MINISTRY OF EDUCATION AND TRAINING
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DETERMINATION OF MICROBIOLOGICAL CHARACTERISTICS OF EXTENDED - SPECTRUM BETA- LACTAMASE PRODUCING *ESCHERICHIA COLI ISOLATED FROM* HEALTHY INDIVIDUALS LIVING IN THE COMMUNITY VU THU DISTRICT,THAI BINH PROVINCE, 2016

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The thesis will be defended to the Institute-level council for evaluating PhD thesis at the National Institute of Hygiene and Epidemiology.

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The thesis can be found at

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LIST OF PUBLICATION RELATED TO THESIS

1. Khong Thi Diep, Pham Ngoc Khai, Do Thi Bich Ngoc, Hoang Thi Thu Ha, 2019," Phylogenetic of ESBL- producing *Escherichia coli* strains isolated from healthy individuals in Vu Thu, Thai Binh, 2016", *Vietnam Journal of Preventive Medicine*, 29 (3):42-47.

2. Khong Thi Diep, Pham Ngoc Khai, Tran Huy Hoang, Pham Duy Thai, Hoang Thi Thu Ha, (2019), "Evaluation the transmission ability of plasmid harboring ESBL gene from ESBL-producing *Escherichia coli* strains isolated from stool samples in healthy individuals to *Escherichia coli* J53 by conjugation", *Vietnam Journal of preventive medicine*, 29 (12):77-83

3. Khong Thi Diep, Pham Ngoc Khai, Hoang Thi Thu Ha, 2019, "Carriage of ESBL-producing *Escherichia coli* in healthy individuals in Nguyen Xa commune, Vu Thu district, Thai Binh province, 2016", *Vietnam Journal of preventive medicine*, 29 (12):111-117

INTRODUCTION

In recent years, spread of antibiotic-resistant bacteria has increased, which is becoming a major threat to public health all over the world.

There are many antibiotic resistance mechanisms of which the antibiotics inhibited by Extended-Spectrum Beta-Lactamases (ESBLs) is a common mechanism. ESBLs are often found in *Enterobacteriaceae* groups, common in *Escherichia coli* (*E. coli*). The ESBL coding genes are often located on large plasmids. Therefore, ESBL coding genes are easily transferred to another bacteria in the same or different species, which leads to an increase in antibiotic resistance.

ESBL-producing *E. coli* are found all over the world, especially in China and Southeast Asia, where high rates of ESBL-producing *E. coli* (over 50%) have been observed in both hospitals and communities.

In Vietnam, most of the studies on ESBL-producing *E. coli* were in hospitals, little is known about microbiological characteristics of ESBL-producing *E. coli* in the community. Improving the understanding of the microbiological characteristics of ESBL-producing *E. coli* in the community and thus the epidemiology of this bacteria will provide a scientific basis to establish a surveillance and prevention system for infection and spread of antibiotic-resistant bacteria in Vietnamese communities.

Therefore, we conducted the study: "**Determination of microbiological characteristics of Extended - Spectrum Beta-Lactamase producing *Escherichia coli* isolated from healthy individuals living in the community, Vu Thu district, Thai Binh province, 2016**"

***Aim of the study*:**

1. To determine the dissemination of Extended-Spectrum Beta-

Lactamase producing *Escherichia coli* in healthy individuals in a rural community, in Vu Thu district, Thai Binh province, 2016.

2. To identify microbiological characteristics of Extended- Spectrum Beta-Lactamase producing *Escherichia coli* isolated from healthy individuals in a rural community, in Vu Thu district, Thai Binh province, 2016.

Scientific findings and practical value of the topic

1. The result of the study showed that the prevalence of ESBL-producing *E. coli* strains that had multi-drug resistance was very high (86.1%), which contributes to the seriousness of the multi-drug resistance in bacteria situation in the communities in Vu Thu. Therefore, interventions are needed to reduce the risk of antibiotic resistance in the healthy individuals.

2. The study found that the prevalence of carrying colistin-resistant gens (*mcr-1*) within ESBL-producing *E. coli* strains isolated from healthy individuals in Vu Thu, Thai Binh was 8% (11/137).

3. This is one of the first studies in Vietnam, which used PFGE, Southern Blot, and conjugation techniques to study ESBL-producing *E. coli* in healthy individuals in rural communities. Our results showed that the combined use of these techniques can assess the ability of transmission of ESBL-producing *E. coli* strains in our setting

STRUCTURE OF THE THESIS

The thesis consists of 126 pages: Introduction (2 pages), overview (34 pages), subjects and research methods (33 pages), research results (30 pages), discussion (24 pages), conclusion (2 pages), recommendations (1 page). In the thesis, there are 44 tables, 11 graphs, 13 figures. The thesis has 127 references; 23 in Vietnamese and 104 in English.

Chapter 1. OVERVIEW

1.1. Carrying and antibiotic resistance of Extended-Spectrum Beta-Lactamase producing *E. coli* in humans

1.1.1. Carrying of Extended - Spectrum Beta-Lactamase producing Escherichia coli in humans.

Currently, ESBL-producing E. coli is increasing in many parts of the world. The bacterium has been detected in many hospitals on most continents, especially in Asian countries like India (79%), China (55%), Thailand (50.8%), and Vietnam (51.6%). The bacterium has also been detected in healthy individuals in communities of several countries such as Switzerland (5.8%), Germany (6.3%), China (50.5%), and Thailand (61.7%).

1.1.2. Antibiotic resistance situation of ESBLs - producing E. coli in humans

The antibiotic resistance of ESBL-producing *E. coli* strains is much higher than that of non-ESBL-producing bacteria. Previous studies showed that antibiotic resistance of ESBL-producing *E. coli* is increasing. This bacterium is resistant not only to common antibiotics at high prevalence of the bacterium but also to colistin and carbapenem. Most ESBL-producing *E. coli* strains are multidrug-resistant bacteria, which are resistant to at least three or more antibiotic groups.

1.2. ESBLs-producing *E. coli* characteristics and research methods of ESBLs-producing *E. coli*.

1.2.1. Biological characteristics

1.2.2. Characteristics and wide dissemination of ESBLs and ESBLs coding genes

ESBLs are enzymes produced by specific bacteria hydrolyzing extended-spectrum cephalosporin. Therefore, ESBLs resistant to beta-lactam antibiotics such as ceftazidime, ceftriaxone, cefotaxime, and oxyimino-monobactam.

ESBLs are often found in Gram-negative bacteria, especially in *Enterobacteriaceae*. There are many types of ESBLs, of which TEM, SHV, and CTX-M are the most common and most important.

These types of ESBL are constantly changing, increasing in number, and more complex than other ESBLs. Currently, more than 500 types of ESBL have been detected. During the 1990s, most reports of ESBL focused on TEM, SHV-type ESBLs, which were associated with cross-infection in hospitals. However, recent reports indicated that the presence of CTX-M type ESBLs is the most common.

1.2.3. Characteristics of phylogenetic group

Phylogenetic of *E. coli* falls into four main phylogenetic groups A, B1, B2, and D. Each group has different characteristics of ecological environment, host, pathogenicity, and antibiotic resistance ability.

1.2.4. Pathogenic characteristics of E. coli in humans

E. coli causes diarrhea, urinary tract infections, sepsis, and pneumonia in newborns. Diarrhea is the most common condition related to the pathogenicity of *E. coli*. The ability and mechanism of causing diarrhea of each *E. coli* group depend on the virulence factors, and toxins.

1.2.5. Ability to spread ESBL-producing E. coli

ESBLs coding genes are mainly located on plasmids, although some are located on transpose, integron. Thus, most of the transmission of antibiotic-resistant genes of ESBL-producing bacteria is often related to these mobile genetic factors. These diverse mechanisms of genetic transmission contribute to the rapid spread of resistance genes.

1.2.6. Research methods for ESBL-producing E. coli

**** Methods of diagnosis of ESBL-producing E. coli***

Clinical microbiological methods include combination disk diffusion test, Minimum Inhibitor Concentrate (MIC), E-test, automatic method using Vitek /BD Phoenix, and Micro scan panel.

Molecular biology methods include oligotyping, Polymerase Chain Reaction (PCR), Restriction Fragment Length Polymorphisms (RFLP), PCR single-strDNA, Ligase chain reaction, sequencing

**** Molecular biology methods research on ESBL-producing E. coli***

Modern methods of studying the origin and transmission ability of ESBL-producing *E. coli* include Pulsed-field Gel Electrophoresis (PFGE), plasmid characteristics analysis, Southern Blotting, conjugation, Multilocus Sequence Typing (MLST) and sequencing.

Chapter 2. METHODS

2.1. Subject, place and time of study

2.1.1. Sampling site

Nguyen Xa Commune, Vu Thu District, Thai Binh Province.

2.1.2. Research time

- Aim 1: 2016
- Aim 2: From 2016 to 2018

2.1.3. Research subjects

- Aim 1: Stool samples collected from healthy individuals at Nguyen Xa commune, Vu Thu district, Thai Binh province
- Aim 2: *E. coli* strains isolated from stool samples of healthy individuals at Nguyen Xa commune, Vu Thu district, Thai Binh province

2.2. Methods

2.2.1. Research design

- Aim 1: Descriptive epidemiological research based on a cross-sectional survey testing stool samples from healthy individuals in a rural commune of Thai Binh to determine the prevalence of ESBL-producing *E. coli* in healthy individuals in Vu Thu district, Thai Binh province.
- Aim2: Descriptive epidemiological research based on analysis and identification of biological characteristics of ESBL-producing *E. coli* strains isolated from stool samples collected from healthy individuals in Nguyen Xa commune, Vu Thu district, Thai Binh province.

2.2.2. Sample selection and sample size

****Sample selection***

+ Sampling site selection: Nguyen Xa commune in Vu Thu district was randomly selected for the present study. In Nguyen Xa, we randomly selected Kien Xa village and 60 households in Kien Xa village for sampling

+ Participants selection: All persons living in the households except those undergoing acute medical treatment and/or antibiotic historical use within three months prior were selected for collection of stool samples.

**** Sample size:*** The sample size to determine the prevalence of *E. coli* carrying in the community is applied by the following formula:

$$n = Z^2_{1-\alpha/2} \frac{p(1-p)}{(p.\varepsilon)^2} \times k$$

- n: Study sample size.
- α /2: Reliability is statistically significant, in this study, it is taken at the threshold α = 0.05; $Z_{1-\alpha/2} = 1.96$.
- p: Estimate the proportion of healthy individual carrying ESBL-producing *E. coli* through a previous trial survey (p: was selected as 65%).
- ε: The expected error coefficient of p, in this study we chose ε = 0.15.
- k: Design coefficient when selecting a beam sample, with k = 2.

With the above data, the calculated sample size was 184 samples. To ensure the sample size, we add more 20% of the participants to the list. Totally, we collected 212 stool samples from 212 individuals from 59 households.

2.3. Variables and indicators

- Variables and indices of the dissemination of ESBL-producing *E. coli* in the community.
- Variables and indices of microbiological characteristics of ESBL-producing *E. coli* strains.

2.4. Materials

Reagents, tests, machines, equipment, and software used in research.

2.5. The techniques used in the study

	Techniques	Place of conduct
1	Stool sampling	Nguyen Xa commune
2	Isolation and identification of *E. coli* from stool samples based on biochemical	Centre for Medical-Pharmaceutical Research and Service, Thai Binh University of Medicine and Pharmacy (TBUMP)
3	Determination of ESBL phenotype of *E. coli* by combination disk diffusion test	
4	Determination of antibiotic-resistant characteristics of ESBL-*E. coli* by disk diffusion method	
5	Determination of ESBL-producing genes coding of ESBL- *E. coli* by multiplex PCR	
6	Determination of colistin-resistant gene coding (*mcr-1*) of ESBL-*E. coli* by realtime PCR	
7	Identify the phylogenetic groups of ESBL-*E. coli* by multiplex PCR	
8	Determination of virulence genes of ESBL- *E. coli* by multiplex PCR	
9	Analysis of the profile of plasmids which carrying ESBL genes by multiplex PCR	
10	Analysis of genotypic relationship between ESBL-*E. coli* strains by PFGE method	National Institute of Hygiene and Epidemiology (NIHE)
11	Locating ESBL- genes by Southern Blot	Osaka Institute of Public Health, Osaka, Japan
12	Evaluating the ability of ESBL genes transmission by conjugation	NIHE, TBUMP

2.6. Data analysis

Apply algorithms commonly used in biomedical research

2.7. Measures to control errors

Measures have been taken to control errors in the study

2.8. Ethical approval

Ethical approval of the study was granted by the Ethics Committee for Biomedical Research of Thai Binh University of Medicine and Pharmacy.

Chapter 3. RESULTS

3.1. Dissemination of ESBL-producing *E. coli* isolated stool samples collected from healthy individual in a rural community in Thai Binh province

3.1.1. Characteristics of participants

The study included 212 participants from 59 households of which 101 were males and 111 were females. Each household had 2 to 7 members. Age of the participants ranged 1 to 89 years and average age was 40.1 years (SD:± 23.08 years). The educational attainment of most participants was secondary school (50%) and high school (25%). The most common occupation of the participants was farming (43.6%).

3.1.2. Dissemination of ESBL-producing E. coli in stool samples collected from healthy individuals.

Table 3.6. Results of screen stool samples on MacConkey with CTX 1µg / ml

Kind of bacteria growth in MacConkey with CTX	Number	Percentage (%)
E. coli	169	79.7
Not E. coli	28	13.2
No have bacteria growth	15	7,1
Total	212	100.0

Results showed that 79.7% of healthy individuals carried CTX-resistant *E. coli*, 13.2% had other CTX-resistant *Enterobacteriaceae*

Table 3.7. Prevalence of ESBL-producing E. coli in stool samples

ESBL- producing *E. coli*	Number	Prevalence (%)
In community (n=212)	137	64,6
Among CTX-resistant *E. coli* (n=169)	137	81,1

The prevalence of ESBL-producing *E. coli* isolated from stool

samples from healthy individuals was 64.6%. ESBL-producing prevalence of CTX- resistant *E. coli* was 81.1%.

ESBL-producing *E. coli* was found in participants at all ages and in almost (55/59) all households selected for the study. There was no difference in the prevalence of carrying ESBL-producing *E. coli* across sex, education level, or occupations.

3.2. Microbiological characteristics of ESBL-producing *E. coli*

3.2.1. Biochemical characteristics of ESBL-producing E. coli

Most of ESBL-producing E. coli strains has fully biological and chemical characteristics of typical *E. coli* on 3 TSI, LIM, and CLIG such as glucose fermentation (100%), no H2S producing (100 %), Indol producing (94.9%), no cellobiose fermentation (100%), and β-glucuronidase hydrolysis (78.8%).

3.2.2. Antibiotic-resistant characteristic of ESBL-producing E. coli

Table 15. Prevalence of resistant to antibiotics of ESBL-producing E. coli

Antibiotic	Sensitivity	Intermediaries	Resistant
	Number (%)		
AMP	0 (0,0)	0 (0,0)	**137 (100,0)**
CAZ	35 (25,5)	59 (43,1)	43 (31,4)
FOX	130 (94,9)	1 (0,7)	6 (4,4)
MEM	135 (98,5)	0 (0,0)	2 (1,5)
STR	20 (14,6)	24 (17,5)	93 (67,9)
KAN	87 (63,5)	21 (15,3)	29 (21,2)
GEN	91 (66,4)	2 (1,5)	44 (32,1)
CIP	82 (59,9)	4 (2,9)	51 (37,2))
NAL	57 (41,6)	2 (1,5)	78 (56,9)
TET	29 (21,2)	2 (1,5)	106 (77,4)
CHL	88 (64,2)	2 (1,5)	47 (34,3)
SXT	26 (19)	0 (0,0)	111(81,0)
FOF	134 (97,8)	1 (0,7)	2 (1,5)

ESBL-producing *E. coli* strains were resistant to common antibiotics at a high rate (from 21.2% to 100%). However, this bacterium is sensitive to cefoxitin, fosfomycin, and meropenem.

All ESBL-producing *E. coli* strains were resistant to antibiotics ranging from 1 to 12 of the 13 antibiotics tested, of which the most common were resistant to ranging from 3 to 9 antibiotics. The prevalence of insensitivity to 3 or more antibiotic groups (MDR) was 86.1%, of these 26.3% were not sensitive to five antibiotic groups, and 22.6% were not sensitive to six antibiotic groups.

3.2.3. Characteristics of ESBLs coding genes in ESBL-producing E. coli

The prevalence of ESBL-producing *E. coli* strains carrying genes coding for CTX-M group was 94.1%, of which *blaCTX-M-9* was predominant with 66.3%, followed by *blaCTX-M-1* (26.3%) and *blaCTX-M-9/CTX-M-1* (1.5%). The prevalence of *blaTEM* was 45.3%. No strain carrying *blaSHV* was detected.

ESBL-producing *E. coli* can carry one gene (55.5%), two genes (41.6%), or three genes simultaneously (0.7%) coding for the ESBL.

Table 3.19. Prevalence of ESBL genotype of ESBL-producing E. coli

ESBL genotype	Number	Percentage (%)
blaCTX-M-1	13	9.5
blaCTX-M-1/CTX-M-9	1	0.7
blaCTX-M-1/CTX-M-9/TEM	1	0.7
blaCTX-M-1/TEM	23	16.8
blaCTX-M-9	58	**42.3**
blaCTX-M-9/TEM	33	24.1
blaTEM	5	3.6
No detected any genotype above	3	2.2
Total	137	100.0

The most common genotype was *blaCTX-M-9 (42.3%)*, followed by *blaCTX-M-9/TEM (24.1%)* and *blaCTX-M-1/TEM (16.8%)*. Other genotypes were low proportions.

Table 3.20. Prevalence of antibiotic resistance of E. coli strains carrying blaCTX-M-1 and blaCTX-M-9 genotypes

Antibiotic	*blaCTX-M-1* (n=36)		*blaCTX-M-9* (n=91)		p
	Number	Percentage(%)	Number	Percentage(%)	
AMP	36	100,0	91	100,0	>0,05
CAZ	**23**	**63,9**	**15**	**16.5**	**<0,05**
FOX	1	2,8	5	5.5	> 0,05
MEM	0	0,0	2	2.2	> 0,05
STR	27	75,0	57	62.6	> 0,05
KAN	**17**	**47,2**	**11**	**12.1**	**< 0,05**
GEN	15	41,7	27	29.7	> 0,05
CIP	23	63,9	25	27.5	< 0,05
NAL	**27**	**75,0**	**44**	**48.4**	**< 0,05**
TET	30	83,3	67	73.6	> 0,05
CHL	**20**	**55,6**	**24**	**26.4**	**< 0,05**
SXT	30	83,3	71	78.0	> 0,05
FOF	1	2,8	1	1.1	> 0,05

E. coli strains carrying the *blaCTX-M-1* genotype have a higher resistance prevalence to antibiotics such as CAZ, KAN, NAL, CHL than that in the *blaCTX-M-9* genotype ($p < 0.05$).

The strains carrying the genotype *blaCTX-M-1* had the lowest prevalence of multi-drug resistance (69.2%). Most of the strains belonged to other genotypes were multi-drug resistance strains (prevalence of multi-drug resistance over 90%). The more ESBL genes the strains carried, the higher prevalence of multi-drug resistance.

The results of the study showed that 11/137 (8.0%) of ESBL-producing *E. coli* strains carried *mcr-1* (a colistin-resistant gene).

3.2.4. Phylogenetic grouping characteristics of ESBL-producing E. coli strains

Phylogenetic analysis showed that the ESBL-producing *E. coli* strains belonged to four phylogenetic groups: A, D, B1, and B2. Of these, A group was highest (43.1%), followed by D group (32.1%), B1 group (14.6%), and the lowest proportion was those in the B2 group (10.2%). There were differences in the level of antibiotic resistance to streptomycin, gentamycin, ciprofloxacin, and chloramphenicol among phylogenetic groups. The prevalence's of multi-drug resistance was not significant difference between phylogenetic groups

3.2.5. Virulence genes characteristics of ESBL-producing E. coli

Table 3.24. Distribution of virulence genes among ESBL-producing E. coli

Diarrhea *E. coli*	Virulence gene	Number	Percentage (%)
EAEC	*AstA*	29	21.1
EPEC	*AstA, bfpA*	6	4.4
	bfpA	8	5.8
	eaeA	6	4.4
	AstA, eaeA	1	0.7
	Total	**21**	**15.6**
ETEC	*AstA, LT, StIa*	1	0.7
	AstA, StIb	1	0.7
	LT, StIa	1	0.7
	StIb	3	2.2
	Total	***7***	***5.0***
EAEC / EPEC	*aggR, bfp*	1	0.7
EAEC /DAEC	*AstA, daaD*	5	***3.6***
Total of strains carrying virulence gene		**63**	**46.0**
	No detection any virulence gene	74	54.0
Total		137	100.0

In this study, virulence genes were found in 46% of ESBL-producing *E. coli* strains. Of these, 21.1% belonged to EAEC, 15.6% belonged to EPEC, 5% belonged to ETEC, 3.6% belonged to EAEC/DEAC, and 0.7% belonged to EAEC/ EPEC.

Table 25. Multi-drug resistance characteristics of ESBL-producing E. coli strains carrying virulence genes

Diarrhea *E. coli*	Non-multi-drug resistance strains		Multi-drug resistance strains	
EAEC	2	6.9	27	93.1
EPEC	1	4.8	20	95.2
EAEC /DAEC	0	0	5	100
ETEC	3	50.0	3	50.0
EAEC / EPEC	0	0	1	100
Not diarrhea *E. coli*	7	9.45	67	90.55

All the EAEC/DAEC and EAEC/EPEC strains were multi-drug resistance. The prevalence of multi-drug resistance in the EAEC, EPEC, and non-virulent strains was high (>90%) whereas the prevalence in ETEC strains was 50.0%. There was no difference in the contribution of the virulence genes among phylogenetic groups.

3.2.6. Genotypic relationship between ESBL-producing E. coli

Among 137 strains of ESBL- producing *E. coli*, 4 strains could not be typed by PFGE. Examination of the remaining 133 PFGE patterns showed that 54.9% strains corresponded to non-genetic-related strains, whereas 45.1 % strains were assigned to clonal groups with >80% of similarity. Of the latter, 32 strains (24%) were closely related with 95-100% of similarity; the 20 of the 32 strains were completely homologous genotype (100% of similarity).

3.2.7. Plasmid profile of ESBL-producing E. coli

The plasmid replicons were determined in 127 (92.7%) of the 137 strains tested, with a total 283 replicons. The ranging of plasmid replicons among the strains from one to six, of which the strains carrying two plasmid replicons were most common (42.3%).

Table 3.27. Prevalence of plasmid types in ESBL-producing E. coli

Plasmid type	Number	Percentage (%)
B/O	30	21.9
FIC	4	2.92
A/C	2	1.46
P	6	4.38
T	2	1.46
FIIA	2	1.46
FIA	34	24.82
FIB	78	56.93
Y	11	8.03
K/B	4	2.92
I1	13	9.49
Frep	71	51.82
X	6	4.38
HI1	5	3.65
N	5	3.65
HI2	6	4.38
L/M	4	2.92
W	0	0

Among 18 plasmid replicons used to determine plasmid characteristics in the ESBL-producing *E. coli* strains, FIB replicon was the most frequent (56.93%), followed by Frep replicon (51.82%), FIA replicon (24.82%), B/O replicon (21.9%) and I1 replicon (9.49%). Other plasmid replicons such as FIC, A/C, P, T, FIIA, Y, K/B, X, HI1, N, HI2, and L/M were detected at low rates. No strain with W plasmid replicon was detected.

The result of the detection of ESBL-genes location by Southern Blotting in 37 strains randomly selected from 137 ESBL-producing *E. coli* strains showed that (67.6% strains containing plasmid that

harboring ESBL coding genes. The proportions of strains contained only plasmid *blaCTX-M-1,* plasmid *blaCTX-M-9,* and plasmid *blaTEM* were 36.4%, 76%, and 75% respectively. Moreover, among the 11 strains carrying both *blaCTX-M* and *blaTEM* genes, two strains carried these genes on the same plasmid while five strains carried these genes on different plasmids.

The result of conjugational transfer of ESBL plasmids from 41 ESBL-producing *E. coli* strains carrying ESBL genes to the laboratory strain *E. coli* J53 showed that 39% (16/41) of strains transferred their ESBLs plasmid to *E. coli* J53 (with red colonies on MacConkey contained cefotaxime and NaN3). All of the transconjugants were confirmed to be ESBLs positive by PCR. The result indicates that we successfully transferred the plasmid carrying ESBL-producing genes from ESBL-producing *E. coli* in our setting to *E. coli* J53 in a laboratory model. The proportions of successful transferred of plasmid *blaCTX-M-1,* plasmid *blaCTX-M-9,* and plasmid *blaTEM* were 20%, 45.2%, and 25% respectively.

Table 3.32. The number of genes that can be transferred on strains carrying two ESBL genes coding

Number of ESBL-gene be transferred	Number	Percentage (%)
2 genes	5	25.0 %
1 gene	2	10.0 %
None of 2 genes	13	65.0
Total	20	100.0

In this study, 20 out of 41 strains used for conjugation carried two ESBL encoding genes simultaneously. Our result showed that conjugational transfer of ESBL plasmids was successful in 7 strains, of which 5/20 (25.0%) strains transferred plasmids two genes.

Chapter 4. DISCUSSION

4.1. Dissemination of ESBL-producing *E. coli* in stool samples

collected from healthy individual in a rural community in Thai Binh province

The prevalence of ESBL-producing *E. coli* in the community in this study (64.6%) are in line with studies in Asia from China (50.5%), Thailand (61.7%), and Ho Chi Minh City (63.1%). The widespread use of antibiotics in treating and in agriculture may one of the causes leading to the appearance and increase of antibiotic resistant bacteria. In addition, the habit of using human and cattle manure in agriculture in Thai Binh combined with the tropical conditions in Vietnam may increase the survival and multiplication of ESBL-producing bacteria in human stools leading to increasing risk of ESBL infection in ruralcommunities. In addition, the high prevalence (68.4%) of ESBL-producing *E. coli* in food samples in this area can be an important source transmission of ESBL-producing *E. coli* in healthy people in the area.

The prevalence of producing ESBL in CTX-resistant *E. coli* strains was very high (81.1%). The result is consistent with the prevalence of ESBL producing in cephalosporin-resistant *E. coli* strains in a study conducted in 30 European countries. Thus, it is possible that producing ESBLs enzyme may be the main mechanism of resistance to 3rd generation cephalosporin of *E. coli* strains.

ESBL-producing *E. coli* was found in 93.2% of households and in 35.6% of the households the bacteria was detected in all household members. This result suggests that members of the same household may spread of ESBL-producing *E. coli* between them. This may happen by sharing of food and drinking water, but could also be due to sharing the same environmental conditions in daily activities such as the water and toilets. These are all conditions that are consistent with the ways of *E. coli* is transmitted such as contaminated food, water, and contact with an infected person.

4.2. Microbiological characteristics of ESBL-producing *E. coli* strains

42.2. Antibiotic-resistant characteristics of ESBL-producing E. coli strains

ESBL-producing *E. coli* was resistant to common antibiotics at a high rate (21.2-100%). In Addition,these strains were resistant to many antibiotics simultaneously, particularly the proportion of strains resistant to between 3 to 9 antibiotics was very high (86.1%). This may be caused by the wide spread use of antibiotics in Vietnam. Although, Ministy of Health has regulations on prescribing and selling prescription drugs, people can still buy antibiotics directly from pharmacies and retail pharmacies without a prescription. Self-treatment is a fairly common condition, even though self-diagnosis is often very inaccurate. Moreover, due to a lack of knowledge of antibiotic use, many people use antibiotics without following the instructions on antibiotic duration and dosage. Therefore, measures are needed to manage both antibiotic prescribtion and use in pharmacies, hospitals and communities to limit the increase of antibiotic resistance, especially multi-drug resistance in the community.

4.2.3. Characteristics of ESBLs coding genes in ESBL-producing E. coli.

The distribution of ESBL-producing genes, including *blaCTX-M* group (94.1%), and *blaTEM* (45.3%) in our study is similar to recent studies in Vietnam. Toghether these results indicate that the trend of distribution of ESBL-producing genes in Vietnam is consistent with that in the world, particularly the widespread distribution of *blaCTX-M* instead of *blaTEM* and *blaSHV*. It is also a proof of the flexible changing, difficult to predict, and difficult to control of antibiotic-resistant bacteria.

ESBL-producing *E. coli* bacteria can carry 1 or more than 1 ESBL

coding gene. In this study, we detected that 42.3% of strains two or more ESBL-producing genes. The emergence of multiple ESBL-producing genes in a bacteria may change the antibiotic-resistant phenotype and lead to increases in the level of multi-drug resistance. Furthermore, we found that 8% of the strains carryied *mcr-1*, a colistin-resistant gene. The carrying of the colistin-resistant gene in multidrug-resistant strains may lead to no effective antibiotics to treat multi-drug resistant strains. Therefore, to limit the spread of reservoirs of dangerous antibiotics-resistant gene in the community measures should be taken to manage the spread of antibiotic-resistant bacteria, especially the strains that carry multiple antibiotic-resistant genes

4.2.4. Phylogenetic grouping characteristics of ESBL-producing E. coli

The majority of ESBL-producing *E. coli* bacteria in healthy individuals was intestinal symbiotic *E. coli* or opportunistic pathogens *E. coli,* which were belonged to groups A (43.1%), D (32.1%), and B1 (14.6%). 10.2% of the strains belonged to group B2, which is highly virulent, capable of causing gastrointestinal, urinary and septicemia diseases. Thus, B2 strains in the healthy individuals may be a potential risk of disease to healthy people in the community

4.2.5. Virulence genes characteristics of ESBL- producing E. coli

Results of identifying 11 virulent genes representing 6 groups of diarrhea *E. coli* showed that 63 strains (46%) carried virulence genes, of which, the most common were EAEC strains (21.1%), and EPEC (15.6%). Further, we detected 6 simultaneous expression strains belonging to groups of EAEC/DEAC (5 strains) and EAEC/EPEC (1 strain). Carrying virulence genes at a high rate, combined with carrying of multiple virulence genes simultaneous from multiple diarrhea *E. coli* groups may lead to an increase in the risk of diarrhea in healthy individuals in the community. Furthermore, most of the strains carrying virulent genes are multi-drug resistant strains. Thus,

the strains carrying both virulence and multi-drug resistance genes may be a potential risk of causing multi-drug resistant diarrhea disease. Especially when the bacteria lives in the intestinal tract and isexcreted in feces in the tropical weather in Vietnam it may very easily spread and may cause an outbreak of multi-drug resistant diarrhea in the community.

4.2.6. Genotypic relationship between ESBL-producing E. coli

Analysis of PFGE results showed that the genotype of ESBL-producing *E. coli* strains was diverse. However, those strains falling within the same cluster had a close relatedness among them: 21.1% of strains were closely related with the genetic similarity from 80 % to 95%, whereas 32 strains (24%) had a high degree of relatedness with 95-100% similarity, and these strains were contribute in 15 genotype groups. Of 15 groups, 1 had 4 strains while 14/15 groups had 2 strains. Analyzing the origin of these strains into 15 genotypic groups, nine of the 15 groups contained strains isolated from members of the same household and 6/15 groups containing strains isolated from members of different households. The strains that have a relatively high degree of relatedness may have the same genetic origin, and come from the same source of contamination (food, drinking water), or may be cross-transmission between individuals. These results indicate that the cross-transmission of some clones not only happens between family members but also among healthy individuals in the same community but from different households. Thus interventions are needed to prevent the spread of these bacteria both within households and in the community.

4.2.7. Plasmid profile of ESBL- producing E.coli

Plasmid is one of the mobile genetic factors that play an important role in the spread of antibiotic-resistant genes. In this study, plasmid replicons were found in 92.7% of ESBL-producing *E. coli* strains with a total of 283 plasmids (mean 2.23, range 1-6). Among

ESBL-producing *E. coli* strains carrying plasmid, the most frequent rates were the strains that carried 2 plasmids (42.3%), or 3 plasmids (27.7%). Carrying multiple plasmids simultaneously in a strains means the bacteria carry multiple antibiotic-resistant genes, including the ESBL encoding gene. Moreover, the plasmid can be transmitted vertically among bacteria in the same bacterial species or horizontal-transmitted among bacteria in the same or different bacterial species. Thus, the presence of these bacteria in healthy individuals in the community may a reservoir and potential for spread of multi-drug resistant bacteria in the community.

A variety of plasmid replicon types (17/18 types tested) were detected in these strains. Among them, the most frequently ones were plasmid replicon types belonged to IncF group (FIB: 56.93%; Frep: 51.82%; FIA: 24.82%, FIC: 2.92%); followed by I group (B/ O: 21.9%; K/B: 2.92%, I1: 9.49%); other plasmid replicon types such as P, T, Y, K / B, X, HI1, N, HI2, L/M were detected in low rates while plasmid replicon type W was not found in any strain. These results are in line with those of other authors and it seems to be well consistent with the characteristics of the IncF, which is known as the most common plasmid containing ESBL coding genes and widely distributed among *E. coli* strains.

Molecular epidemiological studies of ESBL-producing bacteria showed that most of the ESBL-producing genes in Enterobacteriaceae are located on large plasmids (50kb to > 500kb). However, in some cases, ESBL-producing genes are located on the bacterial chromosome. In this study, Southern Blotting was used to determine the location of ESBL-producing genes in 37 ESBL-producing *E. coli* strains, including 4 strains carrying *blaCTX-M-1* gene, 7 strains carrying blaCTX-M-1/TEM, 21 strains carrying blaCTX-M-9, 4 strains carrying blaCTX- M-9/TEM and 1 strain carrying blaTEM. The Southern hybridization of S1-PFGE gel

results showed the majority (67.6%) of the ESBL-producing *E. coli* carried the ESBLs genes on a large plasmid (ranging from 56.7 kb to 157 kb in size). Plasmid is known as the main factor for the transmission of antibiotic-resistant genes from one bacterium to another, even from non-pathogenic bacteria to pathogenic bacteria. Thus, the high rate of hosting ESBL genes on plasmid could be a potential reservoir and source of dissemination of plasmid-mediated ESBL in the community setting.

The plasmid may host only *blaCTX-M* gene, or *blaTEM* gene, or both *blaCTX-M/TEM* genes simultaneously. The proportion of the plasmid detected to host *blaCTX-M-1, blaCTX-M-9,* and *blaTEM* genes were 36.4%, 76%, and 75%, respectively. These results indicate that the plasmid-mediated transmission of *blaCTX-M-9* and *blaTEM* may be higher than *blaCTX-M-1*.

Among 11 strains that carried 2 ESBL-producing genes, 6 of strains carried both genes on the plasmid. Carrying two genes on plasmids simultaneously, especially on the same plasmid, can increase the ability to spread the ESBLs gene by plasmid-mediated transmission.

Previous studies demonstrated that antibiotic-resistant genes can be transferred between bacteria by conjugation. In this study, we found that 39.0% of the ESBL- producing *E. coli* strains transferred plasmids hosting ESBLs genes to *E. coli* J53 by conjugation. The rate of plasmid transmission of other bacteria to *E. coli* J53 is higher in our study than that in the study conducted by Tran Huy Hoang. It is possible that the donor strains in our study were *E. coli*, therefore the plasmid transmission between *E. coli* strains is easier than between other bacteria and *E. coli*. These results suggest that antibiotic-resistant genes can be transferred easily between *E. coli* strains. This is an issue of concern because the transfer of plasmids carrying the ESBL genes between bacteria can lead to the rapid spread of

antibiotic-resistant genes among *E. coli* strains and from *E. coli* to other pathogenic bacteria.

Among strains that carried 2 ESBL genes, the rate of simultaneous transmission of both genes (25%) was higher than that of only 1 gene (10%). The number of strains carrying 2 genes in our experiment is small, and it is not possible to accurately assess the rate of gene transmission among strains that carrying multiple antibiotic-resistant genes. However, this result suggests that the bacteria tend to transmit multiple genes at the same time to other bacterial strains. Our results are in line with the result of others. The transmission of multiples genes is one of the important reasons for the wide spread of antibiotic-resistant bacteria in the community, especially the spread of multi-drug resistant strains due to the simultaneous transmission of multiple antibiotic-resistant genes.

The demonstration that ESBL-producing *E. coli* strains isolated from healthy individuals in our setting could spread to the gram-negative bacteria through conjugation-transfer indicate that the potential and dangerous spread of ESBL genes in Vietnam does not only occur in hospitals but also in the community. This is a situation that causes many difficulties for the control and prevention of the spread of antibiotic-resistant bacteria in general and ESBL-producing *E. coli* strains in particular. The results also help us to better understand the transmission characteristics of ESBL-producing bacteria in Vietnam.

CONLUSSION

1. Dissemination of ESBL-producing *E. coli* isolated stool samples collected from the healthy individual in a rural community in Thai Binh province

The prevalence of ESBL-producing *E. coli* isolated from stool samples collected from healthy individuals in Nguyen Xa commune, Vu Thu district, Thai Binh was high (64.6%). Prevalence of ESBL-

producing among of CTX-resistant *E. coli* was very high (81.1%). There is no difference in the prevalence of carrying ESBL-producing *E. coli* between males and females or across education level or occupation.

2. Microbiological characteristics of ESBL-producing *E. coli*

- ***Antibiotic-resistant characteristics:*** ESBL-producing *E. coli* strains were resistant to ampicillin, and were highly resistant to other common antibiotics (21.2% - 81.0%). The prevalence of multi-drug resistance among ESBL-producing *E. coli* was very high (86.1%).

- ***Characteristics of ESBLs coding genes:*** 91.4% of the strains carried the CTX-M coding genes, of which the most common was *blaCTX-M-9* (66.3%). The percentage of *blaTEM* was 45.3%. No strains carrying *blaSHV* were detected. ESBL-producing *E. coli* can carry from 1 to 3 ESBL coding genes, of which, most strains carried 1 gene (55.5%), or 2 genes (41.6%) with the majority of the *blaCTX- M/TEM* genotype.

- ***Characteristics of colistin-resistant gene:*** The prevalence of *mcr-1* among ESBL-producing *E. coli* was 8%.

- ***Phylogenetic grouping characteristics:*** 89.8% of ESBL-producing *E. coli* were intestinal symbiotic *E. coli* or opportunistic pathogens *E. coli*, which belonged to groups A (43.1%), D (32.1%) and B1 (14.6%). While 10.2% belonged to group B2, which is highly virulent, and capable of causing disease.

- ***Virulence genes characteristics:*** Virulence genes were detected in 46% of ESBL-producing *E. coli* strains. Of these, the most common virulence genes belonged to the EAEC group (21.1%) and EPEC groups (15.6%).

- ***Genotypic relationship between ESBL-producing E. coli:*** The genotype of ESBL-producing *E. coli* strains was diverse. However, PFGE results showed that ESBL-producing *E. coli* strains spread among members of the same household (with 9 genotypes including

20 strains with 95-100% genetic similarity between members of the same genotype group) and between healthy individual in a community (with 6 genotypes including 12 strains with 95-100% genetic similarity among members of the same genotype group).

- ***Plasmid profile of ESBL- producing E.coli***

+ Plasmid replicons hosting the ESBL coding gene were found in 92.7% of ESBL-producing *E. coli* strains with a total of 283 plasmids (mean 2.23, range 1-6). The most frequent rates were plasmid replicon types belonging to the IncF group (FIB: 56.93%; Frep: 51.82%; FIA: 24.82%, FIC: 2.92%); followed by I group (B/O: 21.9%; K/B: 2.92%, I1: 9.49%); other plasmid replicon types such as P, T, Y, K/B, X, HI1, N, HI2, L/M were detected at low rates.

+ The plasmid may host only *blaCTX-M*, or *blaTEM*, or both *blaCTX-M/TEM* genes simultaneously. The prevalence of detection of the plasmid hosting blaCTX-M-1, *blaCTX-M-9*, and *blaTEM* genes were 36.4%, 76%, and 75%, respectively.

+ In the laboratory model, it was demonstrated that ESBL-producing *E. coli* strains isolated from healthy individual in a community setting can transfer plasmids hosting the ESBL coding gene to *E. coli* J53.