MINISTRY OF EDUCATION AND TRAINING

MINISTRY OF HEALTH

NATIONAL INSTITUTE OF HYGIENE & EPIDEMIOLOGY

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MOLECULAR BIOLOGICAL CHARACTERISTICS OF *Escherichia coli* CARRYING *mcr-1* GENE RESISTANT TO COLISTIN ISOLATED FROM HUMAN, DOMESTIC ANIMALS, FOODS AND WATER AT THANH HA COMMUNE, HA NAM PROVINCE IN 2015

SUMMARY OF PhD THESIS ON MEDICINE

HA NOI - 2022

THIS THESIS WAS PERFORMED AND COMPLETED AT THE NATIONAL INSTITUTE OF HYGIENE AND EPIDEMIOLOGY

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National Institute of Hygiene & Epidemiology

on ,2022

This doctoral thesis can be found at:
1. The National Library
2. The Library of National Institute of Hygiene and Epidemiology

LIST OF PUBLISHED SCIENTIFIC ARTICLES RELATED TO THE THESIS

1. **Nguyen Thi Tuyet Mai**, Pham Duy Thai, Tran Thi Van Phuong, Nguyen Hiep Le Yen, Vu Thi Tuong Van, Dang Duc Anh, Tran Nhu Duong, Nguyen Thi Lan Phuong, Vu Thi Thu Hien, Tran Huy Hoang (2017), "Genotypic relationship of *Escherichia coli* strains carrying colistin resistant gene (*mcr-1*) isolated in Hanoi and Ha Nam in 2015-2016". Journal of Vietnam Preventive Medicine, Vol. 27(9): 57-64.
2. **Nguyen Thi Tuyet Mai**, Pham Duy Thai, Tran Thi Van Phuong, Nguyen Hiep Le Yen, Vu Thi Tuong Van, Nguyen Thi Lan Phuong, Vu Thi Thu Hien, Tran Huy Hoang (2017). "Application of PCR technique to detect colistin-resistant *mcr-1* gene on bacterial strains isolated from animal feces samples collected in Ha Nam in 2016". Journal of Vietnam Medicine. Vol. 456 (2): 90-94.
3. Bich Vu Thi Ngoc, Thanh Le Viet, **Mai Nguyen Thi Tuyet**, Thuong Nguyen Thi Hong, Diep Nguyen Thi Ngoc, Duyet Le Van, Loan Chu Thi, Hoang Tran Huy, John Penders, Heiman Wertheim, H. Rogier van Doorn: (2022) "Characterization of Genetic Elements Carrying *mcr-1* gene in *Escherichia coli* isolated from the Community and Hospital Settings in Vietnam" *Microbiology spectrum, 10*(1):1356-21.
4. **Nguyen Thi Tuyet Mai**, Vu Thi Ngoc Bich, Pham Duy Thai, Hoang Thi An Ha, Hoang Thi Mai Huong, Tran Huy Hoang, Vu Thi Tuong Van (2022): "Antibiotic resistant characteristics of *E. coli* strains carrying gene *mcr-1* isolated from human, animals, foods and environment at Thanh Ha commune, Thanh Lien district, HaNam province in 2015". *Journal of Vietnam Medicine.* 32 (3): 125-35.

INTRODUCTION

Unreasonable use of antibiotics in hospitals, in livestock and aquaculture leads to antibiotic resistance (AR) of bacteria increasing continuously and getting worse. The presence of *E.coli* strains carrying *mcr-1* gene causing disease in humans and animals has been and is an urgent warning for public health. Especially, when these pathogenic bacteria are resistant to carbapenem and colistin at the same time, the treatment of infections caused by gram-negative bacteria becomes an enormous challenge. Foods contaminated with *mcr-1*-carrying bacteria strains, the spread of these strains through the food chain (meat, vegetables, water) is the risk for the emergence and increase of Enteric gram-negative bacteria strains carrying the colistin-resistant *mcr-1* gene in humans and domestic animals. However, at present, in the North of Vietnam, there is no adequate research on this issue in the whole community including people, domestic animals, surrounding natural environment (food and water) - where bacteria carrying resistance genes can be spread from contamination sources in community. Therefore, assessing the status of *E.coli* strain carrying *mcr-1* gene located in human gastrointestinal tract, located in the "reservoirs" of domestic animals and in the environment (food, water), evaluate the genomic transmission route of these resistant strains by determining their molecular biology is essential for synchronous control of the risk of antibiotic resistance and increasing colistin resistance.

These scientific evidences will help to identify some risk factors that can create particularly emergence antibiotic-resistant strains of bacteria in Vietnam, providing a scientific basis for proposing measures to prevent and reduce the proliferation of colistin-resistant bacteria strains. Stemming from that urgent practical situation, we conduct the research on:

“Molecular biological characterisics of *Escherichia coli* carrying colistin resistant *mcr-1* gene islolated from human, domestic animals, foods and water at Thanh Ha commune, Ha nam province in 2015” with 2 following objectives:

1. *Determine the rate of E.coli strains carrying mcr-1 gene resistant to colistin isolated from human and animal feces, from foods and water at Thanh Ha commune, Thanh Liem district, Ha Nam province in 2015.*
2. *Determine some molecular biological characteristics of isolated E. coli strains carrying mcr-1 gene.*

NEW SCIENTIFIC CONTRIBUTIONS AND PRACTICAL VALUE OF THE THESIS

The research has identified some sequence types (STs) of *E.coli* strains carrying *mcr-1* gene circulating in the community (56 different types of STs, 3 most common ST types found are ST10, ST48 and ST206).

The research has detecmined the plasmid replicons of *E.coli* strains carrying colistin-resistant *mcr-1* gene circulating in the community, included, in particular, 7 types of single replicons carrying *mcr-1* gene (IncI2, IncP, InX4, IncFIA, IncHI1B, IncN and IncX1). 20 types of plasmids containing different replication units with combinations of IncFIA, IncFIB, IncHI1B, IncHI2, IncN, IncY, IncX1 and IncI2 plasmids were found in *E.coli* strains isolated from human, animal, and water samples. The most common combination was found between IncHI2 with other replicans and between IncH and IncF. Thereby elucidating the spreading mechanism of these strains in the community mainly through these plasmids.

The study has also determined the structure of some common plasmid replicons and *mcr-1* gene-carrying transposons of *E.coli* strain circulating in the community, thereby providing further insights into the genomics of these strains.

Research results of this thesis provided scientific evidence for the transmission of colistin-resistant *mcr-1* gene in *E.coli* strains in the community. Based on this scientific evidence, the Antibiotic Resistance Surveillance Program will introduce the effective measures to prevent antibiotic resistant gene transmission route in the community.

STRUCTURE OF THE THESIS

The thesis is introduced in 118 pages, excluding administrative sections, lists of published articles, references and appendices.

The main part of thesis includes: Introduction (3 pages), Literature Review (35 pages); Study Subjects and methods (23 pages); Research Results (27 pages); Discussion (23 pages); Conclusion (2 pages); contributions of thesis (1 page) and Recommendation (1 page).

Thesis has 18 tables, 4 charts and 18 figures. The thesis used 126 reference articles/documents and 42 pages of appendices.

Chapter 1. LITERATURE REVIEW

1.1. Antibiotic resistance

Antibiotics have the effect of inhibiting or killing bacteria, but if they can grow in an environment containing antibiotics, it is considered antibiotic resistance.

1.1.1. Antibiotic resistant mutations

When mutations occur, antibiotics have eliminated all susceptible populations and only antibiotic-resistant bacteria predominate. Resistance mutations can be occurred through the following mechanisms: altering the action target; altering metabolic pathways through altered regulatory nets; reduces the permeability of the plasma membrane, reduces drug absorption; efflux pump.

1.1.2. Horizontal Gene Transfer (HGT)

Conjugation usually uses mobile genetic elements (MGEs) as a means to share important genetic information including antibiotic resistance. The most important MGEs are *plasmids* and *transposons*, thus play an important role in the development and spread of clinically resistant bacteria.

Plasmid: Plasmids are circular DNA molecules located outside of chromosomes and capable of self-replicating. Some plasmids that play an important role in medical microbiology are plasmids carrying resistance genes for antibiotic and heavy metals (R-plasmids), toxin-producing plasmids, plasmids containing virulence factors (the ability to adhere and penetrate into cells). Plasmid genes can also be transmitted

vertically across generations or horizontally to other bacteria through transformation, conjugation, and transduction. This is one of the main ways to increase the spread of antibiotic resistant genes rapidly in the same and different species of bacteria.

Transposons: Transposons are fragments of DNA containing one or more genes, whose both ends are nucleotide sequences that can be inverted repeat (IR) in opposite directions and can switch positions from one DNA molecule to another, such as from plasmid to chromosome or vice versa or from plasmid to another plasmid.

1.1.3.5. The antibiotic resistant genes

Some antibiotic resistant genes of different groups of antibiotics are introduced in the table below.

Table 1.1 Table summaried the antibiotic resistant genes of common antibiotic groups

Antibiotic groups	Important antibiotic resistant genes
β-lactam	*Amp*C β-lactamase *ESBL: bla*$_{\text{TEM-}}$, *bla*$_{\text{SHV-}}$, *bla*$_{\text{CTX-M-}}$ Carbapenemase: Group A (KPC, SME, IMI, NMC, GES); Group B (NDM, IMP, VIM, SPM, GIM), Group D (OXA-).
Aminoglycosid	Gene methylase: *arm*A, *npm*A, *rmt*A, *rmt*B, *rmt*C, and *rmt*D Gene introduces mutated enzymes: AAC (acetyltransferase), ANT (nucleotidyltransferase or adenyltransferase), APH (phosphotransferase)
Chloramphenicol	CAT: *cat*A and *cat*B. *cml*A và f*lo*R
Glycopeptid	*van*A and *van*B: located on plasmid and/or NST *van*C1, *van*C2/3, *van*D, *van*E, and *van*G: located only on NST
Quinolon	GyrA and GyrB: Gram negative bacteria *parC* and *parE*: all Gram positive bacteria located on NST. *qnr* (*qnr* A,B,C,D,S), *aac(6)-Ib, qepA: located on plasmid*
Sulfonamid và trimethoprim	Resistant to sulfonamid: *folP, sul1, sul2* and *sul3* Resistant to trimethoprim: *folA, dfrA, dfrB, dfrK*
Polymycin	*lpxA, lpxC or lpxD* *mcr-1 to mcr-10*

1.2. Use of antibiotics in livestock

Overuse of antibiotics in livestock in Vietnam is also very common. People often feed pigs and chickens with bran mixed with

antibiotics to prevent disease without control on the dosage. Transmission of antibiotic resistant strains through the food chain is one of the problems that need to be solved today

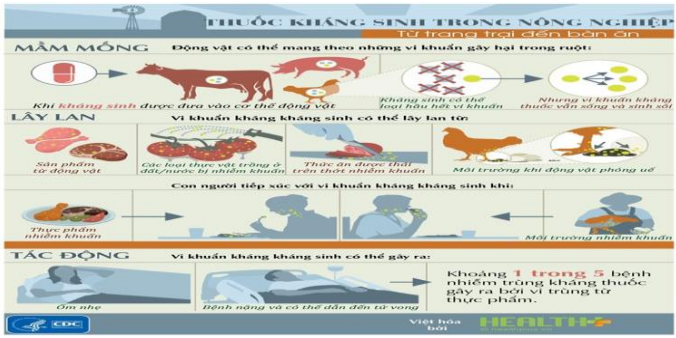


Figure 1.4: Transmission of antibiotic resistance through food chain
(Source: http://healthplus.vn)

1.3. Colistin

1.3.1. Action principle

Bacterial cell membrane is the primary site of action of colistin. By binding to lipopolysaccharides (LPS) and phospholipids in the outer membrane of gram-negative bacteria, colistin is able to kill bacteria with various mechanisms of action. Ca2+ and Mg2+ are two ions that stabilize the LPS molecule. Colistin is a cationic molecule, so by competing for the replacement of these cations from the phosphate groups of the lipid membrane, it leads to disruption of the outer cell membrane, leakage of internal substances, and cause the death of bacteria. In addition, the drug also binds to lipid A, which is part of the endotoxin or LPS molecule, and in many studies used animal model, it has blocked the effects and toxicity of bacteria.

1.3.2. Colistin resistance

In early February 2015, a Chinese scientific team, Liu et al., during the daily surveillance of antibiotic resistance, announced that they found the colistin-resistant *mcr-1* gene in plasmid-transmitted *E.coli* bacteria. Following the first discovery of Liu et al., up to date, at least over 30 countries in the world, in all continents, have conducted studies to detect bacterial strains carrying *mcr-1* gene.

In Vietnam, Surbhi Malhotra-Kumar et al has carried out the screening for *mcr-1* gene detection by PCR and Sanger sequencing on 24 strains of *E.coli* producing ESBL isolated from direct smear of intestines of chickens and pigs at 2 farms in Van Lam, Hung Yen and Hoai Duc, Hanoi in 2014-2015. However, there has not been any research on the molecular biological characteristics to provide a scientific basis on the transmission mechanism of E. coli strains carrying colistin-resistant *mcr-1* gene from different sources in Vietnam.

1.3.3. Some characteristics of gen mcr-1 and plasmid carried by colistin resistant E. coli strains

1.3.3.1 Some characteristics of mcr-1 gene

Structure of plasmid carrying mcr-1 gene

The study of Liu et al. using gene sequencing showed the structure of 4 plasmids carrying *mcr-1* gene from 04 colistin-resistant *E.coli* isolates (Figure 1.7)

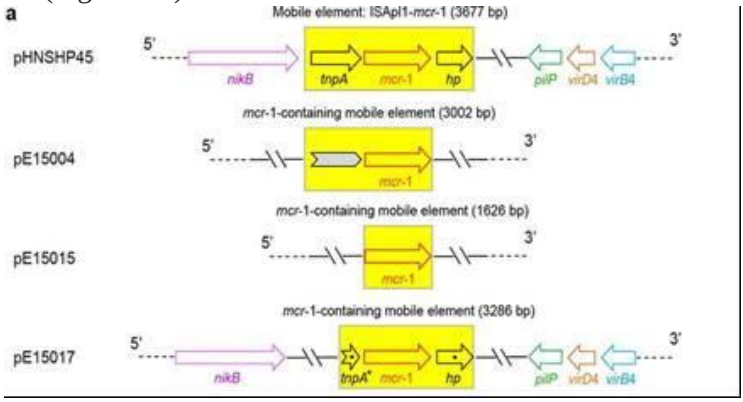


Figure 1.7: The structure of 04 plasmid carrying *mcr-1* gene by sequencing the isolated *E. coli* strains

pHNSHP45: plasmid of *E.coli* strain isolated from pig feces; three other plasmids (pE15004, pE15015 and pE15017) were isolated from three clinical strains of *E.coli* (E15004, E15015 and E15017).

Research on plasmid typing carrying *mcr-1* showed that the most common plasmid types found today are: IncHI2, IncI2 and IncP.

1.4. Molecular biology techniques used in detection of antibiotic resistance genes

1.4.1. Plasmid analysis

This is also the most important antibiotic resistance mechanism because the majority of antibiotic resistance genes in bacteria are located on plasmids and can be transmitted within species of other bacteria through conjugation.

1.4.2. Study on the transmission of antibiotic resistant plasmids

Through conjugation, the plasmids can be transferred from the donor cell to the recipient cell through the pili by a pusher pump. In Vietnam, this technique is also used to analyze the characteristics of bacteria carrying plasmids resistant to antibiotics.

1.4.3.Using PCR technique to detect antibiotic resistant gene

Principle: The synthesis of DNA in the cell is replicated by a semi-conservative mechanism. With a specific primer pair, a certain DNA fragment is synthesized after each thermal cycle of PCR. The replicated specific gene fragment will be detected on agar electrophoresis.

1.4.4. Pulsed field gene electrophoresis (PFGE)

Principle: This technique uses suitable enzymes to cut DNA into different segments, which are electrophoresed to separate fragments from 1 to 1000kb. This technique allows to determine the origin and relationship of bacterial strains in different regions and times by the comparison of DNA fragments of bacterial strains.

1.4.5. Gene sequencing technique

This is the most accurate method for determining the antibiotic-resistant properties of bacteria. New generation sequencing methods were first introduced in 2005 to overcome the limitations of previous traditional sequencing methods. Although the next-generation gene sequencing method will only produce shorter sequence reads and lower accuracy than the Sanger-sequenced DNA fragments, it compensates with a higher number of reads that covers the entire region of sequenced DNA and as the results, these will be reassembled to obtain a final DNA sequence with high accuracy, thus also known as Whole Genome Sequence (WGS). The accuracy of the WGS technique is highly dependent on the data analysis obtained from the sequencing process.

Data reliability is often based on the coverage. Coverage is used for the purpose of comparing genomes to find 20X of nucleotide differences.

1.5. Problems to be solved in the research

- What is the contamination level of E. coli strains carrying the mcr-1 gene in the community (water environment, food)?
- Whether strains of E. coli carrying colistin-resistant mcr-1 gene exist in the digestive tracts of healthy individuals exposed to environments and food contaminated with these strains of bacteria?
- What is the level of antibiotic resistance of E. coli strains carrying colistin-resistant mcr-1 gene?
- - What are the molecular characteristics of E. coli strains carrying colistin-resistant mcr-1 gene? What is the transmission mechanism of these strains?

Chapter 2

STUDY SUBJECTS AND METHODS

2.1. Study subjects, place

- Sampling site: Thanh Ha commune, Thanh Liem district, Ha Nam province. Thanh Ha commune includes 7 villages: An Hoa, Duong Xa, Quang Trung, Ung Liem, Hoa Ngai, Mau Chu, Thach To. The place where performed all microbiological testing is the Laboratory of Antibiotic Resistance, Department of Bacteriology, National Institute of Hygiene and Epidemiology.
- The place where whole genome sequencing (WGS) was performed: Laboratory of Antibiotic Resistance, Department of Bacteriology, National Institute of Hygiene and Epidemiology.

2.2. Research design

The cross-sectional descriptive design was used for the entire study

2.3. Time to perform the research: 2015

All samples used for the study were collected in the study coded VN V1.1 Ha Nam on the "Determination of antibiotic resistance of microbiota in healthy human volunteers in Vietnam and the effects of using the antibiotics in the community" in 2015. All samples after collection were kept in a -70ºC freezer at the Laboratory of Antibiotic Resistance, Department of Bacteriology, National Institute of Hygiene and Epidemiology.

2.4. Study subjects

The research object used for Objective 1 was all strains of *E.coli* isolated from research samples cultured on MAC selective medium containing 0.5 µg/ml of colistin. The research objects for Objective 2 were *E.coli* strains carrying the *mcr-1* gene collected for Objective 1.

2.5. Sample size

Sample size used for Objective 1:

Applied the fomula of calculating sample size for a study the estimated proportion:

$$n = Z^2_{(1-\alpha/2)} \times \frac{p \times (1-p)}{d^2} \quad (1)$$

Of which: n: number of strains needed for research; p: prediction rate; Z1-α/2: the coefficient of confidence, with a confidence level of 95% ($Z_{(1-\alpha/2)}$ = 1.96); d: absolute accuracy (d=0.05)

- With the proportion of *E. coli* strains carrying the colistin-resistant *mcr-1* gene in human feces reported by previous studies as 17.6%, the number of human stool samples required for determining the percentage of *E.coli* carrying the *mcr-1* gene in human feces was determined as 222 (n= 222).
- With the rate of *E.coli* strains carrying the colistin-resistant *mcr-1* gene in animal feces reported by previous studies as 7.9%, the number of animal feces samples for determining the percentage of *E.coli* carrying *mcr-1* gene was estimated as 112 ((n= 112)
- With the rate of *E. coli* strains carrying the colistin-resistant *mcr-1* gene in food of 15% reported by previous studies, the number of food samples for determining the percentage of E.coli carrying *mcr-1* gene in food was calculated as 189 (n=189).
- With the rate of *E.coli* strains carrying colistin-resistant *mcr-1* gene in water samples of 10% reported by previous studies, the number of water samples used for determining the percentage of *E.coli* carrying *mcr-1* gene in water in this study was determined as 138 (n=138)

Based on the above calculated sample sizes, the study selected the sample as follows: The sample of the study was selected from samples of human feces, domestic animal feces, food and water (rainwater, well water,

irrigation water) of 80 households in Thanh Ha commune, Thanh Liem district, Ha Nam province. The number of samples collected for this study was presented in the Table 2.1

Table 2.1: Number of samples of different types used in the research

Sample type	Number
Human feces	265
Domestic animal feces	122
Food samples	179
Water samples	159

Sample size for Objective 2:

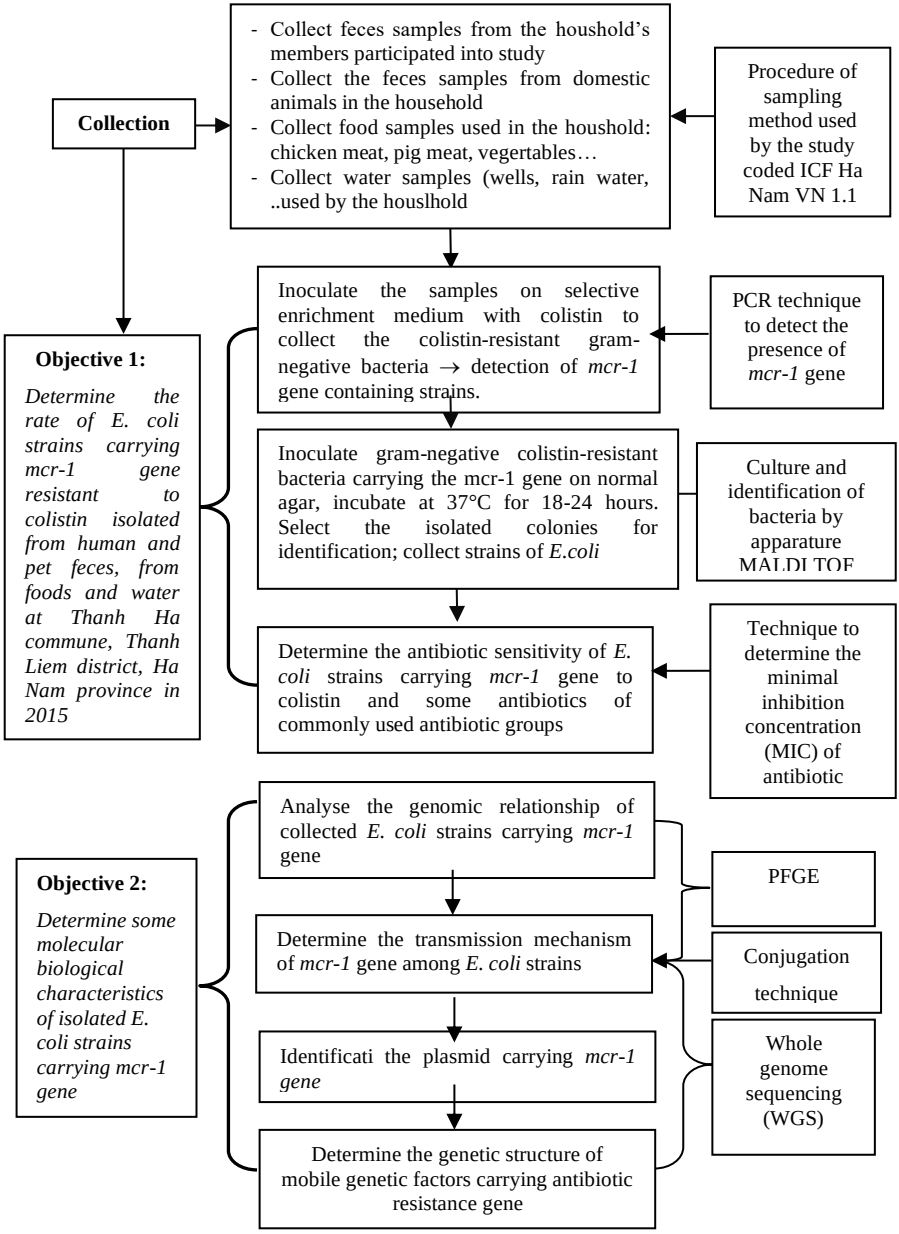
PFGE technique: All colistin-resistant strains of *E.coli* carrying *mcr-1* gene were selected for antibiotic susceptibility assessment using the quantitative technique of minimal inhibitory concentration (MIC) of antibiotic resistance

Whole-genome sequencing (WGS): Used the representative and targeted selection of strains from the group of bacterial strains isolated for performing the PFGE: those having >90% genomic homology from the obtained PFGE results were selected as representative strains. If there is no strain with >90% genomic homology from PFGE, all will be used. The number of *E.coli* strains isolated for sequencing was 98 strains, of which 87 strains carried the *mcr-1* gene.

2.6. Data treatment and analysis

- Data was managed by using excel software
- Determination of molecular biological characteristics: used FinchTIVI, DNA-blast, Bio-numeric-6.5, Inter plasmid Analyzing tool softwares.
- The results are presented in the form of tables, graphs, charts

- Schematic summary of the research steps



Chapter 3. RESULTS

3.1. Rate of *E.coli* strains carrying *mcr-1* gene resistant to colistin

Table 3.2: Rae of tested samples containing *E.coli* strains carrying *mcr-1* gene isolated from human, domestic animals, food and water samples

Sample type	Total number of samples	Number of samples isolated with *Enterobacteriaceae* using MAC + 0.5 µg/ml colistin medium	Rate of samples isolated with *E.coli* carrying *mcr-1* gene	
			Number	Tỷ lệ (%)
Human feces	265	221(83.3%)	97	36.6
Animal feces	122	97 (79.5%)	42	34.4
Food	159	72 (72.5%)	6	3.8
Water	179	156 (87.1%)	15	8.4
Total	725	546 (75.3%)	160	22.1

From a total of 725 samples of human feces, animal feces, food and water inoculated on MacConkey agar containing 0.5 µg/ml colistin, 546 samples produced pink colonies (containing the bacteria of enterobacteriaceae) (75.3%).

All types of collected samples (human feces, animal feces, food, water) were found to be contaminated with *E.coli* strains carrying *mcr-1* gene. The proportion of samples identified with *E.coli* strains carrying *mcr-1* gene was: 36.6% of human feces; 34.4% of animal feces; 3.8% of food samples and 8.4% of water.

3.1.4. Colistin sensitivity of *E.coli* strains carrying resistant *mcr-1* gene

Table 3.3. Results of colistin MIC testing of isolated *E.coli* strains carrying resistant *mcr-1* gene

Sample type	Sample number (n)	MIC for Colistin (µg/ml)			
		≤ 2	4	8	≥16

Human feces	236	137(58.1%)	87 (36.9%)	7 (2.97%)	5(2.12%)
Animal feces	99	71(71.7%)	23 (23.2%)	1 (1.01%)	4 (4.04%)
Food	16	6 (37.6%)	3 (18.6%)	2(12.5%)	5 (31.3%)
Water	47	24 (51.1%)	15 (31.9%)	1 (2.13%)	7(14.9%)
Total	398	238 (59.8%)	128(32.2%)	11 (2.8%)	21 (5.2%)

Results of assessing the sensitivity to colistin of 398 isolated *E.coli* strains carrying *mcr-1* gene using the minimum inhibitory concentration method by microdilution technique showed that the number of strains having genotypic expression without phenotypic expression was accounted for a high proportion in all samples: 71.7% of domestic animal feces, 58.1% of human feces). Among the 160 strains carrying *mcr-1* gene that expressed colistin-resistant phenotypes, the majority showed to have colistin MICs of 4 µg/ml (128/160 strains), accounting for 80% of colistin-resistant strains. *E.coli* strains carrying *mcr-1* gene exhibiting colistin-resistant phenotype were found in all 4 types of samples: human feces, domestic animal feces, food and water (Table 3.3).

3.1.5. Distribution ratio of mcr-1 gene on chromosomes and plasmids of isolated E. coli strains

Table 3.4. Distribution of *E.coli* strains carrying *mcr-1* gene on chromosome and plasmid

Sample type	***mcr-1 subtype***	**Strain carrying *mcr-1* gene on chromosome**	**Strains carrying *mcr-1* gene on plasmid**	**Strains carrying *mcr-1 gene* on chromosome and plasmid**	**Total**
Human feces	*mcr-1.1*	10	35	0	45
Animal feces	*mcr-1.1*	5	23	1	29
Food	*mcr-1.1*	3	0	0	3
Water	*mcr-1.1*	5	4	1	10
Total	*mcr-1.1*	23	62	2	87
		$p < 0.001$			

The colistin-resistant *mcr-1* gene of *E.coli* strains was found on both chromosomes and plasmids. The rate of *mcr-1* genes located on plasmids

accounted for 71.2% (n=62) and 26.4% on chromosomes (n=23). Especially, there are 2 strains with *mcr-1* gene located simultaneously on chromosome and plasmid, of which 01 isolates was obtained from water and other 01 was found from animal feces. 100% of *mcr-1* genes were of subtype *mcr-1.1*

3.2. Molecular biological charactristics of isolated *E. coli* strains carrying *mcr-1* gene

3.2.1. Genotypic relationships among bacterial strains carrying the mcr-1 gene

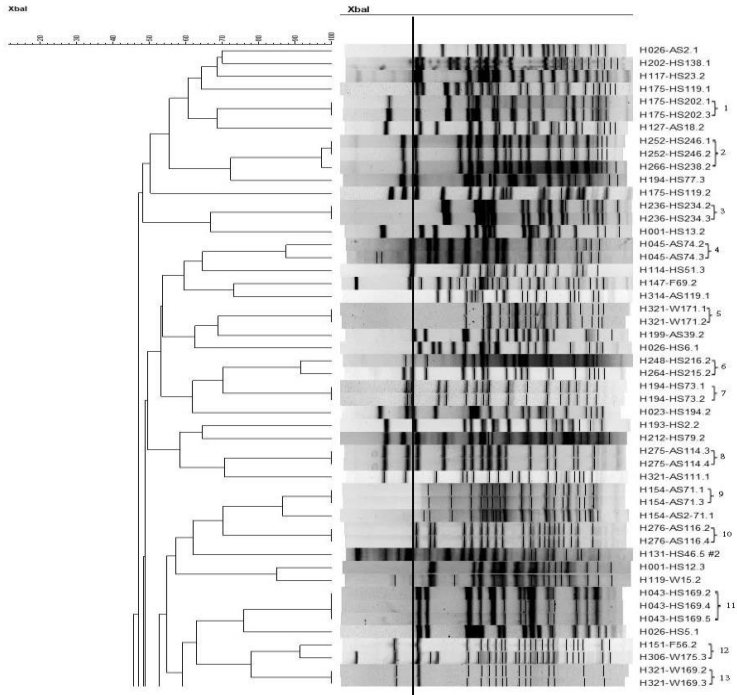


Figure 3.2: Representative image of Phylogenetic tree showing the genotypic relationship of *E.coli* strains carrying *mcr-1* gene isolated in Ha Nam

The results of genotyping of 240 *E.coli* strains carrying *mcr-1* gene by using PFGE technique (Figure 3.3-3.6) showed the diverse genotypes of *E.coli* bacteria carrying *mcr-1* gene in the study. The results of Phylogenetic tree analysis have identified 58 clusters of strains with highly similar genotypes (genotypic similarity of ≥ 90%).

Among the genotyped identical clusters, those with *mcr-1* gene-carrying *E.coli* strains isolated from the same sample were accounted for 68.9% (40 strain clusters). The remaining 18 clusters of similar strains are strains isolated from samples of human feces, domestic animal feces, food and water obtained within the same household and from different households.

3.2.2. Molecular biology of E.coli strains carrying mcr-1 gene detrmined by whole-genome

3.2.3.1. Characteristics of sequence type distribution of isolated E.coli strains carrying the mcr-1 gene

The study selected 98 strains for whole-genome sequencing to further analyze the molecular biological characteristics as well as the distribution of sequence type (STs) of *E.coli* strains in the community.

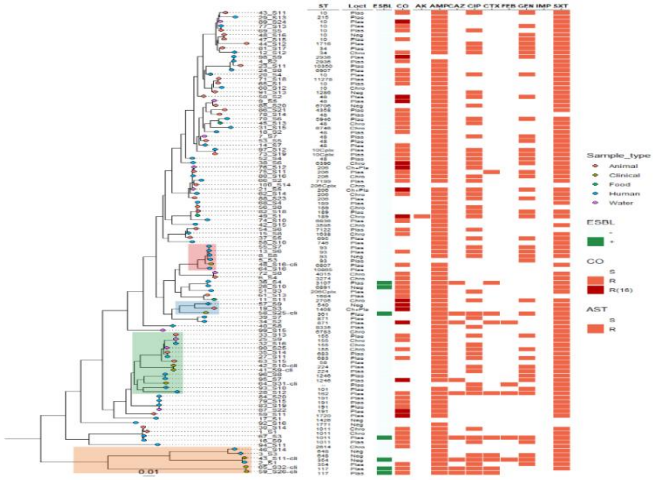


Figure 3.7: Taxonomic tree of core genome and sequence type of *E.coli* strains carrying *mcr-1* gene isolated in Ha Nam

Figure 3.7 reprents the very diverse distribution of genotypes of *E. coli* strains carrying *mcr-1* gene isolated from human feces, domestic animal feces and water environment samples with 56 different sequence types (STs).

3.3. Results of plasmid characterization, plasmid-mediated transmission mechanism and dynamic genetic structure of *E.coli* strains carrying the *mcr-1* gene

Table 3.10: Number and type of plasmids carrying *mcr-1* genes

STT	Replicon_formula (In silico)	Số chủng (short read)	Kích thước (Kb)	Loại mẫu
1	IncFIA	3	150 - 300	Phân người + động vật
2	IncFIA : IncFIC : rep2327	1	50 -100	Phân động vật
3	IncFIB : IncHI1B	2	100 - 150	Phân động vật + nước
4	IncFIB : IncHI1B : IncHI1B	2	150 - 200	Phân người + động vật
5	IncFIB : IncHI1B : IncHI1B : IncN	1	100 - 150	Phân động vật
6	IncFIB : IncHI1B : IncHI2A : IncHI2 : IncN	1	>300	Phân người
7	IncFIB : IncHI1B : IncN	2	50 - 150	Phân người + động vật
8	IncFIB : IncI2	1	<50	Phân người
9	IncFIC : rep2244 : IncHI2A : IncHI2	1	>300	Phân người
10	IncHI1B	3	50 - 100	Phân người + nước
11	IncHI1B : IncHI1B	1	100 - 150	Phân người
12	IncHI1B : IncHI1B : rep2327	1	50 - 100	Phân người
13	IncHI1B : IncHI2A : IncHI2	2	200 - 300	Phân động vật
14	IncHI1B : IncHI2A : IncHI2 : IncN	4	>300	Phân người + động vật
15	IncHI1B : IncX1	2	100 - 150	Phân người + động vật
16	IncHI1B : IncX2	1	100 - 150	Phân người
17	IncHI1B : rep2327	1	100 - 150	Phân động vật
18	IncHI2A : IncHI2	3	200 - 300	Phân người + động vật
19	IncHI2A : IncHI2 : IncI2	2	>300	Phân người
20	IncHI2A : IncHI2 : IncN	1	200 - 300	Nước
21	IncHI2A : IncHI2 : IncY	2	200 - 300	Phân người + động vật
22	IncHI2A : IncI2 : IncN : IncHI2	1	200 - 300	Phân người
23	IncI2	8	50 - 100	Phân người + động vật+ nước
24	IncN	1	50 - 100	Phân động vật
25	IncP	7	50 - 100	Phân người + động vật
26	IncX1	1	<50	Phân người
27	IncX4	5	<50	Phân người + động vật
28	undetected	6	<50	Phân người + động vật

There are several types of plasmids thus carrying *mcr-1* gene-, including single-replicon plasmids and multi-replicon plasmids. The combination of the single replicons produces 20 different types of multireplicon plasmids. The common found single-replicons in the community were IncI2 (n=8, 12.1%), IncP (n=7, 10.6%), InX4 (n=5, 7.6%).

3.3.3. Determining the structure of mobile genetic elements carrying mcr-1 gene

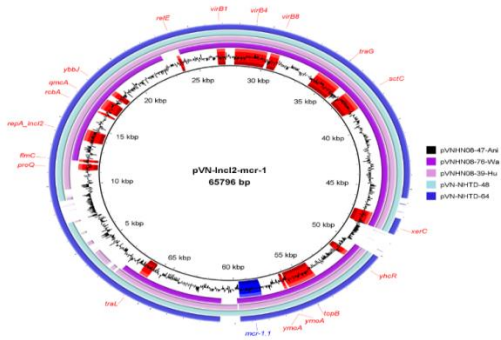


Figure 3.8: Structure of plasmid IncI2 carrying *mcr-1 gene*

Long-read and short-read data were used to compare the sequences of IncI2 plasmid of strains isolated from human feces, animal feces and water samples taken in the study community and clinical samples with pVNHN08-47-Ani-black for animal feces, pVNHN08-76-Wa-violet for water sample, pVNHN08-39-Hu-pink for sample of human feces, pVN-NHTD-48, pVN-NHTD-64-blue and light blue for the clinical patient.

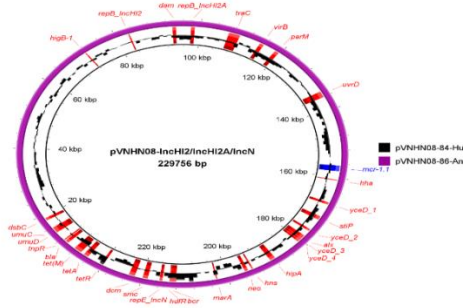


Figure 3.9: Structure of Plasmid IncHI2/IncHI2A/IncN carrying *mcr-1* gene

Long-read and short-read data were used to compare sequences of IncHI2/IncHI2A/IncN plasmids of human and animal feces. pVNHN08-84-Hu-black color is from human feces, pVNHN08-86-An-purple color is from animal

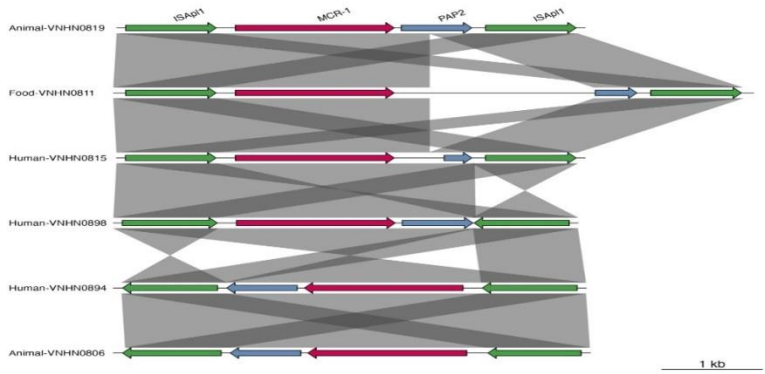


Figure 3.10. Genetic structure of transposons of 6 representative samples of *mcr-1* gene on chromosomes of *E.coli* strains isolated from human, animal, and food feces. *ISApl1: green arrow; gene mcr-1: red arrow; PAP2: blue arrow.*

Chapter 4. DISCUSSION

4.1. Rate of *E.coli* strains carrying colistin-resistant *mcr-1* gene isolated from humans, domestic animals, food and water in Thanh Ha commune, Thanh Liem district, Ha Nam in 2015

4.1.1. Percentage of E.coli strains carrying colistin-resistant mcr-1 gene isolated from humans, domestic animals, food and water

Results of our research in households in Thanh Ha commune, Thanh Liem district, Ha Nam province showed high percentage of samples containing *E.coli* strains carrying colistin-resistant *mcr-1* gene in humans and domestic animals when compared with studies conducted by Liu et al in China with the rates of 36.6% and 34.4%, respectively. *E.coli* strains carrying colistin-resistant *mcr-1* genes were also found in food samples taken from households (3.8%) and water samples including domestic well water, rainwater and irrigation water (8.4%) (Table 3.2).

Our study is the first in Vietnam to synchronously investigate the status of *E.coli* strains carrying *mcr-1* gene both in humans, animals and the environment (water, food) of an area belonging to the countryside region in the North.

The other similar studies conducted in China, Japan, and France on animal feces, farmer's feces, and food samples during period of 2015-2016 showed the proportion of *E.coli* strains carrying *mcr-1* gene of 21%, in particular, 8.47% of *E.coli* carrying *mcr-1* gene in animal feces found in China; and 4.84% of *E.coli* carrying *mcr-1* gene in animal feces and in farmer's feces found in Japan; in France, 5.9% of *E.coli* strains was found carrying *mcr-1* gene in food samples.

4.1.2. The susceptibility to colistin of E.coli strains carrying mcr-1 resistance gene

Among isolated 398 *E.coli* strains carrying *mcr-1* gene, 160 strains (40.2%) were found with colistin-resistant phenotype (MIC >2 µg/ml). The *E.coli* strains expressed colistin-resistant phenotype carrying *mcr-1* gene were found in all four type of samples (human feces, animal feces, water, and food) (Figure 3.1). *E.coli* strains carrying colistin-resistant *mcr-1* gene with MIC = 4 µg/ml were accounted for the highest percentage (80%). 21 strains were determined with MIC >16 (5.2%) including: 5 isolates from human fecal samples, 4 isolates from animal feces samples, 7 isolates from water samples and 5 strains derived from food. Some studies in the world also found high phenotypic expression rate of *E.coli* strains carrying *mcr-1* gene such as the results of study conducted by Katarzyna Ćwiek et al. at the farms in Poland with 52.9% of *E.coli* strains found to carrying *mcr-1* gene with phenotypically expressed and 88.8% had a colistin MIC of 8 µg/ml. Some other studies in the world also found that the colistin MIC of *E.coli* strains carrying *mcr-1* gene isolated from farms were mainly 8 µg/ml.

4.1.3. The prevalence of E.coli strains carrying mcr-1 gene on chromosomes and plasmid

The results of whole genome sequencing of 87 *E.coli* strains carrying *mcr-1* gene of our study identified that the *mcr-1* gene is located on both chromosomes and plasmids, but the proportion of *mcr-1* genes located on plasmids is much higher than that on choromosome (71.3% vs 26.4%, $p<0.001$). All 87 strains of *E.coli* carrying *mcr-1* gene were of subtype *mcr-1.1*. Our study also identified 2 strains (2.3%) of *E.coli* having *mcr-1* gene located on both chromosomes and plasmids, including 1 isolate from animal feces and 1 strain isolated from water.

Many studies have recorded the presence of *mcr-1* gene on plasmids, however some studies also found *E.coli* strains carrying the *mcr-1* gene located on chromosomes.

4.2. Molecular biological characteristics of *E.coli* strains carrying *mcr-1* gene

4.2.1. Genotypic relationship of *E.coli* strains carrying *mcr-1* gene determined by PFGE technique

The PFGE results showed that the *E.coli* strains carrying *mcr-1* gene were very diverse in genotype (Figures 3.3 to 3.6). 61.3% of *E.coli* strains (147/240 strains) found to carrying *mcr-1* gene were closely related to each other with genotypic similarity of ≥ 90%) and were differenciated into 58 genotype groups. 38.7% of strains (93/240) showed to have no genotypic relationship but have diverse genotype distribution.

According to result of analysing genotype relationships by PFGE technique, we found that *E.coli* strains carrying *mcr-1* gene in community both have stable genotyping character, creating different genitype trasmission groups in households, and are diverse with unrelated genotypes. This result is consistent with the distribution of *mcr-1* gene on chromosomes and plasmids clarified above.

4.2.2. Molecular biological characterization of E.coli strains carrying mcr-1 gene determined by whole-genome sequencing

In order to further investigate the transmission mechanism of *E. coli* strains carrying *mcr-1* gene in the community in a rural area of Northern Vietnam, our study selected 87 *E.coli* strains that carrying the *mcr*-1 gene.

4.2.3.1. Distribution characteristics of sequence types of E.coli strains carrying mcr-1 gene

The sequencing results obtained from 87 *E.coli* strains carrying the short-read *mcr-1* gene, according to the core genome and sequence type tree (Figure 3.6) showed 56 different STs found in 94 strains (7 strains isolated from clinical specimens). Commonly encountered STs for all 4 sample types include ST10 (n=10, 11%), ST48 (n=9.9%), and ST206 (n=8.8%). ST10 is a common ST of *E.coli* strains carrying *mcr-1* gene in the region and around the world in all humans, animals and the

environment. ST48 is the most commonly reported ST in China in *E. coli* strains carrying *mcr-1* gene isolated from animals.

E.coli strain carrying *mcr-1* gene of ST206 is a rare strain, sporadically recorded in animals and in patients in clinical practice. However, this strain is the dominant strain found in both humans, animals and in the aquatic environment in our study, and has not been found in clinical isolates.

4.2.3.2. Determination of plasmid characteristics of E.coli strains carrying mcr-1 gene

Computer simulation analysis of *mcr-1* gene-carrying plasmids of 64 strains based on short-read sequencing data showed that the plasmids exist in two forms: the unique replication - plasmids (single-replicon plasmids) and plasmids with multiple replication units combined in the same mobile genetic elements (multi-replicon plasmid).

This is the first study of Vietnam to determine the types of plasmid replication units of *E.coli* strains carrying *mcr-1* gene in all types of samples (human, animal and environment) in the community by using WGS technique. The study of Malhotra-Kumar et al. has sequenced an *E.coli* strain carrying *mcr-1* gene isolated at a farm in Van Lam, Hung Yen, and identified four types of plasmids: IncFII, IncF1A, IncF1B and IncX1 replicon. Research conducted by Yamaguchi et al. on human feces samples in Thai Binh province in 2017 also recorded 5 types of plasmids containing only 1 replication unit, IncI2, IncP1, IncHI2, Inc X4 and IncY.

The research results also showed the plasmid diversity of *E.coli* strains carrying *mcr-1* gene in the community, which is expressed most in the healthy population. The plasmids have very diverse sizes with the smallest plasmid size of < 50 Kb and the largest plasmid size of > 300 Kb. There are 7 types of plasmids containing 1 replicon unit carrying *mcr-1* gene detected from 29 strains in the studied samples including IncI2 (n=8, 12.1%), IncP (n=7, 10.6%), InX4 (n=5, 7.6%), IncFIA (n=3, 4.5%), IncHI1B (n=3, 4.5%), IncN (n=1, 1.5%) and IncX1 (n=1, 1.5%) found in humans, animals and water. The plasmids carrying multi replicon units containing *mcr-1 gene* were detected with a combination of IncHI2, IncN, IncX1 and IncR plasmids. 20 different types of

replicaton units with combinations of IncFIA, IncFIB, IncHI1B, IncHI2, IncN, IncY, IncX1 and IncI2 plasmids were found in 31 strains of *E.coli* isolated from humans, animals and water.

To determine the mechanism of plasmid-mediated transmission, we selected a number of *E.coli* strains carrying *mcr-1* gene located on different plasmids for conjugation techniques.

4.2.3.3. Determination of plasmid mediated transmission mechanism of E. coli strains carrying mcr-1 gene

By conjugation technique, the *mcr-1* gene was successfully transferred from the donor strain to *E.coli* J53 strain among 17 out of 24 strains (70.8%) including strains with plasmids containing a single replicon unit and plasmids containing multi- replicon units (Table 3.12). The results showed that *E.coli* strains carrying *mcr-1* gene located on plasmids in the research community, including plasmids containing one replicon unit and plasmids containing many replicon units, are capable of transmitting the *mcr-1* gene for other strains. The success rate of gene transfer obtained in our trial was higher than that found by the study of Le Quoc Phong et al. It is possible that we selected strains with known presence of *mcr-1* genes located on the plasmid and used different recipient strains that led to this difference.

4.2.3.4. Determination of the genetic structure of E.coli strains carrying the mcr-1 gene

We have sequenced 10 strains representing 6 replicon units of the plasmid using the MinION method (Oxford Nanopore Technologies) of nanopore sequencing. The results of the long-read technique confirmed the findings of the computer simulation results of the short-read technique including 3 types of plasmids containing single replicon unit IncI2 (n=2), IncP (n=1), InHI2 (n=1) and 2 plasmids IncHI2 and IncHI2A containing multiple replicon units in the community. We also used 2 clinically derived strains for comparison. Nanopore was also used to identify the *mcr-1* gene located on the chromosome (n=2).

Our results showed that plasmid IncI2 carrying *mcr-1* gene of *E.coli* strains isolated in samples collected from animals was similar to plasmid IncI2 of *E.coli* strains isolated from samples collected from people in the community (coverage rate: 90%) and similar to IncI2 of

strains isolated from clinical patients (coverage rate: 98%) with a similarity range of 81%-100% (Figure 3.8). We also observed 100% coverage rate when comparing sequences on long-read data of 2 plasmids containing multiple replicon units Inc (HI2:HI2A:N) in isolates of *E.coli* from human fecal and animal feces (Figure 3.9). This result suggests that the presence of *mcr-1* gene-carrying plasmids of *E. coli* strains is due to horizontal transmission in the bacterial community of humans, animals, as well as in the environment and in clinical patients.

We also constructed the structure of transposons of 10 representative strains based on data obtained from the Nanopore engineering platform. Six different structural forms of the *mcr-1* gene with or without ISApl1 have been detected on both chromosomes and plasmids. Among the strains carrying *mcr-1* gene on the chromosome (n=6), there were 5 strains containing the complete structure of the Tn6330 transposon, ISApl1-pap2-mcr-1-ISApl1.

One strain was found having a combination of ISApl1 and IS91 (ISApl1-mcr-1-IS91) (Figure 3.10). There were 2 variants (ISApl1 and IS1A) found in 2 plasmids containing multiple replicon units Inc (HI2:HI2A) and Inc (HI2:HI2A:N) plasmid (sample pVNHN08-95 and pVNHN08-84). We did not detect the full Tn6330 transposon on the plasmid. However, Yamaguchi et al detected the complete Tn6330 transposon ISApl1-pap2-mcr-1-ISApl1 in their study.

In summary, our study found a high prevalence of *E.coli* strains carrying *mcr-1* gene in the community in a rural commune in the North of Vietnam. This genes are located on both chromosomes and plasmids. The similar genomic results also suggest human-animal transmission through food and water contaminated with *E.coli* carrying the *mcr-1* gene. Thereby, we also found that the monitoring of colistin resistance requires synchronous evaluation and monitoring in both humans, animals and polluted environments. Solutions to minimize the increase in the rate of colistin resistance are not only monitoring the clinical use of colistin antibiotics or preventing the use of colistin in livestock, but also need to take measures to reduce contamination in food and in water environment with strains of *E.coli* carrying *mcr-1* gene.

4.3. Limitations and directions for further research

The research is limited in terms of time, funding, sample size, and within the framework of a doctoral thesis, thus cannot solve all issues related to epidemiological, microbiological and molecular biological aspects. Therefore, further studies are needed to elucidate the source of infection, risk factors and demonstrate the risk of spreading of *E. coli* carrying the *mcr-1* gene in the community from various sources such as food, soil, wastewater... In addition, it is also necessary to have intervention studies to come up with solutions to limit the infection and spread of *E.coli* bacteria carrying the *mcr-1* gene in particular and antibiotic-resistant bacteria in general, in the community.

For Objective 2, we wish to be able to compare the molecular biological characteristics of *E.coli* strains carrying *mcr-1* gene in the study with the *E.coli* strains isolated in some other areas in Vietnam in the period of 2016 - 2018 to see if there are genetic changes. However, we were unable to do this because we could collect data on a only small number of clinical *E.coli* strains carrying mcr-1 gene (n=7) for comparison. Therefore, there is a need for further similar studies to be able to evaluate the changes over time of E. coli strains carrying the mcr-1 gene in the community.

CONCLUSION

1. The percentage of *E.coli* strains carrying *mcr-1* gene in Thanh Ha commune, Thanh Liem district, Ha Nam province was very high in both human and animal feces samples, food and water environment with the corresponding rate of 36.6% and 34.4%, 3.8% and 8.4%.

2. The *E.coli* strain isolates were found to carrying *mcr-1* gene located on both chromosomes and plasmids, but the percentage of that located on plasmids was higher (71.3%) compared to that located on chromosomes (26.4%, $p<0.001$).

- Genotypic association analysis by PFGE showed strains of 18 identical genotype groups isolated from different households and/or different types of samples.

- Among the 87 strains of *E.coli* carrying *mcr-1* gene having whole genome sequenced, 56 different STs were differenciated, including STs

common to all 4 types of samples (human/animal feces, food and water) thus as ST10 (n=10, 11%), ST48 (n=9.9%), and ST206 (n=8.8%).

- The investigated plasmids were found of very diverse sizes with the smallest plasmid size of < 50 Kb and the largest plasmid size of > 300 Kb. 7 types of single replicon that carrying *mcr-1* gene were detected from 29 strains in the studied samples including IncI2 (n=8, 12.1%), IncP (n=7, 10.6%), InX4 (n=5, 5, 7,6%), IncFIA (n=3, 4.5%), IncHI1B (n=3, 4.5%), IncN (n=1, 1.5%) and IncX1 (n=1, 1.5%) found in humans, animals and water. There are 20 types of plasmids containing different replicon units with combinations of IncFIA, IncFIB, IncHI1B, IncHI2, IncN, IncY, IncX1 and IncI2 plasmids, found in 31 strains of human and animal and water *E.coli* isolates. The most common combination was found between IncHI2 with other replicons (n=23.4%) and IncH with IncF (n=12.33%).

- The plasmid IncI2 carrying *mcr-1* gene from animals was similar to plasmid IncI2 from humans in the community (coverage rate: 90%) with a homogeneity range of 81%-100%.

- Among the strains carrying *mcr-1* gene on the chromosome (n=6), 5 strains were found containing complete structure of the Tn6330 transposon, namely ISApl1-pap2-mcr-1-ISApl1. Another strain has a combination of SApl1 and IS91 (ISApl1-mcr-1-IS91).