-3 (PG D, *bla*$_{\text{CTX-M-9}}$) (b) Lane 1, VPF3-2 (PG B1, *bla*$_{\text{CTX-M-1}}$); 2, VPF9-3, (PG A, *bla*$_{\text{CTX-M-9}}$); 3, VPF7-1 (PG B1, *bla*$_{\text{CTX-M-9}}$/*bla*$_{\text{TEM}}$); 4, VPFp1-2 (PG A, *bla*$_{\text{CTX-M-9}}$); 5, VPF11-1 (PG D, *bla*$_{\text{CTX-M-9}}$) 6, VPFp2-2 (PG A, *bla*$_{\text{CTX-M-9}}$/*bla*$_{\text{TEM}}$); 7, VPFp2-4 (PG B2, *bla*$_{\text{CTX-M-9}}$/*bla*$_{\text{TEM}}$); 8, VPFp2-8 (PG B1, *bla*$_{\text{CTX-M-9}}$/*bla*$_{\text{TEM}}$). M, *Xba*I-digested chromosomal DNA from *S. enterica* serotype Braenderup strain H9812 used as molecular size markers.

multidrug resistance of the ESBL-EC isolates. The chickens of the flocks in August 2013 received tylosin and chloramphenicol but not β-lactam antimicrobials, but all of the ESBL-EC isolates from the flocks in August 2013 were found to be resistant to chloramphenicol (Table 2, tylosin was not analyzed in this study), indicating that the ESBL-EC isolates cannot be removed from the chicken flocks by the alternative antimicrobials due to multidrug resistance of the isolates. This includes possession of *mcr-1* gene, which was detected in 94.7% of the ESBL-EC isolates from the chicken farm. Moreover, all-in and all-out system to cultivate chickens could also be associated with such high prevalence in the chicken farm. This could be due to difference in floors and cleaning patterns of the pig and chicken farms. While the floors of pig farms were made of concrete and were kept clean by washing every day, the floors of the chicken farm were overlaid by bedding and were not cleaned during the fattening period. This might promote ESBL-EC shed from the chickens to survive in the bedding, which could spread infection among the chicken flocks. Indeed, the bedding collected in the present study was contaminated by ESBL-EC. Since the limited numbers of samples were analyzed in this study, further experimental evidences are required to understand precisely how the chickens are infected with ESBL-EC, and their dissemination.

Pond water samples examined in this study were also contaminated with ESBL-EC. It has already been known that fishes carry ESBL-EC[7], suggesting that the waste water from the animal farms could lead to ESBL-EC infection to fishes in the ponds, which could be consumed by nearby residents. Moreover, when the water is drained into river, ESBL-EC could also spread into the outside environment. Pond and river waters are also used in farming of crops. Thus, by-products from farming could also be associated with spread of ESBL-EC.

ESBL-EC has been considered to be non-pathogenic *per se* or might cause urinary tract infections in human. However, 4 and 15 isolates from chicken and pig farms, respectively, harbored *astA* gene encoding enteroaggregative *E. coli*

heat-stable enterotoxin 1 (EAST1), and 3 isolates from pig farms harbored *eae* gene encoding intimin necessary for intimate attachment of enteropathogenic *E. coli* and enterohemorrhagic *E. coli*. Although we did not confirm expression of their functional peptide and protein, this result suggests that these strains could be potentially virulent, which may cause diarrhea to human. To date, there was less report suggesting an association of ESBL-EC with diarrhea in human[6], but ESBL-EC, which can produce Shiga toxin 2, was isolated from piglets with diarrhea in India[16]. This suggests that, in addition to uropathogenic *E. coli* producing ESBL, ESBL-EC isolates being able to cause human diarrhea might be present in industrial food animals at least in Vietnam and India. Moreover, as shown in Fig. 2, the ESBL-EC isolates except for a strain VPF3-2 from pig farm B carried ESBL genes on their plasmids, suggesting that they could be reservoirs of ESBL genes by conferring the ability to produce ESBL on diarrheagenic *E. coli* or other pathogenic bacteria. Since most of studies targeting ESBL-EC from food animals have not analyzed for their virulence properties, continuous and extensive surveillance including virulence profilings of ESBL-EC in industrial food animals is crucial. Genetic characterization such as PFGE patterns, plasmid profiling and the location of the $bla_{\text{CTX-M}}$ genes revealed that various ESBL plasmids and ESBL-EC clone are distributed in chicken and pig farms in Mekong Delta, Vietnam (Figs. 1 and 2).

In conclusion, this is the first report showing that broiler chickens in large scale-farms and pigs in Vietnam carry multiple-drug resistant ESBL-EC isolates and some of which harbor potentially virulent genes and thus, they could serve as diarrheagenic pathogens to humans. However, there are still several questions which remain to be answered like source of ESBL-EC in industrial food animals in Vietnam, prevalence of ESBL-EC in their meats, association with human infections including both clinical and asymptomatic cases, and so on. Further studies are necessary to unveil these questions.

Acknowledgements

We thank Dr. Rupak K. Bhadra (CSIR-Indian Institute of Chemical Biology, India) for critical reading of the manuscript. This work was supported in part by Grant-in-aid for Scientific Research (KAKENHI) from JSPS and the Japan Agency for Medical Research and Development (AMED)/Japan International Cooperation Agency (JICA) as part of the Science and Technology Research Partnership for Sustainable Development (SATREPS). The authors declare no conflict of interest.

References

1) Cao V, Lambert T, Nhu DQ, Loan HK, Hoang NK, Arlet G, Courvalin, P. Distribution of extended-spectrum beta-lactamases in clinical isolates of *Enterobacteriaceae* in Vietnam. Antimicrob Agents Chemother 46, 3739–4743, 2002.
2) Carattoli A. Animal reservoirs for extended spectrum beta-lactamase producers. Clin Microbiol Infect, 14 Suppl 1, 117–123, 2008.
3) Carrique-Mas JJ, Trung NV, Hoa NT, Mai HH, Thanh TH, Campbell JI, Wagenaar JA, Hardon A, Hieu TQ, Schultsz C. Antimicrobial usage in chicken production in the Mekong Delta of Vietnam. Zoonoses Public Health 62 Suppl 1, 70–78, 2015.
4) Clermont O, Bonacorsi S and Bingen F. Rapid and simple determination of the *Escherichia coli* phylogenetic group. Appl Env Microbiol 66, 4555–4558, 2000.
5) Hinenoya A, Nagita A, Ninomiya K, Asakura M, Shima K, Seto K, Tsukamoto T, Ramamurthy T, Faruque SM, Yamasaki S. Prevalence and characteristics of cytolethal distending toxin-producing *Escherichia coli* from children with diarrhea in Japan. Microbiol Immunol 53, 206–215, 2009.
6) Hoang PH, Awasthi SP, Do Nguyen P, Nguyen NL, Nguyen DT, Le NH, Van Dang C, Hinenoya A, Yamasaki S. Antimicrobial resistance profiles and molecular characterization of *Escherichia coli* strains

isolated from healthy adults in Ho Chi Minh city, Vietnam. J Vet Med Sci 79, 479–485, 2017.
7) Jiang HX, Tang D, Liu YH, Zhang XH, Zeng ZL, Xu L, Hawkey PM. Prevalence and characteristics of β-lactamase and plasmid-mediated quinolone resistance genes in *Escherichia coli* isolated from farmed fish in China. J Antimicrob Chemother 67, 2350–2353, 2012.
8) Jones CH, Tuckman M, Keeney D, Ruzin A, Bradford PA. Characterization and sequence analysis of extended-spectrum-{beta}-lactamase-encoding genes from *Escherichia coli*, *Klebsiella pneumoniae*, and *Proteus mirabilis* isolates collected during tigecycline phase 3 clinical trials. Antimicrob Agents Chemother 53, 465–475, 2009.
9) Jones SL, Nguyen VK, Nguyen TMP, Athan E. Prevalence of multiresistant Gram-negative organisms in a surgical hospital in Ho Chi Minh City, Vietnam. Trop Med Int Health 11, 1725–1730, 2006.
10) Kluytmans JA, Overdevest IT, Willemsen I, Kluytmans-van den Bergh MF, van der Zwaluw K, Heck M, Rijnsburger M, Vandernbroucke-Grauls CM, Savelkoul PH, Johnson BD, Gordon D, Johnson JR. Extended-spectrum β-lactamase-producing *Escherichia coli* from retail chicken meat and humans: comparison of strains, plasmids, resistance genes, and virulence factors. Clin. Infect. Dis 56, 478–487, 2013.
11) Lahlaaoui H, Ben Haj Khalifa A, Ben Moussa M. Epidemiology of Enterobacteriaceae producing CTX-M type extended spectrum β-lactamase (ESBL). Med Mal Infect 44, 400–404, 2014.
12) Le QP, Ueda S, Nguyen TN, Dao TV, Van Hoang TA, Tran TT, Hirai I, Nakayama T, Kawahara R, Do TH, Vien QM, Yamamoto Y. Characteristics of extended-spectrum β-lactamase-producing *Escherichia coli* in retail meats and shrimp at a local market in Vietnam. Foodborne Pathog Dis, 12, 719–725, 2015.
13) Liu YY, Wang Y, Walsh TR, Yi LX, Zhang R, Spencer J, Doi Y, Tian G, Dong B, Huang X, Yu LF, Gu D, Ren H, Chen X, Lv L, He D, Zhou H, Liang Z, Liu JH, Shen J. Emergence of plasmid-mediated colistin resistance mechanism MCR-1 in animals and human beings in China: a microbiological and molecular biological study. Lancet Infect Dis 16, 161–168, 2016.
14) Leverstein-van Hall MA, Dierikx CM, Cohen Stuart J, Vets GM, van den Munckhof MP, van Essen-Zandbergen A, Platteel T Fluit AC, van de Sande-Bruinsma N, Scharinga J, Bonten MJ, Mevius DJ. Dutch patients, retail chicken meat and poultry share the same ESBL genes, plasmids and strains. Clin Microbiol Infect 17, 873–880, 2011.
15) Luvsansharav UO, Hirai I, Niki M, Nakata A, Yoshinaga A, Yamamoto A, Yamamoto M, Toyoshima H, Kawakami F, Matsuura N, Yamamoto Y. Fecal carriage of CTX-M β-lactamase-producing *Enterobacteriaceae* in nursing homes in the Kinki region of Japan. Infect Drug Resist 6, 67–70, 2013.
16) Mandakini R, Dutta TK, Chingtham S, Roychoudhury P, Samanta I, Joardar SN, Pachauau AR, Chandra R. ESBL-producing Shiga-toxigenic *E. coli* (STEC) associated with piglet diarrhoea in India. Trop Anim Health Prod 47, 377–381, 2014.
17) Monstein HJ, Östholm-Balkhed Å, Nilsson MV, Nilsson M, Dornbusch K, Nilsson LE. Mutiplex PCR amplification assay for the detection of *bla*SHV, *bla*TEM and *bla*CTX-M genes in *Enterobacteriaceae*. APMIS 115, 1400–1408, 2007.
18) Nakayama T, Ueda S, Huong BT, Tuyen le D, Komalamisra C, Kusolsuk T, Hirai I, Yamamoto Y. Wide dissemination of extended-spectrum β-lactamase-producing *Escherichia coli* in community residents in the Indochinese peninsula. Infect Drug Resist 8, 1–5, 2015.
19) Nguyen HTT, Tran VTN, James IC, Nguyen TH, Jeremy F, Stephen B, Pham TD. The characterization of ESBL genes in *Escherichia coli* and *Klebsiella pneumoniae* causing nosocomial infection in Vietnam. J Infect Dev Ctries 7, 922–928, 2013.
20) Nguyen HTN, Hoa TTT, Nguyen TQ, Hinenoya A, Nakayama T, Harada K, Asayama M, Warisaya M, Hirata K, Nguyen TP, Yamamoto Y. Spread of antibiotic and antimicrobial susceptibility of ESBL-producing *Escherichia coli* isolated from wild and cultured fish in the Mekong Delta, Vietnam. Fish Pathol 51, S75–S82, 2016.
21) Nhung NT, Cuong NV, Campbell J, Hoa NT, Bryant JE, Truc VN, Kiet BT, Jombart T, Trung NV, Hien VB, Thwaites G, Baker S, Carrique-Mas J. High levels of antimicrobial resistance among *Escherichia coli* isolates from livestock farms and synanthropic rats and shrews in the Mekong Delta of Vietnam. Appl Environ Microbiol 81, 812–820, 2015.
22) Nguyen NT, Nguyen HM, Nguyen CV, Nguyen TV, Nguyen MT, Thai HQ, Ho MH, Thwaites G, Ngo HT, Baker S, Carrique-Mas

J. Use of colistin and other critical antimicrobials on pig and chicken farms in southern Vietnam and its association with resistance in commensal *Escherichia coli* bacteria. Appl Environ Microbiol 82, 3727–3735, 2016.
23) Nguyen VT, Carrique-Mas JJ, Ngo TH, Ho HM, Ha TT, Campbell JI, Nguyen TN, Hoang NN, Pham VM, Wagenaar JA, Hardon A, Thai QH, Schultsz C. Prevalence and risk factors for carriage of antimicrobial-resistant *Escherichia coli* on household and small-scale chicken farms in the Mekong Delta of Vietnam. J Antimicrob Chemother 70, 2144–2152, 2015.
24) Paterson DL and Bonomo RA. Extended-spectrum β-lactamases: A clinical update. Clin Microbiol Rev 18, 657–686, 2005.
25) Pitout JDD, Hossain A, Hanson ND. Phenotypic and molecular detection of CTX-M-β-lactamases produced by *Escherichia coli* and *Klebsiella* spp. J Clin Microbiol 42, 5715–5721, 2004.
26) Rao L, Lv L, Zeng Z, Chen S, He D, Chen X, Wu C, Wang Y, Yang T, Wu P, Liu Y, Liu JH. Increasing prevalence of extended-spectrum cephalosporin-resistant *Escherichia coli* in food animals and the diversity of CTX-M genotypes during 2003–2012. Vet Microbiol 172, 534–541, 2014.
27) Ueda S, Bui TK, Bui TM, Hirai I, Le, DT Yamamoto Y. Limited transmission of blaCTX-M-9-type positive *Escherichia coli* between humans and poultry in Vietnam. Antimicrob Agents Chemother 59, 3574–3577, 2015.
28) Shima K, Yuluo W, Sugimoto N, Asakura M, Nishimura K, Yamasaki S. Comparison of a PCR-restriction fragment length polymorphism (PCR-RFLP) assay to pulsed-field gel electrophoresis to determine the effect of repeated subculture and prolonged storage on RFLP patterns of Shiga toxin-producing *Escherichia coli* O157:H7. J Clin Microbiol 44, 3963–3968, 2006.
29) Usui M, Ozawa S, Onozato H, Kuge R, Obata Y, Uemae T, Ngoc PT, Heriyanto A, Chalemchaikit T, Makita K, Muramatsu Y, Tamura Y. Antimicrobial susceptibility of indicator bacteria isolated from chickens in Southeast Asian countries (Vietnam, Indonesia and Thailand). J Vet Med Sci 76, 685–692, 2014.
30) Zurfluh K, Wang J, Klumpp J, Nüesch-Inderbinen M, Fanning S, Stephan R. Vertical transmission of highly similar $bla_{\text{CTX-M-1}}$-harboring IncI1 plasmids in *Escherichia coli* with different MLST types in the poultry production pyramid. Front Microbiol 5, 519, 2014.