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Potential Transmission Opportunity of CTX-M-producing *Escherichia coli* in Large-scale Chicken Farm in Vietnam.

Running title: ESBL-producing *E. coli* transmission in chicken farm.

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Highlights

- ESBL-producing *E. coli* was detected in 83.3% of Vietnamese chicken farm workers.
- The detected rates of ESBL-producing *E coli* in a chicken farm was higher than community.
- Clonal distribution of ESBL-producing *E. coli* was observed only in the chicken farm.

Abstract

Objectives. Extended spectrum β-lactamase (ESBL)-producing *Escherichia coli* has been spread out worldwide. We investigated *bla*$_{\text{CTX-M}}$-positive *E. coli* in a Vietnamese large-size chicken farm, and evaluated whether there was any different prevalence and molecular characters of *bla*$_{\text{CTX-M}}$-positive *E. coli* between the large size chicken farm and a Vietnamese community.

Methods. Fecal samples were collected from 24 human individuals and 38 chickens of a large-scale chicken farm, and from 51 human and 36 chickens of a community. All samples were collected between June, 2013 and June 2014 in the Bavi province in the Red River Delta region of Vietnam. Molecular characterizations of CTX-M-producing *E. coli* and genetic relatedness among the isolates were evaluated by conventional typing methods. Antimicrobial susceptibility of the isolates was evaluated by disc diffusion test.

Results. The prevalence of *bla*$_{\text{CTX-M}}$-positive *E. coli* was 83.3%, 71.1%, 54.9% and 13.9% in farm workers, farm chickens, community individuals and community backyard chicken, respectively. On average, *bla*$_{\text{CTX-M}}$-positive *E. coli* isolates obtained from the farm chicken were resistant to 8.3 different antibiotics. The average number of detected aminoglycoside modifying enzyme (AME) genes (3.1 genes) and detection rate

of the plasmid colistin resistance gene, *mcr-1* (33.3%) were higher in *bla*$_{\text{CTX-M}}$-positive *E. coli* isolated from the farm chicken than other sampling groups. In addition, two types of indistinguishable pulsed-field gel electrophoresis patterns were observed in six *bla*$_{\text{CTX-M-65}}$-positive *E. coli* and three *bla*$_{\text{CTX-M-55}}$-positive *E. coli* from the farm chicken.

Conclusions. Our results suggested more frequent transmission opportunity of *bla*$_{\text{CTX-M}}$-positive *E. coli* in the Vietnamese large-scale chicken farm.

Keywords: Antimicrobial susceptibility, CTX-M type ESBL, Transmission

1. Introduction

Antimicrobial resistance (AMR) is a global public health concern because it impairs our capability to control infectious diseases (11, 16, 20). Since its emergence in the mid-1980s, extended-spectrum β-lactamase (ESBL) -producing Enterobacteriaceae including *E. coli, Klebsiella pneumoniae* and nontyphoidal *Salmonella* have become one of the most important causes of nosocomial and community-acquired infections (28, 34), and ESBL encoding genes, such as *bla*$_{\text{CTX-M}}$, *bla*$_{\text{TEM}}$ and *bla*$_{\text{SHV}}$, have been spreading in both community and nosocomial settings (27, 29). In Vietnam, a number of studies have reported the alarmingly high prevalence of ESBL-producing

Enterobacteriaceae in clinical isolates (5, 23, 24, 36, 39) and in isolates obtained from asymptomatic healthy individuals (3, 37).

Vietnam is an agricultural country with about 70% of the population living in rural areas. A approximately 8 million households engaged in small-scale poultry production, a common form of livestock cultivation (40). During the last decade, lager-scale poultry farming has steadily increase, accounting for 28% of Vietnam's chicken production in 2007 (4).

It has been suggested that food animal played a role as the reservoir for *bla*$_{\text{CTX-M}}$ (2, 6, 7), and that food animal holds the potential to spread ESBL-producing Enterobacteriaceae to humans (17). Surveillance conducted in Vietnam reported widespread distributions of ESBL-producing Enterobacteriaceae in livestock, fishery products (18), and retail meat (9, 22). However, the quantitative and geographical extent of the problem remains elusive (17).

After emergence of *bla*$_{\text{NDM-1}}$-positive Enterobacteriaceae (14), polymyxins, including colistin, have been regarded as "last hope" to cure Gram-negative 'superbugs' (38). Therefore, having found of the plasmid colistin resistant gene, *mcr-1*, in commensal *E. coli* of food animals had an impact on all over the world (20). In addition to b*la*$_{\text{CTX-M}}$, *bla*$_{\text{NDM-1}}$ and *mcr-1*, certain aminoglycoside modifying enzyme (AME)

genes have been detected on transmissible antibiotic resistance plasmids (30). Consequently, antibiotic resistance plasmid harboring several AMR genes have played essential role in wide distribution of AMR bacteria.

Our previous study reported the limited transmission of *E. coli* producing CTX-M-9-type ESBL between humans and poultry in a Vietnamese community (37). However, the close contacts between human and animal could further increase risk of ESBL-producing enterobacterial transmission and dissemination in large-scale farms. In this study, we investigated the prevalence and molecular characteristics of $bla_{\text{CTX-M}}$-positive *E. coli* in a Vietnamese large-scale chicken farm and the community, and evaluated the transmission of CTX-M-producing *E. coli* among workers and chickens in a large-scale chicken farm.

2. Materials and methods

2.1. Sample collection: A total of 149 fecal swabs, were collected every 6 months three times from June 2013 to June 2014 in the Bavi province in the Red River Delta region of Vietnam. We collected human and chicken fecal samples from the biggest poultry farm which contains 5,000 chickens and which was maintained by eight workers. We collected 24 fecal samples from farm workers (three times from each of the eight

workers), and 38 fecal samples from randomly selected chickens. In a community of the same area as the large-scale chicken farm, we collected 51 and 36 fecal samples from asymptomatic healthy individuals and randomly selected their own chickens, respectively. Informed consent was obtained from all participants before samplings. This study protocol was reviewed and approved by the participating institutes (University of the Ryukyus and National Institute of Nutrition, #195).

2.2. Characterization of CTX-M-producing *E. coli*: We screened for cefotaxime (CTX)-resistant Enterobacteriaceae on MacConkey agar supplemented with 1μg/ml CTX and incubated at 37ºC overnight. Bacterial species of isolates was identified by VITEK2 system (bioMerieux, Marcy l'Etoile, France). ESBL phenotypes of CTX-resistant *E. coli* isolates was confirmed by disc diffusion test described in the Clinical and Laboratory Standards Institute standard, M100-S23 (CLSI M100-S23) (10). Briefly, CTX-resistant Enterobacteriaceae was inoculated on Muller-Hinton agar with antibiotic discs, such as CTX, CTX/clavulanic acid, ceftazidime (CAZ) and CAZ/clavulanic acid. After 16-18 hours of incubation at 37ºC, diameters of zones of inhibition were measured and evaluated as described in the CLSI M100-S23. Phylogenetic group and $bla_{\text{CTX-M}}$ genotypes of the isolated ESBL-producing *E. coli* were determined by sequencing analysis as described previously (3).

2.3. Antimicrobial susceptibility tests. The antimicrobial susceptibility profiles of CTX-M-producing *E. coli* isolates were examined by disc diffusion test with Mueller-Hinton agar using 12 discs (Eiken Chemical Co., Tokyo, Japan) including ampicillin (AMP), ciprofloxacin (CIP), chloramphenicol (CHL), fosfomycin (FOF), cefoxitin (FOX), gentamicin (GEN), kanamycin (KAN), meropenem (MEM), nalidixic acid (NAL), streptomycin (STR), trimethoprim-sulfamethoxazole (SXT), and tetracycline (TET).

2.4. Detection of integrase genes, AME genes and plasmid colistin gene, *mcr-1*. PCR detections of integrase genes (*intI1*, *intI2* and *intI3*), AME genes and plasmid colistin gene, *mcr-1* was performed by as described by previous studies (8, 20, 33). The AME genes included aminoglycoside nucleotidyltransferase (ANT)(3'')-I genes (*aadA21* and *aadA11*), ANT(2'')-I gene (*aadB1*), aminoglycoside acetyltransferase (AAC)(3)-II gene (*aac(3)-II*), AAC(3)-IV gene (*aac(3)-IV*), AAC(6)-I gene (*aac(6)-Ib*), AAC(6)-II gene (*aac(6)-II*), aminoglycoside phosphotransferase (APH)(3)-VI gene (*aph(3)-VI*), and aminoglycoside-resistance methyltransferase genes (*armA* and *rmtB*).

2.5. Pulsed-field gel electrophoresis (PFGE) analysis: Total bacterial DNA was digested by XbaI. PFGE of the digested total DNA was performed to evaluate genetic relatedness among the CTX-M-producing *E. coli* isolates using the pulse-net protocol

(31). XbaI-digested genomic DNA of *Salmonella enterica* subsp. *enterica* serovar Braenderup (ATCC BAA-664) was used as molecular marker. The genetic relatedness among the CTX-M-producing *E. coli* isolates was evaluated by following the criteria proposed by Tenover et al., (35).

2.6. Statistical analysis: Statistical significance was evaluated by the $\chi 2$ test or the Fisher's exact test.

3. Results

3.1. Prevalence of CTX-M-producing *E. coli*: In this study, we set up four sampling groups, *i.e.*, workers for the large chicken farm (farm worker), bred chickens in the large chicken farm (farm chicken), asymptomatic individuals in Bavi, Vietnam community (community individual) and backyard chicken belonging to asymptomatic individuals (community chicken). The prevalence of CTX-M-producing *E. coli* was 83.3% in the farm worker group, 71.1% in the farm chicken group, 54.9% in the community individual group and 13.9% in the community chicken group. Every four phylogenetic groups were detected in the farm worker group and the community individual group; however, phylogenetic group B2 was not detected in the farm chicken group and the community chicken group. Molecular characterization of CTX-M-producing *E. coli* is

summarized in **Table 1**. There was significant difference in the prevalence of CTX-M-producing *E. coli* among the four sampling groups (p<0.05). Detected $bla_{\text{CTX-M}}$ genotypes were $bla_{\text{CTX-M-3}}$, $_{\text{-15}}$, $_{\text{-27}}$, $_{\text{-55}}$ and $_{\text{-64}}$ of $bla_{\text{CTX-M-1}}$ group and $bla_{\text{CTX-M-14}}$, $_{\text{-24}}$ and $_{\text{-65}}$ of the $bla_{\text{CTX-M-9}}$ group. Only one isolate obtained from the community individual group possessed both $bla_{\text{CTX-M-14}}$ and $bla_{\text{CTX-M-15}}$. The detection rates of each $bla_{\text{CTX-M}}$ in the four sampling groups are summarized in **Table 2**.

3.2. Antimicrobial susceptibility profile of CTX-M-producing *E. coli*: The AMR profile of CTX-M-producing *E.coli* was different between the human and chicken isolates (**Figure 1A**). Most of the CTX-M-producing *E.coli* isolates, regardless of the sampling groups, were resistant to AMP, and susceptible to FOF, FOX and MEM. CTX-M-producing *E. coli* isolates from the farm worker, farm chicken, community individual and community chicken sampling groups were resistant to an average of 4.5, 8.3, 4.2 and 6.8 different antibiotics, respectively. Regardless of the sampling groups, antibiotic resistance rates against TET, NAL, CHL, KAN, GEN and CIP in CTX-M-producing *E.coli* isolates from chicken sampling groups were higher than those of human sampling groups. There were also significant differences among the four sampling groups in the resistance ratios against TET, NAL, CHL, KAN, GEN and CIP (p<0.05).

3.3. Prevalence of AMR genes in the CTX-M-producing *E. coli* isolates: Regarding

to AMR profiles, integrase genes such as *intI1*, *intI2* and *intI3*, and AME genes were examined. Among the three integrase genes, *intI1* was detected in the CTX-M-producing *E. coli* isolates from all sampling groups (70.0% of the farm worker group isolates, 74.1% of the farm chicken group isolates, 60.7% of the community individual group isolates and 100.0% of the community chicken group isolates). The *intI2* gene was only detected in 8 of the 27 farm chicken isolates (21.1%), and *intI3* gene was not detected in all sampling groups. Among the examined AME genes, *aadA1*, *aadA2*, *aac(3)-II*, *aac(3)-IV*, *aac(6')-Ib*, *aac(6')-II* and *aadB* were detected (**Figure 1B**), and detection rates were generally higher in the chicken sampling groups. The average numbers of the detected AME genes in each isolate were 1.2 from the farm worker group isolates, 3.4 from the farm chicken group isolates, 1.2 from the community individual group isolates and 2.4 from the community chicken group isolates. The detected AMR gene ratios to the collected sample numbers of the four sampling groups were calculated in order to evaluate potential risk of antimicrobial resistance gene transmission in each sampling group (**Figure 1C**). The detection rates of *aadA1*, *aadA2*, *aac(3)-II*, *aac(3)-IV* and *mcr-1* of the farm chicken group was higher than other groups.

3.4. Genetic relatedness of the CTX-M-producing *E. coli* isolates: We used PFGE to analyze the DNA banding patterns from the CTX-M-producing *E. coli* isolates. Most of

the examined *E. coli* isolates showed genetically not-related PFGE patterns, *i.e.* there were more than three distinguishable DNA fragments among the isolates (35). As shown in **Figure 2**, our results indicated that two types of the DNA banding patterns were indistinguishable among six *E. coli* isolates-possessing $bla_{\text{CTX-M-65}}$ (isolate numbers, TB04, TB05, TB06, TB07, TB08 and TB10) and three *E. coli* isolates-possessing $bla_{\text{CTX-M-55}}$ (isolate numbers, TC02, TC05 and TC13). These $bla_{\text{CTX-M-65}}$-positive and $bla_{\text{CTX-M-55}}$-positive *E. coli* isolates were obtained from the bred chickens in a large-scale chicken farm and were *mcr-1*-negative.

3.5. Transfer of the plasmid *mcr-1* gene among CTX-M-producing *E. coli* isolates:

The plasmid colistin-resistant gene, *mcr-1* was detected in three of the four sampling groups (**Figure 1 B, C**). The prevalence of the *mcr-1* gene was one of the 20 farm worker group CTX-M-producing *E. coli* isolates (5.0%), 9 of the 27 farm chicken group CTX-M-producing *E. coli* isolates (33.3%) and one of the 5 community chicken group CTX-M -producing *E. coli* isolates (20.0%). As same as AME genes, the detected *mcr-1* ratio to each collected sample numbers of the farm chicken group (23.7%) was higher than the farm worker (4.2%), community individual (0.0%) and community chicken (2.8%) groups (**Figure 1C**). Various DNA banding patterns were obtained by PFGE; however, any indistinguishable DNA banding pattern was not observed among

the *mcr-1* positive CTX-M-producing *E. coli* isolates (data not shown).

4. Discussion

In this study, we investigated CTX-M-producing *E. coli* isolated from asymptomatic healthy individuals and chickens in a large-scale chicken farm and a community, the Bavi district, Vietnam. Our results indicated higher prevalence rates of CTX-M-producing *E. coli* in the four sampling groups. The obtained *bla*$_{\text{CTX-M}}$-positive *E. coli* isolates were genetically diverse as observed by the PFGE banding patterns. In this study, eight kinds of *bla*$_{\text{CTX-M}}$ were detected. Most types of *bla*$_{\text{CTX-M}}$s, including *bla*$_{\text{CTX-M-14}}$ and *bla*$_{\text{CTX-M-15}}$, were often observed worldwide, mainly in clinical settings. The *bla*$_{\text{CTX-M-14}}$, *bla*$_{\text{CTX-M-55}}$ and *bla*$_{\text{CTX-M-65}}$ were also detected in food and pets animals in Southeast and East Asian countries (1, 9, 13, 19, 21, 41, 42). Accordingly, CTX-M-producing *E. coli* isolated from the large-scale chicken farm possessed only *bla*$_{\text{CTX-M-14}}$, *bla*$_{\text{CTX-M-55}}$ or *bla*$_{\text{CTX-M-65}}$ (**Table 2**).

Regardless of the sampling group, results obtained in this study indicated that the molecular characteristics of the CTX-M-producing *E. coli* isolates from asymptomatic healthy individuals and chicken were not identical. Therefore this study supports our previous observation that the transmission risk of CTX-M-producing *E.*

coli between human and chicken is low (37). This suggests that the risk of CTX-M-producing *E. coli* transmission between a large-scale chicken farm and surrounding communities is low. However, we observed high prevalence of CTX-M-producing *E. coli* both in the large-scale chicken farm and the community; therefore, monitoring of these antimicrobial-resistance bacteria is essential to prevent the spread of antimicrobial-resistance bacteria in the community.

Previous studies reported low prevalence (<0.2%) of ESBL-producing *E. coli* in chicken farms in the Mekong Delta of Vietnam (25, 26). However, our results indicated extremely high prevalence (71.1%) of CTX-M-producing *E. coli* of the bred chicken of the large-scale chicken farm in the Red River Delta region of Vietnam. In the Red River Delta region, various antibiotics have been used to promote growth of poultry and to prevent the spread of diseases (12). Particularly, , ampicillin, fluoroquinolones, sulfonamides, tetracyclins and colistin have been used for disease prevention in the Red River Delta region (12). Accordingly, resistance rates against the frequently used antibiotics, such as AMP, SXT, TET and CIP, were high in this study. Most of the $bla_{\text{CTX-M}}$ are located on antibiotic resistance plasmid (15, 32). The frequent use of antibiotics adds selective pressure for the CTX-M type ESBL *E. coli* in the large-scale chicken farm. Consequently, clonal transmission of *E. coli* isolates-possessing

bla$_{\text{CTX-M-65}}$ or *bla*$_{\text{CTX-M-55}}$ isolates in **Figure 2** might have occurred among chickens in the chicken farm.

We detected one CTX-M-producing *E. coli* isolates-possessing plasmid colistin-resistant gene, *mcr-1* from a worker for the large-scale chicken farm in addition to 10 CTX-M-producing *E. coli* isolates from chickens. The detection rate of *mcr-1*-positive *E.coli* in chicken and farm workers at the chicken farm could have been enhanced by the use of colistin, which is used in poultry production in the Red River Delta region (12). Our PFGE results indicate that there was no genetic relatedness between the *mcr-1*-positive *E. coli* isolates from human and chicken. Thus, colistin usage in the large-scale chicken farm might select *mcr-1*-positive *E. coli* may have transferred from chicken feces or soil to the workers.

This study described the current prevalence of CTX-M-producing *E. coli* in a large-scale farm and surrounding community. Our results suggested potential transmission risk of AMR bacteria from chicken to human, and among bred chickens. Further epidemiological analysis and higher sample number are essential for identifying risk factor(s) for the transmission of AMR bacteria.

Declarations

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Ethical Approval: University of the Ryukyus and National Institute of Nutrition, #195

Competing Interests: None declared.

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7. Figure legends

Figure 1. Antibiotic resistance characterization of CTX-M-producing *E. coli* isolates. (**A**) Antibiotic resistance profiles of CTX-M-producing *E. coli* isolates from four sampling groups were determined by disc diffusion test using 12 discs including ampicillin (AMP), ciprofloxacin (CIP), chloramphenicol (CHL), fosfomycin (FOF), cefoxitin (FOX), gentamicin (GEN), kanamycin (KAN), meropenem (MEM), nalidixic acid (NAL), streptomycin (STR), trimethoprim-sulfamethoxazole (SXT), and Tetracycline (TET). (**B** and **C**) AME genes and the plasmid colistin-resistant gene, *mcr-1*were detected. The detection ratios of CTX-M-producing *E. coli* isolate numbers to the total isolates numbers obtained from the four sampling groups (**B**), and those to the sample numbers of the four sampling groups (**C**) are shown. Groups are labeled as follows: Farm worker, workers for the large-scale Vietnamese chicken farm; Farm chicken, bred chicken in the large-scale Vietnamese chicken farm; Community individual, asymptomatic healthy individuals in a community; Community chicken, chicken belonged to the asymptomatic healthy individuals in the community.

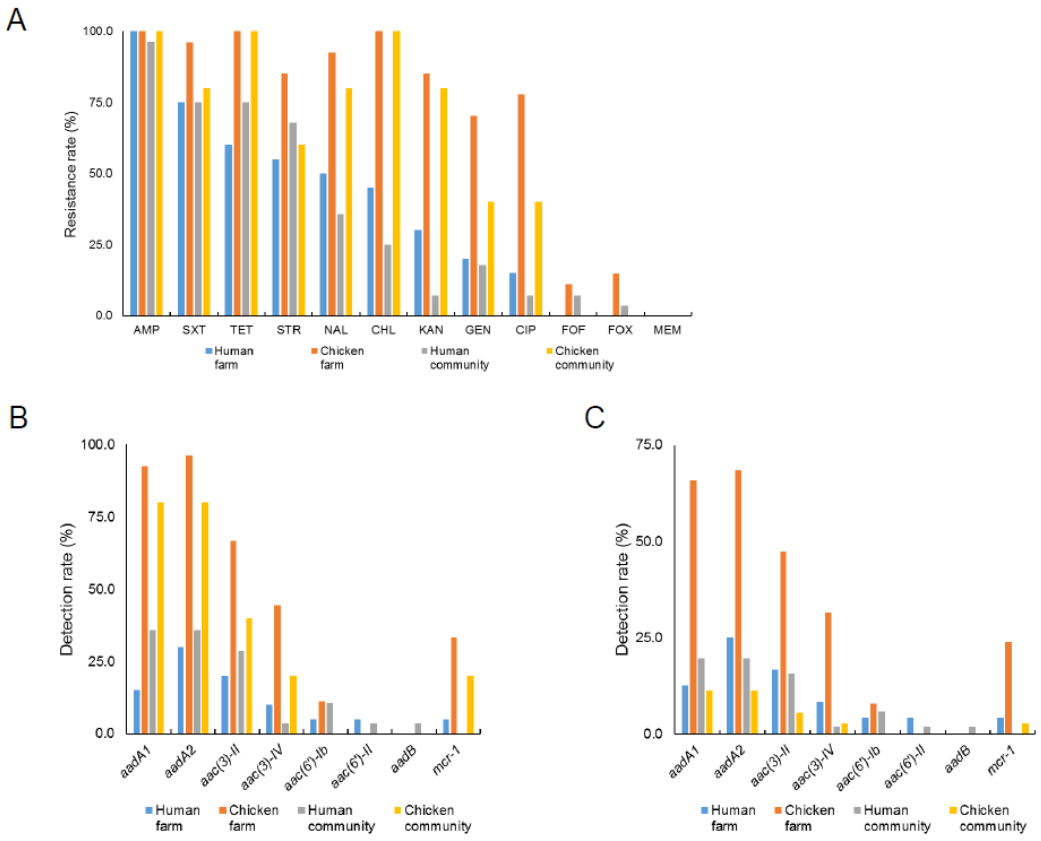

Figure 2. PFGE profiles of CTX-M-producing *E. coli*. XbaI-digested bacterial DNA was developed by PFGE. PFGE banding patterns, which was indistinguishable among equal to 3 or more than 3 isolates, were observed among the CTX-M-producing *E. coli* isolates obtained from bred chicken in the large-scale chicken farm. Black squares indicate resistance to antibiotic or detected integrase and AME genes. Abbreviation are as follows: M, XbaI-digested *Salmonella* Braenderup; Phylo. group, phylogenetic group.

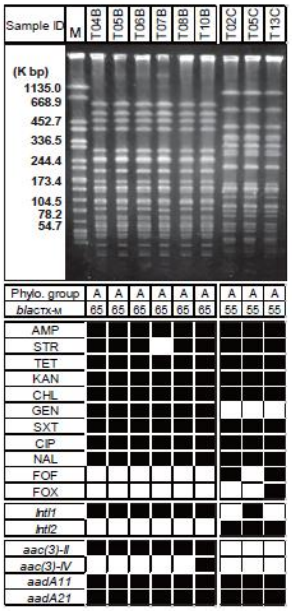

Table 1. Genotyping of *E. coli*-possessing *bla*$_{CTX-M}$s.

		Detected *bla*$_{CTX-M}$ group						Detected phylogenetic group							
		any CTX-M		CTX-M-1		CTX-M-9		A		B1		B2		D	
	Total	n	%	n	%*	n	%*	n	%*	n	%*	n	%*	n	%*
Farm worker	24	20	83.3	14	70.0	6	30.0	6	30.0	6	30.0	1	5.0	7	35.0
Farm chicken	38	27	71.1	9	33.3	18	66.7	14	51.9	5	18.5	0	0.0	8	29.6
Cmty** individual	51	28	54.9	22	78.6	7	25.0	11	39.3	6	21.4	1	3.6	10	35.7
Cmty** chicken	36	5	13.9	1	20.0	4	80.0	4	80.0	0	0.0	0	0.0	1	20.0

*; Detection rates of *bla*$_{CTX-M}$ (%) and phylogenetic group (%) are indicated in percentage of the detected *E.coli*-possessing any kind of *bla*$_{CTX-M}$.

**; cmty, community.																							

Table 2. Detection rates of *bla*$_{CTX-M}$ genotypes.

		CTX-M-1group				CTX-M-9 group											
		3		15		27		55		64		14		24		65	
	Total	n	%	n	%*	n	%*	n	%*	n	%*	n	%*	n	%*	n	%*
Farm worker	20	1	5.0	0	0.0	12	60.0	1	5.0	0	0.0	5	25.0	0	0.0	1	5.0
Farm chicken	27	0	0.0	0	0.0	0	0.0	9	33.3	0	0.0	11	40.7	0	0.0	7	25.9
Cmty** Indvidual	28	0	0.0	1	3.6	18	64.3	3	10.7	0	0.0	6	21.4	0	0.0	1	3.6
Cmty** chicken	5	0	0.0	0	0.0	0	0.0	0	0.0	1	20.0	3	60.0	1	20.0	0	0.0

*; Detection rates of each *bla*$_{CTX-M}$ (%) are indicated in percentage of the detected *E.coli*-possessing any kind of *bla*$_{CTX-M}$.

**; cmty, community.																								