博士論文

Antimicrobial-resistant bacteria isolated from urban rodents and house shrews in Vietnam and Indonesia

(ベトナムおよびインドネシアにおけるげっ歯類ならびにスンクスより分離された薬剤耐性菌の解析)

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Abbreviations

16S rRNA: 16S ribosomal RNA

AMR: Antimicrobial resistance

AVMA: American Veterinary Medical Association

CLSI: Clinical & Laboratory Standards Institute

DEC: Diarrheagenic *Escherichia coli*

DHL: Deoxycholate Hydrogen Sulfide Lactose Agar

E. coli: *Escherichia coli*

EAEC: Enteroaggregative *Escherichia coli*

EEM broth: Enterobacteriaceae Enrichment Mannitol broth

EIEC: Enteroinvasive *Escherichia coli*

ESBL: Extended-spectrum beta-lactamases

EPEC: Enteropathogenic *Escherichia coli*

ETEC: Enterotoxigenic *Escherichia coli*

FAO: The Food and Agriculture Organization

LB: Luria Bertani

LIM: Lysine Indole Motility

mcr: Mobilized colistin resistant

MDR: Multi-drug resistance

MIC: Minimum inhibitory concentration

MRSA: Methicillin-resistant *Staphylococcus aureus*

MSSA: Methicillin susceptible *Staphylococcus aureus*

OIE: The World Organisation for Animal Health

PCR: Polymerase Chain Reaction

RV: Rappaport-Vassiliadis

S. aureus: *Staphylococcus aureus*

STEC: Shiga toxin-producing *Escherichia coli*

TSA: Trypticase soy agar

TSI agar: Triple sugar Iron agar

VP: Voges-Proskauer

WHO: The World Health Organization

Abstract

AMR is a global public health concern for both clinical and veterinary medicine. AMR could be used as an indicator for misuse and overuse of antimicrobial agents. The intensive use of antimicrobial agents in humans, livestock and companion animals may affect infection of wildlife animals with AMR bacteria. Small mammals might acquire AMR bacteria in the environment through their habitats. To estimate AMR bacteria present in the environment and risk of public health, I have conducted a study of AMR bacteria in small mammals dwelling in urban areas in Hanoi, Vietnam and Bogor, Indonesia.

1. Antimicrobial-resistant *E. coli* isolated from rodents in Vietnam

In Vietnam, antimicrobials are extensively used in human medicine and animal farming. Consequently, the high levels of AMR *E. coli* have been reported in food, animals and humans. There was a concern about AMR *E. coli* contaminated to the environment in the city. Urban rodents were considered as a suitable indicator for the presence of those bacteria in the environment.

To investigate the prevalence and characteristics of AMR *E. coli* including ESBL-producing and colistin-resistant *E. coli*. Feces or cloacal swabs were collected from 144 urban rodents caught in Hanoi, Vietnam. Antimicrobial susceptibility tests were conducted according to the Kirby-Bauer disc diffusion method using ten antimicrobial discs. The isolates of ESBL-producing *E. coli* were confirmed by the synergy test of clavulanate diffusion from an amoxicillin-clavulanate disc with cefotaxime and ceftazidime. The resistance genes of *β*-lactams, sulfonamide and tetracycline were detected by multiplex PCR. The colistin resistance genes were amplified by PCR and identified into *mcr-1*, *mcr-2* and *mcr-3* by sequencing analysis.

A total of 59 AMR *E. coli* was isolated from urban rodents. The prevalence of AMR isolates was 45.2% in hospitals (14/31), 41.9% in markets (36/86) and 33.3% in a cargo station

(9/27). There was no statistical difference in the prevalence of AMR *E. coli* between hospitals, markets and a cargo station. The most common resistant phenotype was observed against ampicillin (79.7%: 47/59), followed by tetracycline (78%: 46/59), nalidixic acid (67.8%: 40/59) and sulfamethoxazole-trimethoprim (59.3%: 35/59). Other resistant phenotypes were observed lower than 50%. MDR *E. coli* were identified in 42 isolates. The most frequent resistance gene was tetracycline resistance genes, followed by beta-lactams and sulfonamides resistance genes. Furthermore, four MDR *E. coli* were confirmed as ESBL-producing *E. coli*, of which three were carried $bla_{\text{CTX-M-1}}$ gene. The colistin resistance gene of *mcr-1* was found in five MDR *E. coli*.

This study has been revealed the prevalence of AMR *E. coli*, including MDR, ESBL-producing and colistin-resistant *E. coli* in Hanoi, Vietnam. It indicated an extensive usage of antimicrobial agents in humans in this area.

2. Antimicrobial-resistant *E. coli* isolated from rodents and house shrews in Indonesia

In Indonesia, misuse and overuse of antimicrobials are common in humans, livestock and aquaculture, leading antimicrobial resistance in bacteria. The Regional Resistance Surveillance Program administered by 12 Asia-pacific countries showed that Indonesia was the most prevalent country of ESBL-producing *E. coli* in clinical cases. However, few studies have examined AMR *E. coli* isolated from small mammals and environment in Indonesia.

A total of 87 small mammals (79 rodents and eight house shrews) were captured in Bogor, Indonesia. The sensitivity test and the identification of colistin-resistant *E. coli* were performed the same as the methods described in the first chapter.

Overall, 20 AMR *E. coli* were identified in small mammals. Of which, 18 and two AMR *E. coli* were isolated from rodents and house shrews, respectively. The most common AMR in *E. coli* was resistant to tetracycline (85%: 17/20) and ampicillin (75%: 15/20). Other

resistant phenotypes were ranged from 5% to 35%. Eight out of 20 AMR *E. coli* were identified as MDR *E. coli*. The most frequent resistance gene was tetracycline resistance genes, followed by β-lactam genes. ESBL-producing and colistin-resistant *E. coli* were not found from small mammals in Indonesia.

This study could elucidate the increase of MDR *E. coli* in the environment in Bogor, Indonesia. In the city, small mammals dwelling proximity to humans might acquire AMR *E. coli* contaminated in the food or water during their feeding activities, indicating that mammals including humans might be infected with AMR *E. coli* contaminated in the environment.

3. Diarrheagenic *E. coli*, *Salmonella* spp. and *S. aureus* from small mammals in Vietnam and Indonesia

Although most of *E. coli* strains are harmless, there are many case reports that the pathogenic strains caused diarrhea. DEC is transmitted by fecal-oral route and causes diarrhea. In addition, *Salmonella* spp. and *S. aureus* were also concerned as foodborne diseases in the developing and developed countries. Vaccine for the infection with these bacteria is not effective or not available. In general, the treatment of foodborne diseases is currently use of antimicrobial agents, except for STEC and *S. aureus* infections.

DEC was analyzed for the presence of virulence genes associated with the group of ETEC, EPEC, STEC, EIEC and EAEC by multiplex PCR. *Salmonella* spp. was identified by the bacterial culture method and analyzed against ten antimicrobial agents using the disc diffusion method. *S. aureus* was identified by the bacterial culture method and then confirmed by the detection of 16S rRNA and specific gyrase genes using PCR. Susceptibility test for *S. aureus* was examined by the disc diffusion method using five antimicrobial agents and MIC test for cefoxitin and vancomycin. MRSA was confirmed by the MIC of cefoxitin and the detection of methicillin resistance gene.

Two DEC were detected in this study. Of which, an EIEC was a susceptible *E. coli*. Another was an EAEC, which showed an MDR phenotype, resistance to ampicillin, nalidixic acid, sulfamethoxazole-trimethoprim and tetracycline. All of four *Salmonella* spp. isolates were susceptible to all of ten antimicrobial agents examined. Eight and 16 *S. aureus* were isolated from 144 rodents and 87 small mammals captured in Hanoi, Vietnam and Bogor, Indonesia, respectively. The most prevalent in 24 *S. aureus* isolates were resistant to ciprofloxacin, followed by sulfamethoxazole-trimethoprim. All of the *S. aureus* isolates were susceptible to vancomycin. One out of 24 *S. aureus* isolate was identified as MRSA. The remaining isolates were MSSA.

The findings show the prevalence of DEC, *Salmonella* spp. and *S. aureus* isolated from small mammals in Vietnam and Indonesia. Control of transmission is the implementation of preventive methods of these pathogens.

Small mammals are not purposefully treated with antimicrobials. This study showed the prevalence of AMR *E. coli*, including MDR, ESBL-producing and colistin-resistant *E. coli* in small mammals. It was assumed that they are carrying AMR bacteria as a consequence of exposure to resistant bacteria contaminated in the environment. In addition, foodborne pathogens such as DEC, *Salmonella spp*. and a pathogen for a potential clinical infection (*S. aureus*) were also detected in the feces of small mammals. Since small mammals are dwelling proximity to humans in the urban areas, preventing the infestation of small mammals and improving hygiene are the key drivers to avoid unexpected transmission of AMR bacteria and foodborne pathogens to humans.

General introduction

AMR is currently considered the most significant global threat to humans and animals in the 21st century. Recently, there has been growing interest in AMR bacteria isolated from wildlife. AMR infection of wildlife animals has been implicated as indication of AMR contamination in the environment. To estimate the infestation with AMR bacteria in the environment, I have conducted a study on the prevalence of AMR bacteria in small mammals in urban areas.

1. Antimicrobial resistance in bacteria

Bacteria are becoming resistant by the mutations of pre-existing DNA or by the acquisition of foreign DNA containing antimicrobial resistance genes. Of which, horizontal gene transfer (HGT) is an essential factor in the maintenance and spread of AMR genes. Acquirement of foreign genetic material in bacteria is observed in three major routes, transformation (acquire naked DNA from the environment), conjugation (through bacterial pilus) and transduction (phage mediated). Transformation is a type of HGT that bacteria could naturally combine naked DNA to develop resistance. Conjugation plays a vital role in the transition of mobile genetic elements such as plasmids and transposons, which carry AMR genes. The emergence of resistant bacteria in the environment is often by conjugation [1, 2]. The resistance of bacteria in the environment is also related to phage mediated transduction. Phages are the most abundant microorganism found in the environments including ocean, lakes, soil, wastewater and so on [3]. The phage-mediated transduction is the driver key of HGT, between pathogens in the environment, animals and humans [4]. In addition, one of the most efficient in accumulating gene cassettes is integrons, particularly class 1 integron. These are genetic elements that can capture and express any open reading frame. Integrons are usually associated with other mobile elements such as plasmids and transposons, which have ability to transfer to a wide range of pathogens [5].

The acquisition of genetic materials in bacteria confers a variety of mechanisms resistance to antimicrobials. The mechanisms to target antimicrobial classes are shown in Table A.

- **Modification of antimicrobial molecule:** Bacteria produce enzymes which modify the antimicrobial molecule, leading that the antimicrobial is incapable of interaction with the target. Modification of the antimicrobial is often observed in classes of *β*-lactams, macrolides and chloramphenicol. The *β*-lactams is one of antimicrobial groups characterized by the possession of a *β*-lactam ring. The intact *β*-lactam ring is necessary for the action of penicillins, cephalosporins, carbapenems and monobactams. Bacteria produce enzymes *β*-lactamases which inactivate the *β*-lactam ring, causing resistance to *β*-lactams [6]. The resistant to chloramphenicol generally occurred by the presence of chloramphenicol acetyltransferase, which could inactivate acetyl of the antimicrobial [7]. Resistance to aminoglycosides is based on aminoglycoside modifying enzymes [8].
    - **Efflux pumps:** Since the antimicrobials are flushed out of the cells by efflux pumps, it would not have any adverse effect on bacteria. The efflux pumps are conferring resistance to antimicrobials in bacteria. This mechanism has been carried in both Gram-negative and Gram-positive bacteria. For example, *nor*A and *nor*B are the most important efflux pumps which resistant to quinolone in *S. aureus* [9]. *tet*(A) is the most popular efflux pump resistant to tetracycline in *E. coli* [10]. Resistant to fluoroquinolones, *β*-lactamases and tetracyclines is commonly observed by the efflux mechanism [11].
    - **Changes in the target site:** Bacteria have a mechanism to modify the target site of antimicrobials. The modifications can be carried out by mutation. An example is a mutation in DNA gyrase for resistance against quinolones. Another example of a

mutation in genes encoding the domain V of the 23S rRNA of Gram-positive bacteria causes resistance to oxazolidinones (linezolid and tedizolid) [11].

There are some other mechanisms of antimicrobial resistance such as overproduction of the target gene (dihydrofolate reductase overproduction for resistance to sulfonamide) [12], target protection (*qnr* genes for quinolone resistance) [13], resistance due to the adaptation of a cell with environmental conditions over a long period (resistance to daptomycin and vancomycin) [11]. However, the above-discussed mechanisms are the primary underlying mechanisms of resistance widely encountered in bacteria. There are still many unknown mechanisms of resistant to antimicrobials in bacteria.

2. Situation of antimicrobial resistance in the world

Since the 1940s, antimicrobials were introduced into clinical and started the extensive usage through this century. There are several ways in which antimicrobials enter the environment, such as antimicrobial use in humans and animals. The misuse and overuse of antimicrobials have cause selection pressure on microorganism by contaminating the environment. Microorganisms frequently exchange their AMR genes between species and species to their survival.

The consumption of antimicrobials has a relationship with AMR bacteria in the environment. β-lactams (penicillins, cephalosporins and carbapenems) are widely prescribed as antimicrobials. Other antimicrobials, such as tetracycline, macrolides and fluoroquinolones, are currently increasing the use for the treatment. According to WHO, 80% of antimicrobials are used in the community and 20% in hospital settings [14]. Most of antimicrobials absorbed by humans were eliminated in an active form in urine and feces [15]. In countries without wastewater treatment plant (WTTP), the eliminated antimicrobials are disseminated directly into the environment. On the other hand, antimicrobials were found as residues in secondary

treated effluents in countries with WTTP [16]. And then, the residues of antimicrobials were biodegraded, absorbed in sewage sludge, or eliminated in the plant effluent [16]. Sewage sludge can be used as fertilizer in crop fields. The effluent is discharged into the aquatic environment. Thus, the residues of antimicrobials released and contaminated into the environment. Even though the concentration of antimicrobial residues in the environment is usually low [17], AMR bacteria in the environment are more concerning in HTG between microorganisms.

The State of the World's Antibiotics 2015 estimated that two-third of total antimicrobials was used in domestic animals and the remaining one-third was in human annually [18]. The overall consumption of antimicrobials was increasing continuously [18]. Tetracyclines, penicillins and sulfonamides are the most commonly used antimicrobials for domestic animals in veterinary care. Other antimicrobials, such as β-lactams, aminoglycosides, chloramphenicol, macrolides and glycopeptides are concerned with the intensive use in domestic animals. The purposes of antimicrobial usage in domestic animals are generally to treat infectious diseases, to prevent and control common diseases. Antimicrobials are often dispensed in drinking water or feed to the entire farm for the treatment and prevention of bacterial infections. On the other hand, feed supplements contained antimicrobials are intended to promote the growth or food animals' productivity. Previous reports showed that the use of antimicrobials for animal farming has led to the rise of AMR bacteria in animal farms as well as of contaminated surrounding waters and soils. Due to the facilities of swine production, the prevalence of tetracycline resistance genes has been reported in lagoons and groundwater [19]. The multi-drug resistant bacteria have been reported from the environment around poultry [20]. Furthermore, the use of antimicrobials in agriculture has led to resistance in the environment [21]. These reports suggested that antimicrobials usage in animal farming and agriculture is applying selection pressure on bacteria in the environment, causing resistant bacteria.

WHO reported a high-level AMR found worldwide in January 2018 [22]. An estimate of 500,000 people across 22 countries was suspected of AMR bacterial infections annually. The most common AMR bacteria were observed to *E. coli*, *Klebsiella pneumoniae, S. aureus,* and *Streptococcus pneumoniae,* followed by *Salmonella* spp. The patients with sepsis were zero to 82% caused by AMR bacteria and remains by susceptible bacteria. WHO confirms that increase AMR bacteria is a serious threat to global public health in this report.

The US Center for Disease Control and Prevention estimated that more than two million people were annually affected by AMR infections in 2013. At least 23,000 people died because of those infections [23].

In Europe, it was estimated that approximately 400,000 people were infected with MDR bacteria. The number of deaths was observed in 25,000 each year. The most common MDR bacteria were *S. aureus*, *E. coli*, *Enterococcus faecium*, *Streptococcus pneumoniae*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* [24].

Since systematic data collection of AMR bacteria has not yet been undertaken in WHO Southeast Asia region [25], the reports of AMR bacteria were limited in the southeast Asian countries. Due to the dispensing antimicrobials without prescription, inappropriate use of antimicrobials, misuse and overuse of antimicrobials, antimicrobial resistance occurs in Vietnam [26]. Further, the hospital-acquired infections (HAIs) with AMR bacteria was reported [27, 28]. The highest prevalence of carbapenem resistance was observed in *Acinetobacter baumannii* (89.2%), followed by *Pseudomonas aeruginosa* (55.7%) and *Klebsiella pneumonia* (14.9%) [28]. The situation of carbapenem resistance in HAIs leads to a significant rate of death, because of exceeding healthcare-related infections and higher health-care costs.

Misuse and overuse of antimicrobials in humans, livestock and aquaculture are leading to antimicrobial resistance in Indonesia [29]. ESBL-producing *E. coli* (20%) and *Klebsiella*

(28%) were common in Indonesia by surveillance from 2005 [30]. Indonesia was then reported as the most prevalent country of ESBL-producing *E. coli* and *Klebsiella* among 12 Asia pacific countries in 2016 [31]. Approximately 6% of Enterobacteriaceae showed the resistance to imipenem in clinical cases from 2001 to 2012 [32]. The prevalence of Enterobacteriaceae, *Pseudomonas aeruginosa* and *Acinetobacter baumannii* resistant to carbapenem were 27.6, 21.9 and 50.5%, respectively in the intensive care units [33]. Although AMR is increasing among hospitalized patients and arose the nation's issue, unnecessary prescription of antimicrobials and self-medication are still common in Indonesia [29].

3. Global action plan on antimicrobial resistance

As an estimated, 0.7 million of people died by AMR bacterial infections in 2013 [34]. The number of deaths will increase up to 10 million by 2050. AMR will be the highest risk for causes of death, in comparison with cancer and diabetes [34]. In 2015, WHO released the global action plan on AMR to tackle the growing problem of AMR [35]. This guideline was the formulation of the global action plan for the member nations. The plan emphasized the need for a practical "one health" approach to coordinate the international actions between human, veterinary, agriculture, environment and finance. With the alarm about AMR, the Vietnamese government has been released the strategy to combat AMR. Vietnam became the first country of the nations in WHO Western Pacific Region to approve a national action plan for AMR combating [36]. Similarly, Indonesian government declared the national action plan to combat AMR in animals, humans and environment sectors as the guideline of WHO. In 2016, WHO, FAO and OIE published a self-assessment questionnaire to track progress in addressing AMR. WHO sent the survey to all governments through its regional and country offices. Each of countries is required to approach the development process of national action plans, work with multiple sectors and take key actions against AMR. As a result, a total of 147 countries had

responded to the questionnaire in May 2017 [37]. Most countries have made significant progress in developing national action plans and implementing actions in human and animal health, crop production, food safety and the environment.

4. AMR bacteria in small mammals

WHO released the first global report on surveillance of antimicrobial resistance in 2014, showing that there were high rates of resistance in bacteria causing common healthcare-associated and community-acquired infections in many countries [38]. So far, resistant bacteria were focused on the clinical fields of humans and veterinary. In recent years, some studies have been reported small mammals as the sentinel of AMR bacteria transmission in the environment [39, 40]. Although the source of AMR bacteria in small mammals was not yet clear, small mammals dwelling close to livestock and humans showed a high prevalence of AMR bacteria [41-43]. In general, small mammals without companion animals are not being expected to contact directly to antimicrobials. These reports suggested that AMR bacteria may spread from livestock and humans to small mammals through food or environment.

AMR genes, particularly in resistance plasmids, could be acquired to susceptible bacteria and spread quickly among microbial communities. The transferable resistant plasmids in ESBL-producing and colistin-resistant bacteria pose threats to humans. Infection with ESBL-producing or colistin-resistant bacteria makes it difficult to select treatment due to the lack of alternative antimicrobials. Rodents could carry ESBL-producing bacteria [44-46], while the study of small mammal carrying colistin-resistant bacteria has never been reported.

Pathogens have detected from rodents were diarrheagenic *E. coli* (DEC), *Salmonella* and *S. aureus* [47]. Rodents carried diarrheagenic *E. coli* were reported in Vancouver, Canada (STEC group) [46] and Buenos Aires, Argentina (EPEC and STEC group) [48]. Even though

the prevalence of DEC strains associated with antimicrobial resistance was low, the risk of DEC strains is still concerned for public health.

Rodents frequently showed asymptomatic infection with *Salmonella* spp. [49]. Rodents infected with *Salmonella* spp. spread the pathogens to the environment by fecal contamination. In general, one to 15% of infected rodents were estimated to be shedding *Salmonella* [50-54]. Rodents and house shrews carrying *Salmonella* spp. were reported in worldwide, Mekong Delta, Vietnam [55], Thailand [56], West Midlands, UK [57], Argentina [58], Vancouver, Canada [46], and California, USA [59]. Rodents and house shrews could act as a source of *Salmonella* infection in humans by contaminating food, water or the environment. Hygiene practices are essential to reduce the risk of salmonellosis in humans.

S. aureus is a potential risk for human health. It has been occurred several community-associated and nosocomial outbreaks globally [60]. The inappropriate use and overuse of antimicrobials in human, livestock and companion animals cause *S. aureus* resistant to antimicrobials, including MRSA strains [61]. Resistance to methicillin is due to the acquisition of methicillin resistance gene (*mec*A) which encodes a penicillin-binding protein (PBP 2a) with a low affinity for β-lactams [62]. Companion animals, livestock and wildlife animals were reported as the reservoirs of *mec*A positive staphylococci [63-68]. Rodents and house shrews also could be the reservoirs of MRSA strains [69], including rodents living in swine farms in the Netherlands [70], urban rats in Canada [71], rodents and house shrews in China [72].

Although the previous reports suggested that small mammals might be the reservoir and spreaders of AMR bacteria, few evidences have shown the ability of small mammals act as carrier of AMR bacteria, meaning that the stability and amplification of AMR bacteria in small mammals are still unknown.

5. Objective of this study

This thesis focuses on the investigation of the distribution and contamination of AMR bacteria in urban areas using small mammals, particularly in rodents and house shrews. Rodents and house shrews have adapted for their habitat in human living or livestock farms and have never been treated with antimicrobials. Therefore, to know the prevalence of AMR bacteria in urban areas, small mammals are able to use the indicator as sentinels in the distribution of AMR bacteria. Feces from small animals have been reported as a bioindicator for AMR *E. coli* [39, 73]. These studies showed that small mammals were useful bioindicators for the distribution of AMR bacteria in the environment and indicated the potential risk of AMR bacteria transmission to mammalian hosts, including humans. In this study, I conducted to investigate the prevalence and characteristics of AMR bacteria in urban rodents and house shrews in Hanoi, Vietnam and Bogor, Indonesia, resulting to elucidate the contamination of AMR bacteria in those cities.

Table A. Mechanisms of antimicrobial resistance

Antimicrobial class	**Representative antimicrobials**	**Function**	**Mechanism of resistance**
β –lactams	Penicillins Cephalosporins Cefotaxime Carbapenems	Inhibition of bacterial cell wall synthesis	- Enzymatic destruction of β-lactam rings - Target (PBP) modification - Reduced intracellular accumulation
Aminoglycosides	Streptomycin Gentamycin Tobramycin Amikacin	Inhibition of protein synthesis	- Antimicrobial structure modification - Target modification - Reduced uptake
Quinolones	Ciprofloxacin Ofloxacin Norfloxacin	Inhibition of DNA replication	- Efflux - Modification of target by mutations - Protection of target
Glycopeptides	Vancomycin	Inhibition of the last stages of cell wall assembly by preventing cross-linking reactions	- Target modification - Productions of false targets
Tetracyclines	Tetracycline	Inhibition of translation	-Efflux

			- Modification of ribosomal proteins
Rifampicins	Rifampin	Inhibition of transcription	- Altered β-subunit of RNA polymerase
Macrolides	Azithromycin, Erythromycin	Inhibition of protein synthesis	- Enzymatic modification - Efflux - Ribosomal mutations - 16S rRNA methylation
Sulfonamides	Sulfamethoxazole Trimethoprim Co-trimoxazole	Inhibition of folic acid synthesis	- Modification of target

Chapter I

AMR *E. coli* isolated from rodents in Vietnam

Introduction

Rodents are dwelling worldwide and cover more than 40% of Mammalia species diversity [47]. In their living habitats, rodents might gain AMR bacteria, including AMR *E. coli* from the environment [46]. Previous studies have highlighted the rodents as an indicator for the presence of AMR bacteria in the environment [39, 40, 46, 74-77]. Those studies have been revealed the prevalence of AMR *E. coli* carried by rodents in both urban and rural areas. The prevalence of AMR *E. coli* showed varied globally [39, 46, 75-77]. Rodents living close to humans or livestock farms were likely to gain a high level of AMR bacteria [39]. The study in Mekong Delta, Vietnam has reported a high profile of AMR *E. coli* in small mammals, including MDR and ESBL producing *E. coli* [76]. The prevalence of MDR *E. coli* in small mammals living close to farms was eight times higher than living in forests and rice fields, indicating that livestock farms were contaminated by AMR and MDR *E. coli* than forest and rice fields.

Rodents have adapted to living in urban areas, particularly in a density of human population and buildings, and are increasing contact with human waste and infrastructure (e.g., garbage and sewage). The residue of antimicrobials was reported in the environmental water and the wastewater from hospitals and slaughterhouse retail stores in Vietnam [78, 79]. Although there are several reports of AMR *E. coli* in the environment, the prevalence of AMR *E. coli* isolated from rodents has not been revealed yet in Vietnam.

In recent years, an increasing number of ESBL-producing *E. coli* was detected from food, poultry and healthy persons in Vietnam [80-82], while the reports of ESBL-producing *E. coli* in the environment were limited. Besides ESBL-producing bacteria, colistin-resistant bacteria have focused in recent years. Since *mcr-1* gene in *E. coli* was identified in China 2015 [83], colistin-resistant bacteria have been reported worldwide from food, animals and humans

[84]. Colistin-resistant bacteria in chicken and healthy humans were also reported in Vietnam [85, 86].

To elucidate the prevalence and contamination of AMR *E. coli* in the environment, AMR *E. coli* were isolated from rodents captured in Hanoi, Vietnam and conducted the detection of antimicrobial susceptibility and AMR genes.

Materials and methods

Sample collection

One hundred forty-four rodents were captured using live traps at eight locations in Hanoi, Vietnam in October 2017, March and June 2018 (Table 1.1). Sampling was conducted in cooperation with the National Institute of Hygiene and Epidemiology and Hanoi Center for Disease Control. Rodents species were identified by DNA sequencing of the mitochondrial cytochrome *b* gene [87]. Rodents were euthanized by isoflurane inhalation following the guideline of the American Veterinary Medical Association (AVMA). Rectal swabs and/or rectal feces were collected and frozen using dry ice and then stored at -80˚C for the bacteria isolation [88].

Isolation of E. coli from rodents

Rectal swabs and/or feces from rodents were soaking in 1 ml of LB broth (Dickinson and Co., Franklin Lakes, NJ, USA). The samples were plated using an inoculation loop onto DHL Agar (Eiken Chemical Co., Ltd., Tokyo, Japan), and incubated at 37°C for 24 hrs. A colony showing typical *E. coli* morphology was picked up from each rodent samples and was identified to *E. coli* by the following biochemical tests: TSI agar (Becton, Dickinson and Company), LIM test (Eiken Chemical Co., Ltd), VP test (Eiken Chemical Co., Ltd), citrate utilization tests, and oxidase test (Merck KGaA, Darmstadt, Germany) [89]. These media were performed as procedures described in figure 1.1. Bacteria fermented glucose, lactose and/or sucrose (yellow on both slant and butt of TSI gar), gas (+), H2S (-), motility, indole (+), lysine (+), VP (-), citrate (-) (no color change), oxidase (-) was considered to be *E. coli*. PCR method was performed to detect *yaiO* gene for *E. coli* confirmation [90]. JCM 384 strain was used as a standard strain of *E. coli* in the biochemical tests and PCR confirmation.

Antimicrobial susceptibility test

The resistance of *E. coli* isolates against ten antimicrobial agents was determined by the Kirby-Bauer disc diffusion method, the procedures as described in Figure 1.2. The antimicrobial agents and the concentrations were as follows: ampicillin (10 µg), cefodizime (30 µg), gentamicin (10 µg), tetracycline (30 µg), ciprofloxacin (5 µg), cefotaxime (30 µg), amoxicillin-clavulanate (20 µg of amoxicillin and 10 µg of clavulanate), nalidixic acid (30 µg), chloramphenicol (30 µg), sulfamethoxazole-trimethoprim (23.75 µg of sulfamethoxazole and 1.25 µg of trimethoprim) (Eiken, Chemical Co., Ltd). The breakpoints (zone sizes of inhibition) of CLSI or manufacturer guidelines were used to determine susceptibility or resistance of isolates [91]. The standard strain (*E. coli* JCM 384) were included in the antimicrobial susceptibility tests for all of *E. coli* isolates.

Confirmation of ESBL-producing E. coli

The potential of ESBL-producing isolates was confirmed by the double-disk synergy test using cefotaxime, amoxicillin-clavulanate and ceftazidime. Amoxicillin-clavulanate disc was placed in the center of the plate. And then, the remaining two discs were placed at a distance of 3 cm from amoxicillin-clavulanate disc. Incubation of the plates was performed under the condition of 37°C for 24 hrs. Any distortion or increase in the zone towards amoxicillin-clavulanate disc was considered as positive for the ESBL production (Figure 1.3). If the distortion zone was not clear, repeat the test with the distance of 2 cm from amoxicillin-clavulanate disc [92]. The standard strain *E. coli* JCM 384 was used as a negative control for the ESBL production.

Detection of AMR gene

DNA extraction of *E. coli* isolates was performed using the boiling method. The colonies of *E. coli* from TSA agar plate were dissolved in 100 µl of distilled water, boiled at 95°C for 15 min and centrifuged at 5000 rpm for 10 min. A 50 µl of supernatant was collected into a 0.6 ml tube and stored at -30°C. The major *β*-lactamase genes (*bla*$_{\text{CTX-M}}$ group (*bla*$_{\text{CTX-M-1}}$, *bla*$_{\text{CTX-M-2}}$, *bla*$_{\text{CTX-M-8/25}}$, *bla*$_{\text{CTX-M-9}}$), *bla*$_{\text{TEM}}$, *bla*$_{\text{SHV}}$ and *bla*$_{\text{CMY-2}}$), sulfonamides (*sul1*, *sul2* and *sul3*), quinolone (*qnr*A) and tetracycline [*tet*(A), *tet*(B) and *tet*(C)] were tested by single or multiplex PCR. PCR sets and primers (Life Technologies Japan Ltd.) for the detection of AMR genes were described in Table 1.1 [42, 93, 94]. PCR reactions were all performed in a 25 µl reaction mixtures containing 12.5 µl of Gotaq (Promega Corporation, Madison, WI, USA), 0.3 µM of each primer and 2 µl of DNA template using the thermal profiles as following:

- Multiplex PCR 1: one cycle consisting of 15 min at 94°C, 35 cycles consisting of 1 min at 94°C, 1 min at 52°C and 1 min at 72°C, and one cycle consisting of 10 min at 72°C
- Single PCR 2, 3 and 4: one cycle consisting of 15 min at 94°C, 30 cycles consisting of 1 min at 94°C, 1 min at 55°C, and 1 min at 72°C, and one cycle consisting of 10 min at 72°C
- Single PCR 5: one cycle consisting of 15 min at 94°C, 30 cycles consisting of 1 min at 94°C, 1 min at 62°C, and 1 min at 72°C, and one cycle consisting of 10 min at 72°C
- Multiplex PCR 6: one cycle consisting of 15 min at 94°C, 30 cycles consisting of 1 min at 94°C, 1 min at 55°C, and 1 min at 72°C, and one cycle consisting of 10 min at 72°C
- Multiplex PCR 7: one cycle consisting of 15 min at 94°C, 30 cycles consisting of 1 min at 94°C, 1 min at 64°C, and 1 min at 72°C, and one cycle consisting of 10 min at 72°C

Detection of colistin-resistant E. coli

All of AMR *E. coli* isolates were examined to detect *mcr-1*, *-2* and *-3* genes as described in the previous studies [83, 85, 95]. Amplified fragments were confirmed by Sanger sequencing. Raw sequencing data were analyzed using the Mega software version 7.0. The nucleotide sequences determined in this study have been deposited to the GenBank (accession numbers MN519790 - MN519794). Colistin susceptibility test was conducted by the broth microdilution and macro dilution methods using colistin sulfate (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan). According to the breakpoint for Enterobacteriaceae in the European Committee on Antimicrobial Susceptibility Testing (EUCAST), the isolates showed > 2 µg/ml of MIC were determined as resistance.

Results

Prevalence of AMR E. coli isolated from urban rodents in Vietnam

A total of 144 rodents were trapped at eight locations, including three markets, two hospitals and one cargo station in the city of Hanoi, Vietnam. Rodents species were identified as one *Rattus argentiventer* (ricefield rat), 135 *Rattus norvegicus* (brown rat) and eight *Rattus rattus* (black rat) (Table 1.2). As a result, fifty-nine AMR *E. coli* were isolated from 58 *R. norvegicus* and one *R. rattus*. AMR *E. coli* isolates were obtained 45.2% (14/31), 41.9% (36/86) and 33.3% (9/27) from rodents in hospitals, market settings and a cargo station, respectively. No statistically significant differences were observed between the prevalence of AMR *E. coli* in hospitals, markets and a cargo station. The highest antimicrobial resistance showed resistant to ampicillin (79.7%: 47/59) and tetracycline (78%: 46/59), followed by nalidixic acid (67.8%: 40/59), sulfamethoxazole-trimethoprim (59.3%: 35/59), chloramphenicol (45.8%: 27/59), ciprofloxacin (44.1%: 26/59) and cefotaxime (30.5%: 18/59) (Table 1.3). MDR (resistance to three or more antimicrobial classes) were identified in 42 out of 59 isolates (71.2%) from *R. norvegicus*.

Prevalence of AMR genes from urban rodents in Vietnam

In 59 AMR *E. coli* isolates, the highest frequency of gene detection was observed in bla_{TEM} (69.5%: 41/59) followed by *tet*(A) (64.4%: 38/59), *sul2* (35.6%: 21/59), *sul3* (23.7%: 14/59), *sul1* (18.6%: 11/59) and *tet*(B) (11.9%: 7/59) (Table 1.3). In contrast, genes of bla_{SHV}, *qnr*A, and *tet*(C) were not detected from AMR *E. coli* isolates. There were several discrepancies in AMR *E. coli* isolates between the phenotypes and genotypes. In the 50 isolates phenotypically resistant to β-lactams (ampicillin, amoxicillin-clavulanate, cefodizime and/or cefotaxime), nine isolates had none of the β-lactamase genes (Tables 1.3 and 1.4). None of the

quinolone-resistance gene was detected from 26 and 40 isolates resistant to ciprofloxacin and nalidixic acid, respectively. Four out of the 35 isolates phenotypically resistant to sulfamethoxazole-trimethoprim was not detected sulfonamide resistance genes, and vice versa. Two out of 46 isolates with phenotypically resistant to tetracycline were not harbored the resistance genes.

Detection of ESBL-producing E. coli from urban rodents in Vietnam

Four isolates were confirmed as ESBL-producing *E. coli* by using the synergy test of clavulanic acid diffusion from an amoxicillin-clavulanate disc with ceftazidime and cefotaxime (Table 1.5). Of those, three isolates were isolated from Ha Dong hospital and one from the Ha Dong market. All ESBL-producing *E. coli* showed MDR phenotypes, which were resistant to at least six antimicrobial agents tested, and carried bla_{TEM} gene. The other ESBL genotypes were observed in three strains which harbored CTX-M-1 enzyme.

Detection of colistin-resistant E. coli from urban rodents in Vietnam

Five AMR *E. coli* were harbored *mcr-1* gene, while none of *mcr-2* and *mcr-3* genes were detected in 59 AMR isolates. The *mcr-1* fragments were confirmed by sequencing analysis. These sequences were deposited in the GenBank, with the length of 250 bp from IDs HN-71 and HN-109 and 259 bp from IDs HN-102, HN-120 and HN-137 (accession nos. MN519790 - MN519794). In comparison with mcr-*1* gene which reported as the first colistin-resistant *E. coli* strain SHP45 (accession no. KP347127) in the world, four sequences in this study were identical. All isolates carried *mcr-1* gene showed 4 µg/ml of MIC with colistin sulfate. The AMR profile was observed resistant to five and six antimicrobial classes. Interestingly, one isolate (HN-71) from Thanh Cong market had the resistant phenotype of all ten antimicrobial agents tested in this study.

Discussion

In the present study, 59 AMR *E. coli* (41%) were isolated from 144 urban rodents in Hanoi, Vietnam (Table 1.2 and 1.3). The prevalence of AMR *E. coli* isolated from rodents was no significant difference between hospitals, markets and the cargo station in Hanoi. Among AMR isolates, the most prevalent antimicrobial resistance was resistant to *β*-lactams (84.7%) followed by tetracycline (78%), quinolones (72.9%), sulfonamide (59.3%), chloramphenicol (45.8%) and gentamicin (22%) (Table 1.3). These results followed the previous reports of AMR *E. coli* found in urban rodents, which might reflect the distribution of AMR bacteria in the environment in recent years [39]. In this study, 71.2% (42/59) of the AMR *E. coli* isolates were resistant to at least three classes of antimicrobial agents (MDR). Of which, the prevalence of MDR *E. coli* in hospital settings (85.7%: 12/14) was higher than those observed in market settings (72.2%: 26/36) and a cargo station (44.4%: 4/9). This result suggested that the risk of MDR *E. coli* present in the environment in hospital settings to the public health should have more concerned. In my study, rodents carrying MDR *E. coli* in the urban city of Hanoi, Vietnam seem to be higher than had been found in other regions. For example, MDR *E. coli* isolated from rodents were 41.5% in Vancouver, Canada [46], 58.2% in Berlin, Germany [75], 66.7% in Nairobi, Kenya [96], and 27.2% in Mekong Delta, Vietnam [76]. The resistance profile of antimicrobials in AMR *E. coli* varied in the previous studied regions. The prevalence of tetracycline resistance ranged from 3.3 to 85.5%, and ampicillin resistance ranged from 15 to 87.6 % [46, 73, 75, 76, 96]. Resistance to ampicillin was the most common phenotype of *E. coli* isolated from urban rodents in Hanoi (79.7%). These results were consistent with other studies that ampicillin resistance was the most common among antimicrobials resistance phenotype [42, 97]. Our data was also in accordance with the ampicillin resistance profile in the Mekong Delta, the south Vietnam (85.9%) [76] (Table 1.7). These results might suggest the long-term use of *β*-lactam antimicrobials in both human and veterinary medicine in

Vietnam. Tetracycline resistance profile in Hanoi (78%) was observed two times higher in AMR *E. coli* isolated from small mammals in comparison with Mekong Delta (34.5%), indicating that the urban city was more contaminated with tetracycline resistant bacteria.

In the urban area, rodents might acquire MDR *E. coli*, including colistin-resistant *E. coli* throughout their habitats. Our data reveal that five AMR *E. coli* (8.5%: 5/59) isolated from urban rodents carried of *mcr-1* gene and the MIC shows those isolates resistance to colistin sulfate (Table 1.6). So far, the was no report of colistin-resistant *E. coli* among small mammals. This is a novel study of rodents carries colistin-resistant *E. coli*. The prevalence was lower in comparison to the prevalence of colistin-resistance *E. coli* in chicken (59.4%) and healthy chicken farmers (20.6%) in southern Vietnam [86] (Table 1.7). In Vietnam, colistin is limited the use of treatments except for the severe cases of infectious diseases [98], while it is one of the most common antimicrobials which contained in feed formula for growth promotion [99]. Another study conducted in the healthy persons living in rural area in northern Vietnam showed a much higher prevalence of colistin-resistance *E. coli* (80.6%) compare to the prevalence in urban rodents in this study [85]. Although colistin contamination was lower in Hanoi comparison to rural area, *mcr* genes were widespread in Vietnam. It requires to watch the use and contamination of colistin carefully.

ESBL-producing *E. coli* were identified in four isolates (6.8%: 4/59) in this study (Table 1.5). The prevalence of ESBL-producing *E. coli* isolated from urban rodents in Hanoi was lower than those observed in Hong Kong (13.9 %) [100] and Germany (16%) [74]. The ESBL-producing *E. coli* in this study was also lower than those observed in livestock (20%) and healthy persons (35.2%) in Vietnam [82]. Regarding ESBL enzymes, we found the $bla_{\text{CTX-M-1}}$ group in three ESBL-producing *E. coli* isolated from rodent feces in this study. It is similar to those observed in Germany that $bla_{\text{CTX-M-1}}$ was also detected from rodent feces in the urban areas [101]. The prevalence of CTX-M-1 enzymes in urban rodents was lower than those

reported in humans (33%) [102] and the food distribution system (29.8%) [81] in Vietnam. Our data contributed to the graphical distribution of CTX-M enzymes among hosts and locations in the world.

There were some exceptions observed between AMR phenotypes and the presence or absence of resistance genes in the previous studies [42, 103]. This study showed the correlation between AMR phenotypes and the presence or absence of resistance genes regarding the resistance to *β*-lactams, sulfamethoxazole-trimethoprim and tetracycline. The lack of *β*-lactamase genes (bla_{TEM}, bla_{SHV}, bla_{CTX-M}, bla_{CMY-2}) in *β*-lactamase resistant *E. coli* may be caused of other genes since the *β*-lactamase genes were assigned to nine distinct structures of TEM, SHV, CTX-M, PER, VEB, GES, BES, TLA and OXA [104]. The plasmid-located genes such as $bla_{CTX-M-1}$ group, bla_{CMY-2}, and *mcr-1* might be disseminated widely to the environment. Another plasmid-located gene, *qnr*A encoding quinolone-resistant was not found in AMR *E. coli* isolates, even 26 and 40 isolates resistant to ciprofloxacin and nalidixic acid, respectively. The resistance to quinolones might cause by chromosomal mutation [75] or efflux pumps [105] in this study.

Overall, the prevalence of AMR *E. coli* isolated in urban rodents (41%) was similar to those observed in rodents living close to farms (45%) and much higher than rodents living in forest and rice fields (5.8%) in Vietnam [76] (Table 1.7). Our data indicated the prevalence of AMR bacteria in the environment, including MDR, ESBL-producing and colistin-resistant *E. coli* using urban rodents as indicators in Hanoi, Vietnam. The study indicated that antimicrobial agents were highly use in the urban areas in comparison to the rural areas.

Figure 1.1

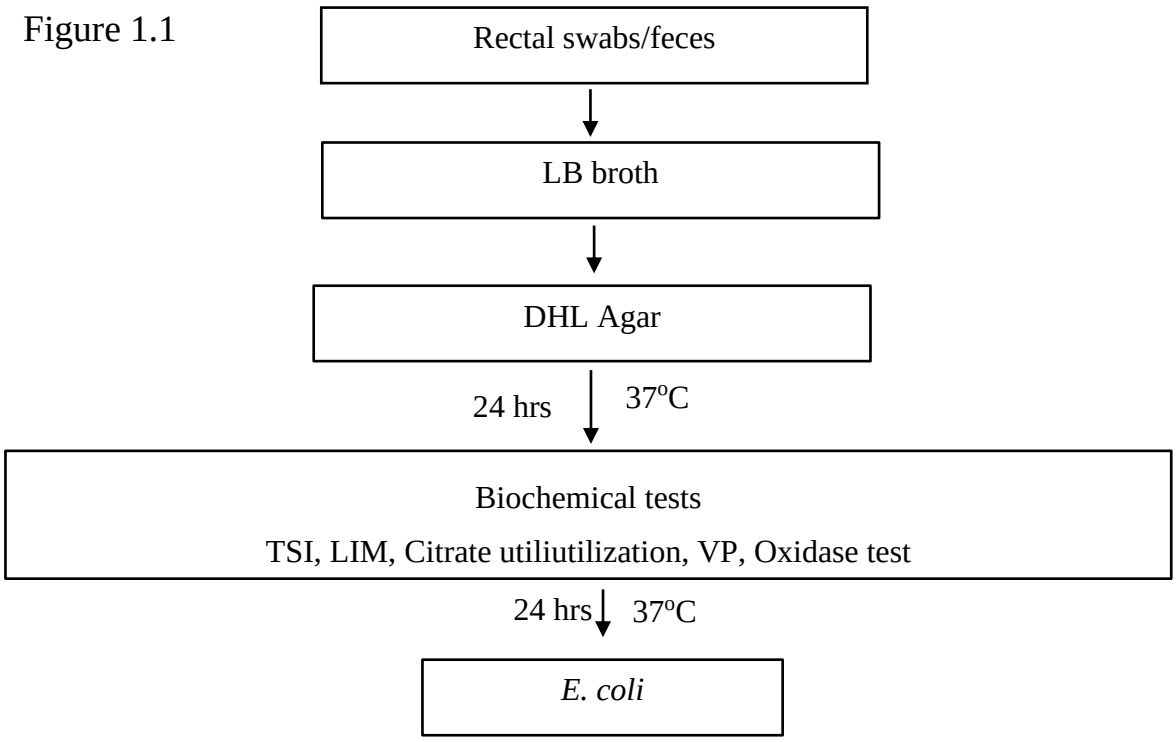

Figure 1.1: Flow diagram showing culture and identification procedures of *E. coli* isolates from urban rodents in Hanoi, Vietnam.

Figure 1.2

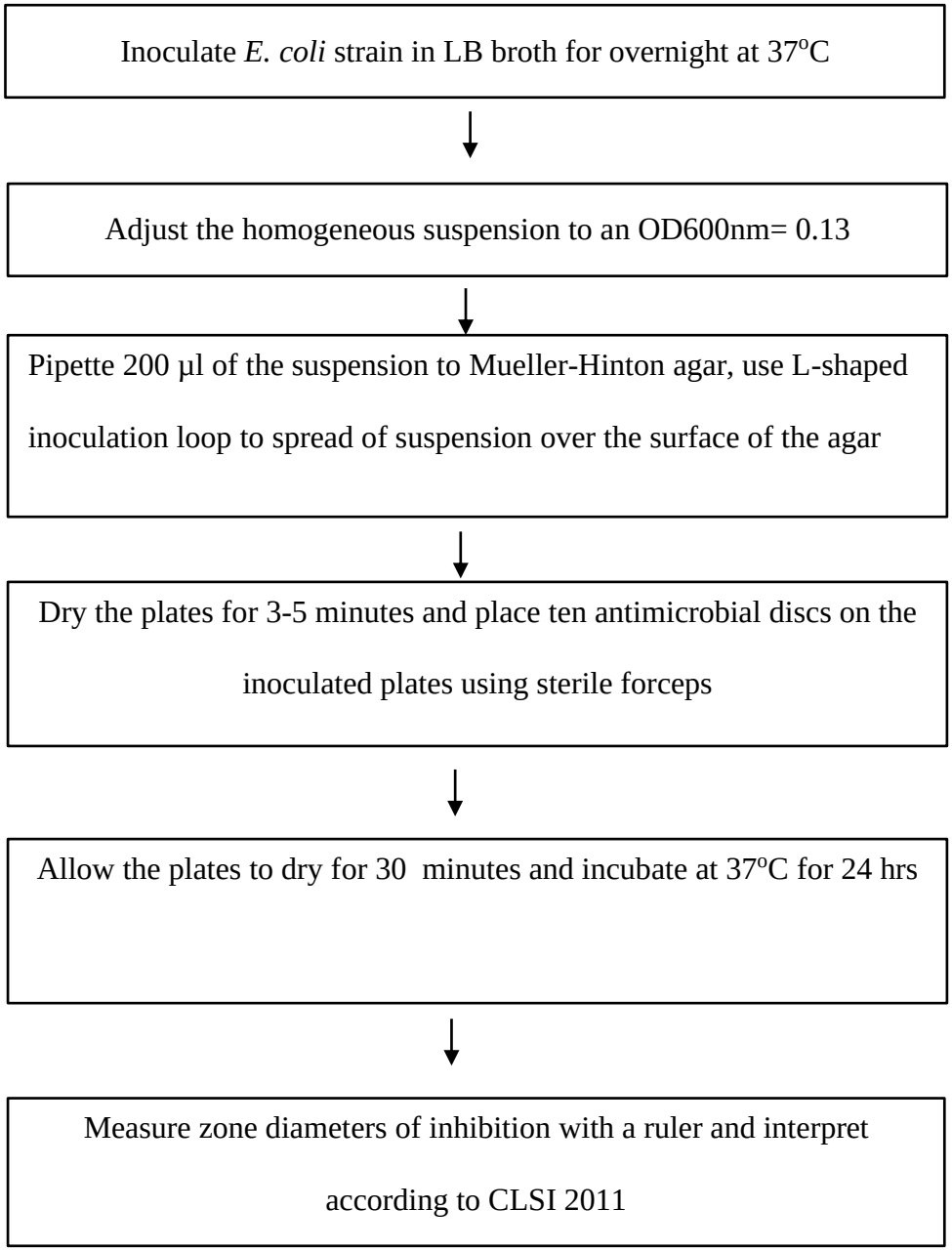

Figure 1.2. Antimicrobial susceptibility test by disc diffusion methods for *E. coli* isolated from rodents in Hanoi, Vietnam.

Figure 1.3

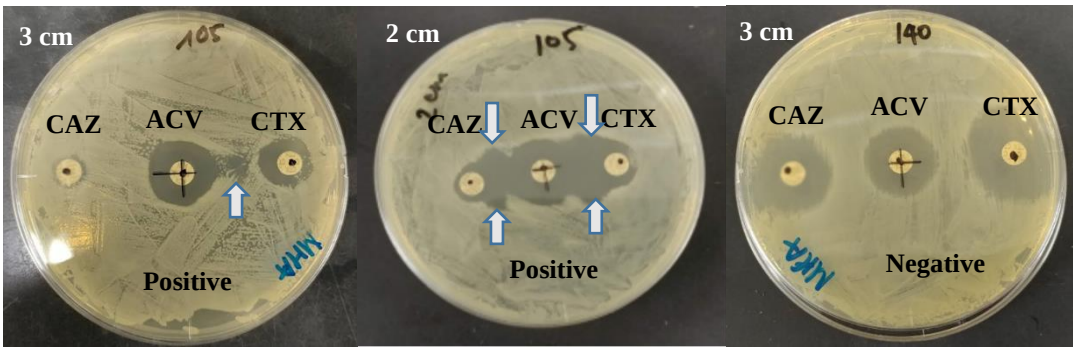

Figure 1.3. Double-disk synergy test for the confirmation of ESBL-producing *E. coli*

Table 1.1. PCR conditions used for detection of antimicrobial resistance genes in *E. coli* isolates

PCR	Gene	Primer	Primer sequence (5' → 3')	Product size (bp)	Reference
1	*bla*$_{TEM}$	GKTEMF GKTEMR	TTAACTGGCGAACTACTTAC GTCTATTTCGTTCATCCATA	247	[42]
	bla$_{SHV}$	SHV-F SHV-R	AGGATTGACTGCCTTTTTG ATTTGCTGATTTCGCTCG	393	
	bla$_{CMY-2}$	CMYF CMYR	GACAGCCTCTTTCTCCACA TGGACACGAAGGCTACGTA	1000	
2	*bla*$_{CTX-M-1}$	CTXM1-F3 CTXM1-R2	GACGATGTCACTGGCTGAGC AGCCGCCGACGCTAATACA	499	[94]
3	*bla*$_{CTX-M-2}$	TOHO1-2F TOHO1-1R	GCGACCTGGTTAACTACAATCC CGGTAGTATTGCCCTTAAGCC	351	
4	*bla*$_{CTX-M-8/25}$	CTXM825F CTXM825R	CGCTTTGCCATGTGCAGCACC GCTCAGTACGATCGAGCC	307	
5	*bla*$_{CTX-M-9}$	CTXM914F CTXM914R	GCTGGAGAAAAGCAGCGGAG GTAAGCTGACGCAACGTCTG	474	
6	*sul1*	sul1-F sul1-R	CGGCGTGGGCTACCTGAACG GCCGATCGCGTGAAGTTCCG	433	[42]
	sul2	sulII-L sulII-R	CGGCATCGTCAACATAACCT TGTGCGGATGAAGTCAGCTC	721	
	sul3	sul3-GKa-F sul3-GKa-R	CAACGGAAGTGGGCGTTGTGGA GCTGCACCAATTCGCTGAACG	244	
7	*tet*(A)	TetA-L TetA-R	GGCGGTCTTCTTCATCATGC CGGCAGGCAGAGCAAGTAGA	502	[42]
	tet(B)	TetBGK-F2 TetBGK-R2	CGCCCAGTGCTGTTGTTGTC CGCGTTGAGAAGCTGAGGTG	173	
	tet(C)	TetC-L TetC-R	GCTGTAGGCATAGGCTTGGT GCCGGAAGCGAGAAGAATCA	888	
8	*qnrA*	qnrA qnrB	GGGTATGGATATTATTGATAAAG CTAATCCGGCAGCACTATTA	670	[93]

Table 1.2. No. of antimicrobial-resistant *E. coli* isolated from urban rodents in Hanoi, Vietnam

Location		GTVT hospital			Ha Dong hospital		Dong Tam market	Thanh Cong market		Ha Dong market		Phung Khoang market	Thai Ha market		Giap Bat cargo station
Latitude, Longitude		21°1'33.50"N, 105°48'11.17"E			20°58'17.16"N, 105°46'30.66"E		20°59'48.52"N, 105°50'42.56"E	21°1'21.51"N, 105°48'54.17"E		20°58'11.61"N, 105°46'46.86"E		20°59'11.32"N, 105°47'37.87"E	21°0'49.40"N, 105°49'20.76"E		20°58'48.77"N, 105°50'29.24"E
Rat species		Rn[a)]	Ra[b)]	Rr[c)]	Rn[a)]	Rr[c)]	Rn[a)]	Rn[a)]	Rr[c)]	Rn[a)]	Rr[c)]	Rn[a)]	Rn[a)]	Rr[c)]	Rn[a)]
No. of resistant isolates / no. of samples (%)	Oct. 2017	0/9	0/1	0/1	-	-	3/12	1/17	-	-	-	-	-	-	3/16
	Mar. 2018	-	-	-	12/13	0/2	-	6/15	-	3/12	1/2	-	-	-	6/11
	Jun. 2018	2/5	-	-	-	-	-	8/9	0/1	2/2	-	10/10	2/4	0/2	-
	Subtotal	2/14	0/1	0/1	12/13	0/2	3/12	15/41	0/1	5/14	1/2	10/10	2/4	0/2	9/27
	Location total	14/31 (45.2)					36/86 (41.9)								9/27 (33.3)
	Total	59/144 (41)													

a) *Rattus norvegicus*

b) *Rattus argentiventer*

c) *Rattus rattus*

Table 1.3. Prevalence of antimicrobial-resistant *E. coli* isolated from urban rodents in Hanoi, Vietnam

Antimicrobial agents[a)]	Antimicrobial classes	No. of resistant isolates (%)								
		GTVT hospital n=2	Ha Dong hospital n=12	Dong Tam market n=3	Thanh Cong market n=15	Ha Dong market n=6	Phung Khoang market n=10	Thai Ha market n=2	Giap Bat cargo station n=9	Subtotal	Total n=59
ABP	Beta-lactams	2 (100)	11 (91.7)	2 (66.7)	12 (80)	5[b)] (83.3)	8 (80)	1 (50)	6 (66.7)	47 (79.7)	50 (84.7)
ACV		0	2 (16.7)	0	4 (26.7)	2 (33.3)	4 (40)	0	1 (11.1)	13 (22)	
CDZ		0	3 (25)	0	2 (13.3)	1 (16.7)	7 (70)	0	1 (11.1)	14 (23.7)	
CTX		0	4 (33.3)	1 (33.3)	1 (6.7)	2 (33.3)	7 (70)	0	3 (33.3)	18 (30.5)	
CIP	Quinolone	1 (50)	7 (58.3)	0	7 (46.7)	3 (50)	7 (70)	0	1 (11.1)	26 (44.1)	43 (72.9)
NA		0	10 (83.3)	1 (33.3)	11 (73.3)	4 (66.7)	9 (90)	1 (50)	4 (44.4)	40 (67.8)	
CP	Chloramphenicol	1 (50)	7 (58.3)	1 (33.3)	6 (0.4)	3 (50)	7 (70)	0	2 (22.2)	-	27 (45.8)
GM	Aminoglycoside	0	5 (41.7)	0	5 (33.3)	1 (16.7)	2 (20)	0	0	-	13 (22)
ST	Sulfonamide	2 (100)	9 (75)	1 (33.3)	9 (60)	3 (50)	9 (90)	0	2 (22.2)	-	35 (59.3)
TC	Tetracycline	2 (100)	9 (75)	2 (66.7)	14 (93.3)	5[b)] (83.3)	9 (90)	0	5 (55.6)	-	46 (78)
Multi-drug resistant		2 (100)	10 (83.3)	1 (33.3)	12 (80)	4 (66.7)	9 (90)	0	4 (44.4)	-	42 (71.2)

a) ABP: Ampicillin, ACV: Amoxicillin - Clavulanate, CDZ: Cefodizime, CTX: Cefotaxime, CIP: Ciprofloxacin, NA: Nalidixic acid, CP: Chloramphenicol, GM: Gentamicin, ST: Sulfamethoxazole - Trimethoprim, TC: Tetracycline

b) Including an isolate from *Rattus rattus*

Table 1.4. No. of antimicrobial resistance genes detected in 59 antimicrobial-resistant *E. coli* isolated from urban rodents in Hanoi, Vietnam

AMR genes	Antimicrobial classes	No. of isolates positive for AMR genes (%)									
		GTVT hospital n=2	Ha Dong hospital n=12	Dong Tam market n=3	Thanh Cong market n=15	Ha Dong market n=6	Phung Khoang market n=10	Thai Ha market n=2	Giap Bat cargo station n=9	Subtotal	Total n=59
bla$_{TEM}$	Beta-lactams	2 (100)	10 (83.3)	1 (33.3)	12 (80)	4[a)] (66.7)	8 (80)	0	4 (44.4)	41 (69.5)	41 (69.5)
bla$_{CTX-M-1}$		0	2 (16.7)	0	0	1 (16.7)	0	0	0	3 (5.1)	
bla$_{CMY-2}$		0	0	0	1 (6.7)	0	0	0	0	1 (1.7)	
bla$_{SHV}$		0	0	0	0	0	0	0	0	0	
*qnr*A	Quinolone	0	0	0	0	0	0	0	0	-	0
sul1	Sulfonamide	0	3 (25)	0	7 (46.7)	0	1 (10)	0	0	11 (18.6)	35 (59.3)
sul2		2 (100)	5 (41.7)	1 (33.3)	7 (46.7)	3 (50)	2 (20)	0	1 (11.1)	21 (35.6)	
sul3		1 (50)	5 (41.7)	1 (33.3)	2 (13.3)	1 (16.7)	2 (20)	0	2 (22.2)	14 (23.7)	
tet(A)	Tetracycline	2 (100)	8 (66.7)	2 (66.7)	9 (60)	4[a)] (66.7)	9 (90)	0	4 (44.4)	38 (64.4)	44 (74.6)
tet(B)		0	1 (8.3)	0	5 (33.3)	1 (16.7)	0	0	0	7 (11.9)	
tet(C)		0	0	0	0	0	0	0	0	0	

a) Including an isolate from *Rattus rattus*

Table 1. 5. Characteristics of extended-spectrum *β*-lactamase-producing *E. coli* isolated from urban rodents in Hanoi, Vietnam

ID	Location	Antimicrobial resistant phenotype[a)]	*β*-lactamase gene
HN-100	Ha Dong hospital	ABP-CDZ-CTX-CIP-NA-CP-GM-ST-TC	bla_{TEM}, $bla_{CTX\text{-}M\text{-}1}$
HN-101		ABP-CDZ-CTX-CIP-NA-CP-ST	bla_{TEM}, $bla_{CTX\text{-}M\text{-}1}$
HN-105		ABP-CDZ-CTX-CIP-NA-TC	bla_{TEM}
HN-133	Ha Dong market	ABP-CDZ-CTX-CIP-NA-CP-GM-ST-TC	bla_{TEM}, $bla_{CTX\text{-}M\text{-}1}$

a) ABP: Ampicillin, ACV: Amoxicillin - Clavulanate, CDZ: Cefodizime, CTX: Cefotaxime, CIP: Ciprofloxacin, NA: Nalidixic acid, CP: Chloramphenicol, GM: Gentamicin, ST: Sulfamethoxazole - Trimethoprim, TC: Tetracycline

Table 1.6. Characteristics of colistin-resistant *E. coli* isolated from urban rodents in Hanoi, Vietnam

ID	Location	Colistin resistance gene	MIC[a)] (µg/mL)	Other resistant phenotype[b)]
HN-71	Thanh Cong market	*mcr-1*	4	ABP-ACV-CDZ-CTX-CIP-NA-CP-GM-ST-TC
HN-102	Ha Dong hospital	*mcr-1*	4	ABP-CIP-NA-CP-GM-ST-TC
HN-109	Ha Dong hospital	*mcr-1*	4	ABP-CTX-NA-CP-GM-ST-TC
HN-120	Thanh Cong market	*mcr-1*	4	ABP-CIP-NA-CP-ST-TC
HN-137	Phung Khoang market	*mcr-1*	4	ABP-CIP-NA-CP-ST-TC

a) Minimum inhibitory concentration (MIC)

b) ABP: Ampicillin, ACV: Amoxicillin - Clavulanate, CDZ: Cefodizime, CTX: Cefotaxime, CIP: Ciprofloxacin, NA: Nalidixic acid, CP: Chloramphenicol, GM: Gentamicin, ST: Sulfamethoxazole - Trimethoprim, TC: Tetracycline

Table 1.7. Comparison with previous studies

E. coli	Rodents					Healthy person (Rural areas in Vietnam)	Livestock (Rural areas in Vietnam)
	Hanoi, Vietnam	Mekong Delta, Vietnam		Vancouver, Canada	Berlin, Germany		
		Forest and rice field	Farms				
Reference	This study	Nhung et al. 2015		Himsworth et al. 2015	Guenter et al. 2012	Nguyen et al. 2019, Trung et al. 2017, Yamamoto et al. 2019	Nguyen et al. 2019, Trung et al. 2017
AMR (%)	41	5.8	45	6.5	26	-	-
Ampicillin	79.7	85.9		53.7	23.6	-	-
Tetracycline	78	34.5		58.6	25.5	-	-
MDR	71.2	27.2		41.5	58.2	-	-
ESBL	6.8	-		4.9	16	35.2	20
Colistin (%)	8.5	-		-	-	20.6 – 80.6	59.4

-: Not applicable

Chapter II

AMR *E. coli* isolated from rodents and house shrews in Indonesia

Introduction

As mentioned in General introduction, the extensive use of antimicrobials is common in Indonesia, increasing more AMR bacteria in the environment. Indonesia was the most prevalent country of ESBL-producing *E. coli* (71%) in hospitalized patients, according to the Regional Resistance Surveillance Program administered by 12 Asia-pacific countries [31]. MDR *E. coli* was reported in 57.3% of swine samples [106], whereas reports of AMR *E. coli* contaminated in the environment have been limited in Indonesia. The density of population is quite high in Java island, Indonesia together with small mammals like rodents [107]. Small mammals dwelling in the city of Indonesia might become suitable indicators for the presence of AMR *E. coli* in the environment. So far, there was only one report that the surveillance of AMR *E. coli* among small mammals was conducted in Indonesia, 1988, over three decades ago. The report showed that *E. coli* isolated from rodents (*R. rattus* and *R. tiomanicus*) were resistant to *β*-lactams, chloramphenicol, sulfonamides and tetracycline [108].

Antimicrobial usage in livestock is dominantly in poultry farms in comparison to other sectors in Indonesia. According to the surveillance on 360 broiler farms (small to medium scale) conducted by the Ministry of Agriculture, most of the broiler farms (81.4%) routinely used antimicrobials for disease prevention and growth promotion [109]. The most common antimicrobial used in those farms were enrofloxacin (49.4%), amoxicillin (35.3%) and colistin (35.3%) [109]. Those surveillances revealed only in wide usage of antimicrobials, not the prevalence of AMR bacteria in farms. A study in 2014, *E. coli* isolated from chickens were resistant to all ten antimicrobial agents tested. The most prevalent AMR *E. coli* was observed resistant to oxytetracycline (61.5%), followed by ampicillin (43.6%) and quinolones (35.9 - 42.3%) [110]. Another study in 2019 has been reported the first detection of *mcr-1* gene in West Java, Indonesia that 89.7% of colistin-resistant *E. coli* isolated from broiler meat supply

chain were carried *mcr-1* gene [111]. These results indicated that increasing phenotypes of antimicrobial resistance occurred by the heavy use of antimicrobials in livestock farms.

Regarding studies in other livestock, there was a report that 8.6% of ESBL-producing *E. coli* isolated from cattle feces in the slaughterhouse in Bogor, Indonesia 2016 [112]. These isolates were identified in MDR *E. coli*, which were resistant to at least four antimicrobial classes. A study in 2018 revealed the prevalence of MDR *E. coli* (57.3%) isolated from 96 swine farms in Indonesia [113]. The common prevalence of resistant phenotype in the study was observed resistant to erythromycin (85.4%), and cephalothin (58.5%). As food contamination, AMR *E. coli* has been detected in fresh milk (67.2%: 86/128 samples). Of which, the most common resistant phenotype was observed resistant to ampicillin (95.3%) and chloramphenicol (39.5%).

With increasing in the prevalence of AMR *E. coli* reported in food, livestock and humans, AMR *E. coli* might widely spread in the environment. Since there was a lack of the prevalence of AMR *E. coli* in small mammals for three decades, the recent surveillance might be required to know the contamination and the risk analysis of AMR in urban city, Indonesia. In this study, I investigated the prevalence of AMR *E. coli* and their characteristics including AMR genes, ESBL-producing and colistin-resistant *E. coli* isolated from rodents and house shrews in Bogor, Indonesia.

Materials and methods

Sample collection

Small mammals were captured using live traps at four markets in Bogor city, Indonesia, in October 2019 (Table 2.1). Sampling was conducted in cooperation with Indonesian Research Center for Veterinary Science (BB Litvet), Bogor, Indonesia. House shrews were identified by morphology observation. Identification of rodent and house shrew species was performed by the DNA sequence of the mitochondrial cytochrome *b* gene [87, 114]. Small mammals were euthanized by isoflurane inhalation, as recommended using the guideline of the AVMA. Rectal feces were collected and frozen using dry ice and then stored at -80˚C until apply the microbiological culture [88].

Isolation of E. coli from small mammals

Feces were soaked in 1 ml LB broth (Dickinson and Co., Franklin Lakes, NJ, USA). The samples were plated using full loops (10 mm inoculate loop) onto DHL Agar, and incubated at 37°C overnight. Five colonies showing typical *E. coli* morphology from each sample were identified by the following biochemical tests: TSI agar, LIM, VP, citrate utilization, and oxidase [89]. The confirmation of *E. coli* was conducted by detecting *yaiO* gene by PCR [90]. Finally, non-duplicated *E. coli* isolate was chosen for further tests which were described in chapter I.

Antimicrobial susceptibility test

The resistance of *E. coli* isolates against ten antimicrobial agents was determined by the Kirby-Bauer disc diffusion method, the procedures as described in Figure 1.2. The antimicrobial agents and the concentrations were as follows: ampicillin (10 µg), cefodizime

(30 µg), gentamicin (10 µg), tetracycline (30 µg), ciprofloxacin (5 µg), cefotaxime (30 µg), amoxicillin-clavulanate (20 µg of amoxicillin and 10 µg of clavulanate), nalidixic acid (30 µg), chloramphenicol (30 µg), sulfamethoxazole-trimethoprim (23.75 µg of sulfamethoxazole and 1.25 µg of trimethoprim) (Eiken, Chemical Co., Ltd). The breakpoints (zone sizes of inhibition) of CLSI or manufacturer guidelines were used to determine the susceptibility or resistance of isolates [91]. The standard strain (*E. coli* JCM 384) were included in the antimicrobial susceptibility tests for all of *E. coli* isolates.

Detection of AMR gene

DNA extraction of *E. coli* isolates was performed using the boiling method. The colonies of *E. coli* from TSA agar plate were dissolved in 100 µl of distilled water, boiled at 95°C for 15 min and centrifuged at 5000 rpm for 10 min. A 50 µl of supernatant was collected into a 0.6 ml tube and stored at -30°C. The major *β*-lactamase genes (*bla*$_{\text{CTX-M}}$ group (*bla*$_{\text{CTX-M-1}}$, *bla*$_{\text{CTX-M-2}}$, *bla*$_{\text{CTX-M-8/25}}$, *bla*$_{\text{CTX-M-9}}$), *bla*$_{\text{TEM}}$, *bla*$_{\text{SHV}}$ and *bla*$_{\text{CMY-2}}$), sulfonamides (*sul1*, *sul2* and *sul3*), quinolone (*qnr*A) and tetracycline [*tet*(A), *tet*(B) and *tet*(C)] were tested by single or multiplex PCR. PCR sets and primers (Life Technologies Japan Ltd.) for the detection of AMR genes were described in Table 1.1 [42, 93, 94]. PCR reactions were all performed in a 25 µl reaction mixtures containing 12.5 µl of Gotaq (Promega Corporation, Madison, WI, USA), 0.3 µM of each primer and 2 µl of DNA template using the thermal profiles of PCR were described in chapter I.

Detection of colistin-resistant E. coli

All of AMR *E. coli* isolates were examined to detect *mcr-1*, *-2*, and *-3* genes as described in chapter I.

Results

Prevalence of AMR E. coli isolates from rodents and house shrews in Indonesia

A total of 87 small mammals, including 79 rodents (*Rattus norvegicus*) and eight house shrews (*Suncus murinus*), were captured at four markets in urban areas of Bogor, Indonesia, 2019 (Table 2.1). Twenty isolates were identified to AMR *E. coli*. Of which, 18 and two AMR *E. coli* were isolated from rodents and house shrews, respectively. The most common AMR was resistant to tetracycline (85%: 17/20), and ampicillin (75%: 15/20), followed by sulfamethoxazole-trimethoprim (35%: 7/20), nalidixic acid (30%: 6/20), ciprofloxacin (20%: 4/20), chloramphenicol (10%: 2/20) and gentamicin (5%: 1/20). None of AMR *E. coli* was observed resistant to cefotaxime, cefodizime, and amoxicillin-clavulanate. In total eight out of 20 (40%) isolates were identified in MDR *E. coli*, seven and one from rodents and house shrew, respectively (Table 2.2).

Detection of AMR genes from rodents and house shrews in Indonesia

The most frequent resistance gene was bla_{TEM} (75%: 15/20) followed by *tet*(A) (70%: 14/20), *sul3* (40%: 8/20), *tet*(B) (15%: 3/20) (Table 2.3). Genes of bla_{SHV}, $bla_{\text{CTX-M}}$, $bla_{\text{CMY-2}}$, *qnr*A, *sul1, sul2* and *tet*(C) were not detected in any isolates. Though ten isolates were showing quinolone-resistant phenotypes (4 and 6 isolates resistant to ciprofloxacin and nalidixic acid, respectively), no quinolone-resistance gene (*qnr*A) was found. On the other hand, one isolate carrying a sulfonamide resistance gene was without showing phenotype of resistant to sulfamethoxazole-trimethoprim. In resistance to β-lactams and tetracycline, the resistance phenotype corresponded to resistance genotypes (Tables 2.2 and 2.3).

Detection of ESBL-producing E. coli in Indonesia

In the results of antimicrobial sensitivity tests, there was no candidates of ESBL-producing *E. coli* in 20 AMR *E. coli* isolated from rodents and house shrews in Bogor, Indonesia.

Detection of colistin-resistant E. coli in Indonesia

There was no detection of *mcr-1, -2, -3* genes in 20 AMR *E. coli* isolated from rodents and house shrews in Bogor, Indonesia, indicating that there was no colistin-resistant *E. coli.*

Discussion

A total of 20 AMR *E. coli* was identified from 79 rodents and eight house shrews in Bogor, Indonesia. The most common resistant phenotypes observed were resistance to tetracycline (85%) and ampicillin (75%), followed by sulfamethoxazole-trimethoprim (35%), nalidixic acid (30%), ciprofloxacin (20%), chloramphenicol (10%) and gentamycin (5%) (Table 2.2). The prevalence of *E. coli* resistant to tetracycline was almost similar to those observed in pathogenic clinical isolates in Indonesia, which ranged from 64% to 88% [106, 115] (Table 2.4). The resistance to tetracycline also had been frequent in *E. coli* isolated from rodents in Hanoi, Vietnam, which was described in chapter I and in Trinidad and Tobago [116]. A previous study in Indonesia showed 13.3% of the resistance to tetracycline in AMR *E. coli* isolated from rodents [108]. It indicated that the environment might be contaminated with tetracycline resistant bacteria because of the longtime usage of tetracycline in the Java island, Indonesia. The tetracycline was used as the first line of antimicrobials in the livestock industry for disease therapy or growth promotion in Indonesia [106]. The feed with supplemental antimicrobials had an effect on increasing AMR bacteria among gut microflora in animals [117]. In this study, the surveillance conducted in the markets was dealing with livestock products. Our results indicated the possibility that small mammals were directly exposed to animal excreta containing AMR *E. coli* or indirectly to the genetic exchange of bacteria in the environment.

All isolates of AMR *E. coli* were susceptible to amoxicillin-clavulanic acid and cephalosporins (cefodizime, cefotaxime) belonging to *β*-lactam class. In contrast, isolates resistant to ampicillin (75%) belonging to *β*-lactam class same as amoxicillin-clavulanic acid and cephalosporins were most frequently occurred in small mammals in this study. The prevalence of ampicillin resistance in this study was increased in comparison to those of the study in 1988 reporting 20% of resistant ampicillin in AMR *E. coli* isolated from rodents [108]

and showed similar to the previous studies which were survey on AMR bacteria in small mammals conducted in England (90%) [118]. Furthermore, the prevalence of ampicillin resistance in this study was similar to those observed in pathogenic isolates from patients ranged from (73 - 78%) [115, 119] and much higher in comparison to isolates from chicken (43.6%) in Indonesia [110] (Table 2.4). Showing the phenotype resistant to ampicillin in *E. coli* isolated from rodents and house shrews may be affected by the intensive use of ampicillin (69 - 76%) for humans in Indonesia [120].

Since AMR bacteria were reported widely in the environment [121], small mammals might be exposed to AMR bacteria, particularly in MDR bacteria. In this study, the prevalence of MDR isolates from small mammals in Bogor (40%) was similar to those observed in Vancouver, Canada (41.5%), Berlin, Germany (58.2%) and Nairobi, Kenya (66.7%) [46, 75, 96]. Furthermore, the prevalence of MDR *E. coli* in small mammals was also similar to those observed in a swine farm (57.3%) and clinical cases (71%) in Indonesia [31, 106]. The prevalence of MDR *E. coli* in this study was two times higher than those observed in the report of Indonesia in 1988 [108]. The overuse or uncontrolled use of antimicrobial treatment for humans and veterinary increased the contamination of MDR *E. coli* in the environment.

Between resistant phenotypes and genotypes, there were controversial disagreements. One isolate carried of *sul3* gene has the susceptible phenotype of sulfamethoxazole-trimethoprim. Further, six isolates with the phenotype of resistant to quinolones did not harbor *qnr*A gene. The resistance to sulfamethoxazole-trimethoprim may refer to the gene of trimethoprim resistance in the isolate [122]. The resistance to quinolone may cause by other mechanisms such as a mutation in chromosome or efflux pumps [105]. Detection of bla_{TEM} and *tet*(A) genes was in accordance with resistant to ampicillin and tetracycline. These genes were detected most frequently in AMR *E. coli* isolated from small mammals in Bogor, Indonesia. The bla_{TEM} gene was more prevalent detected in *Klebsiella* than other pathogens in

clinical samples [123] and in *E. coli* isolated from animals [124]. A study on swine farms showed that *tet*(A) gene was also detected frequently in tetracycline resistance genes [106]. These results revealed that bla_{TEM} and *tet*(A) were widely disseminated through the environment, suggesting that genetic exchange between environmental bacteria in environment could be explained by the high prevalence of β-lactams and tetracycline resistance genes.

In this study, there was no candidate for ESBL-producing *E. coli* in AMR *E. coli*. *mcr* genes were also not found in this study. Since ESBL-producing and colistin-resistance bacteria usually associated with plasmid-mediated, data from this study suggested that plasmids of ESBL-producing Enterobacteriaceae and colistin resistance may not yet widely distribute in the environment in Bogor, Indonesia.

In conclusion, AMR *E. coli*, including MDR *E. coli* were observed in small mammals in Bogor, Indonesia. In the city, small mammals such as rodents and house shrews living proximity to humans, and they might ingest food or water containing resistant *E. coli* strains in the environment. Although the risk of small mammals carried AMR *E. coli* to human health remains unclear, this study elucidated increasing MDR *E. coli* in small mammals and contamination of AMR *E. coli* in the environment in Bogor, Indonesia.

Table 2.1. No. of antimicrobial-resistant *E. coli* isolated from small mammals in Bogor, Indonesia

Location	Anyar market	Bogor market	Jambu Dua market	Merdeka market	Subtotal	Total
Latitude, Longitude	6°35'31.4"S 106°47'33.1"E	6°36'14.0"S 106°47'58.2"E	6°34'12.3"S 106°48'28.2"E	6°35'32.0"S 106°47'10.2"E		
No. of resistant isolates/ no. of rodents (%)	7/45 (15.6)	6/21 (28.6)	3/7 (42.9)	2/6 (33.3)	18/79 (22.7)	20/87 (23)
No. of resistant isolates/ no. of house shrews (%)	-	2/8 (25)	-	-	2/8 (25)	

Table 2.2. Prevalence of antimicrobial-resistant *E. coli* isolated from small mammals in Bogor, Indonesia

Antimicrobial agents[a)]	Antimicrobial classes	No. of resistant isolates (%)					
		Anyar market n=7	Bogor market n=8	Jambu Dua market n=3	Merdeka market n=2	Subtotal	Total n=20
ABP	Beta-lactams	4 (57.1)	7 (87.5)	3 (100)	1 (50)	15 (75)	15 (75)
ACV		0	0	0	0	0	
CDZ		0	0	0	0	0	
CTX		0	0	0	0	0	
CIP	Quinolone	1 (14.3)	2 (25)	1 (33.3)	0	4 (20)	6 (30)
NA		2 (28.6)	2 (25)	1 (33.3)	1 (50)	6 (30)	
CP	Chloramphenicol	0	1 (12.5)	1 (33.3)	0	-	2 (10)
GM	Aminoglycoside	0	0	1 (33.3)	0	-	1 (5)
ST	Sulfonamide	0	5 (62.5)	2 (66.7)	0	-	7 (35)
TC	Tetracycline	6 (85.7)	8 (100)	2 (66.7)	1 (50)	-	17 (85)
Multi-drug resistant		1 (14.3)	5 (62.5)[b)]	2 (66.7)	0	-	8 (40)[b)]

a) ABP: Ampicillin, ACV: Amoxicillin - Clavulanate, CDZ: Cefodizime, CTX: Cefotaxime, CIP: Ciprofloxacin, NA: Nalidixic acid, CP: Chloramphenicol, GM: Gentamicin, ST: Sulfamethoxazole - Trimethoprim, TC: Tetracycline

b) Including one isolate from a house shrew

Table 2.3. No. of antimicrobial resistance genes detected in 20 antimicrobial-resistant *E. coli* isolated from small mammals in Bogor, Indonesia.

AMR genes	Antimicrobial classes	No. of isolates positive for AMR genes (%)					
		Anyar market n=7	Bogor market n=8	Jambu Dua market n=3	Merdeka market n=2	Subtotal	Total n=20
bla$_{TEM}$	Beta-lactams	4 (57.1)	7 (87.5)	3 (100)	1 (50)	15 (75)	15 (75)
bla$_{CTX-M}$		0	0	0	0	0	
bla$_{CMY-2}$		0	0	0	0	0	
bla$_{SHV}$		0	0	0	0	0	
*qnr*A	Quinolone	0	0	0	0	-	0
sul1	Sulfonamide	0	0	0	0	0	8 (40)
sul2		0	0	0	0	0	
sul3		1 (14.3)	5 (62.5)	2 (66.7)	0	8 (40)	
tet(A)	Tetracycline	6 (85.7)	5 (62.5)	2 (66.7)	1 (50)	14 (70)	17 (85)
tet(B)		0	3 (37.5)	0	0	3 (15)	
tet(C)		0	0	0	0	0	

Table 2.4. Comparison with previous studies

E. coli	Small mammals				Hospitalized patients in Indonesia	Livestock in Indonesia
	Bogor, Indonesia	Four islands (Java, Rataka, Sertung, Panjang), Indonesia	Vancouver, Canada	Berlin, Germany		
Reference	This study	Graves et al. 1988	Himsworth et al. 2015	Guenter et al. 2012	Subekti et al. 2003, Duerink et al. 2007,	Usui et al. 2014, Purwanto et al 2019, Kallau et al. 2018, Sudarwanto et al. 2016
AMR (%)	23	46.9	6.5	26	-	-
Ampicillin	75	20	53.7	23.6	73 - 78	43.6
Tetracycline	85	13.3	58.6	25.5	64 - 88	85.3
MDR	40	20	41.5	58.2	-	57.3
ESBL	0	-	4.9	16	71	8.6
Colistin (%)	0	-	-	-	-	-

- : Not applicable

Chapter III

Diarrheagenic *E. coli, Salmonella* spp. and *S. aureus* from small mammals in Vietnam and Indonesia

Introduction

AMR is a global health threat for both humans and animals. In chapter I and II, the prevalence of AMR *E. coli* isolated from small mammals have been revealed. Small mammals in Vietnam and Indonesia may carry AMR pathogens, which can cause infectious disease in humans. There have been reported that small mammals carried bacterial pathogens, which could associate with AMR [46, 71, 73]. Enterobacteriaceae such as *Salmonella* spp. and DEC have been identified from rodents in some cities, Piraeus, Greece [73], Vancouver, Canada [46], Buenos Aires, Argentina and so on [48]. Another bacterial pathogen such as *S. aureus* also detected in small mammals in Vancouver, Canada [71] and Guangzhou, China [72]. So far, studies on small mammals carried of these pathogens in Vietnam and Indonesia were limited or no interested.

DEC infection showed high mortality in infants and young children, both developing and developed countries [125]. According to the virulence genes, DEC belongs to five major intestinal pathogen groups: ETEC, EPEC, STEC, EIEC and EAEC [126]. STEC is well-known as foodborne pathogens distributed worldwide. With the ability to produce Shiga-toxin (*stx*), STEC could cause life-threatening hemolytic uremic syndrome (HUS) in humans, especially in children under five years old [126]. Patients infected with STEC group should avoid the treatment with antimicrobials due to HUS. EAEC is also an important group causing foodborne illness. EAEC group has occurred several foodborne outbreaks in India, Japan, Serbia and Mexico [127-130]. The transmission of DEC has been reported through the food or water contaminated with feces of humans and animals [126]. While some studies have been reported that STEC and EPEC were associated with rodent feces, these studies have been limited in geographically [48, 131]. So far, small mammals acquired DEC has not yet been revealed in Southeast Asia.

Salmonella spp., which is the causative pathogen of salmonellosis, were reported in small mammals [46, 96]. Salmonellosis is important in public health, causing 95.1 million cases and 50,000 deaths annually, according to the Global Burden of Diseases, Injuries, and Risk Factors Study 2017 [132]. *Salmonella* is classified into two species, *Salmonella bongori* and *Salmonella enterica* and has been identified over 2,500 serovars. All the serovars could cause foodborne disease in humans. Most of *Salmonella* serovars colonized in the gastrointestinal tract of humans and animals. Further, *Salmonella* could be present in the intestinal tract of wild birds, reptiles and occasionally insects [133]. Small mammals often infected with *Salmonella* spp. asymptomatically [80]. The infection cycles of *Salmonella* spp. are usually completed in small mammals, because of their habitats. The food and water sources are infested with small mammals and contaminated by their droppings and urine [47]. *Salmonella* spp. and other foodborne pathogens could be introduced onto soils, water, vegetables and fruits by small mammals.

According to WHO, it is estimated that there are more than 600 million people acquired foodborne illness annually [134]. Staphylococcal foodborne illness is one of the most common foodborne diseases globally and caused by *S. aureus* infection [135]. Food consumption contaminated with *S. aureus* caused a rapid onset (usually 3-5 hours) due to the enterotoxin released during the growth of the organism [135]. So, the duration of onset depends on the amount of toxin ingested [136]. The symptoms of staphylococcal foodborne illness at onset were hypersensitivity, nausea, vomiting and abdominal cramps with or without diarrhea. Since the toxin is not affected by antimicrobials, antimicrobials are not useful for the treatment of staphylococcal foodborne illness [137].

Besides staphylococcal foodborne illness, *S. aureus* could cause a wide spectrum of diseases such as bacteremia-sepsis, endocarditis, pneumonia, osteomyelitis, arthritis and skin diseases [138]. Owing to the combination of enterotoxin, invasiveness and antimicrobial

resistance, *S. aureus* has emerged as a major pathogen in both hospital and community-acquired infections [135, 138]. The widespread use of antimicrobials in humans, livestock and companion animals applies selective pressure on *S. aureus*, resulting MRSA [61]. Resistant to methicillin occurs the acquisition of *mec*A gene encoding a penicillin-binding protein (PBP 2a) and shows resistant to all of beta-lactams [62]. There are several reports that staphylococci carried *mec*A gene were detected from wildlife animals [68]. Livestock-acquired MRSA (LA-MRSA) in rodents were reported from Netherlands in 2009 [70], Canada in 2014 [71], Germany, France and the Czech Republic in 2018 [61] and China in 2019 [72]. Due to the intrinsically resistant to beta-lactams of MRSA strains, MRSA infection is more difficult to treat than other staphylococcal infections [137, 139].

The purpose of this study was to monitor the contamination of AMR pathogens in the environment using the fecal prevalence of diarrheagenic *E. coli*, *Salmonella* spp. and *S. aureus* in rodents and house shrews in the city of Vietnam and Indonesia.

Materials and methods

Sample collection

A total of 231 small mammals were trapped in Vietnam and Indonesia. Trapping methods and identifications of species were described in chapter I and chapter II.

Isolation of E. coli and Salmonella spp.

E. coli isolates were collected by the bacterial culture methods as described in chapters I and II. For *Salmonella* isolation, rectal swabs and/or feces were soaked in 1 ml of LB broth. The samples were transferred using a full loop (10 mm inoculate loop) to Enterobacteriaceae EEM broth (Nihon SeiYuku Co., Ltd., Japan). The media were then incubated at 37°C for 24 hrs to enrich the organisms. After incubation, 1 ml of enriched samples in EEM broth was transferred to 9 ml RV broth, and a full loop was plated onto DHL Agar. Both media (RV broth and DHL Agar) were incubated at 37 °C overnight. Incubated RV broth was inoculated onto DHL agar and incubated at 37 °C for 24 hrs. Up to five colonies showing typical *Salmonella* morphology from each sample of small mammal were identified by the following biochemical tests: TSI agar, LIM, VP, citrate, and oxidase [89]. The procedures of bacterial culture for *Salmonella* were described in figure 3.1. Bacteria fermented glucose only (red slant/ yellow butt of TSI gar), gas (+), H2S (+), motility (+), indole (-), lysine (+ or -), VP (-), citrate (+), oxidase (-) was considered to be *Salmonella*. Confirmation and classification of the *Salmonella* to Enterica group were conducted by direct PCR amplification of *hil*A gene [140].

S. aureus isolation and gene detection

Rectal swabs and/or feces were soaked in 1 ml LB broth. The samples were plated using full loops (10 mm inoculate loop) onto MSA agar (Merch KGaA, Darmstadt, Germany), and transferred using a full loop to enrichment broth containing 10 g/L tryptone, 75 g/L sodium

chloride, 10 g/L mannitol, and 2.5 g/L yeast extract [71]. Both media were incubated at 37°C overnight. After 24 hrs, a full loop of inoculated enrichment broth was plated onto MSA agar for 24-72 hrs at 37°C. MSA agar was observed the growth of bacteria until 72 hrs. After gram staining, up to five colonies showing typical *S. aureus* morphology were selected from each sample of small mammal by the observation under microscopy. Catalase test was used to detect staphylococci. Colonies with catalase-positive were sub-cultured to TSA agar for 24 hrs at 37^{o}C. After 24 hrs, the agglutination tests (Denka Seiken co., Ltd, Tokyo, Japan) were examined to identify as *S. aureus*. Procedures of *S. aureus* detection were described in Figure 3.2. The confirmation was conducted by the detection of 16S rRNA and the gyrase gene of *S. aureus* [93]. The *luk*S/F-PV gene encoding the Panton-Valentine leucocidin (PVL) toxin and *mec*A gene were detected by the PCR methods [141].

Antimicrobial **susceptibility test**

Antimicrobial susceptibility tests for *E. coli* and *Salmonella* spp. were conducted using the same antimicrobials and methods in the chapter I. For *S. aureus* isolates, antimicrobial susceptibility test was conducted on Mueller Hinton II agar (Becton, Dickinson and Company) according to the Kirby-Bauer disc diffusion method using disc and titer details as follows: chloramphenicol (30 µg), ciprofloxacin (5 µg), gentamicin (10 µg), tetracycline (30 µg) and sulfamethoxazole-trimethoprim (1.25 µg of trimethoprim and 23.75 µg of sulfamethoxazole) (Eiken, Chemical Co., Ltd). The MIC test was performed according to the microdilution method using antimicrobials of cefoxitin (Combi-Blocks Inc., CA, USA) and Vancomycin hydrochloride (Merck KGaA, Darmstadt, Germany). In this study, *S. aureus* JCM 28624 was used as a quality control strain.

Identification of diarrheagenic E. coli

In this study, multiplex PCR was used for identification of DEC strain. PCR primers used in this study are described in Table 3.1. The target genes are associated with DEC, including *elt* (heat labile enterotoxin), *est* (heat stable enterotoxin), *bfpA* (bundle-forming pilus), *eae* (*E. coli* attaching and effacing), *stx* (Shiga toxin), *ipaH* (invasion plasmid antigen H), *aatA* (outer membrane protein), *aaiC* (secreted protein) and *aggR* (transcriptional activator).

The PCR mixture was adjusted to a total 25 µl, containing with 12.5 µl of Gotaq (Promega Corporation, Madison, WI, USA), 0.3 µM of each primer (Table 3.1), 2 µl of DNA template. Multiplex PCR was divided into three sets. The first set was contained four primer sets for the detection of *eae*, *aat*A, *elt* and *aai*C genes. The second set was contained three primer sets for *ipa*H, *stx* and *est* genes. The third set was contained two primer sets for *bfp*A and *aag*R genes. All of PCR sets were performed condition as follows; 95^{o}C for 5 min, 30 cycles of 95^{o}C for 20 sec, 52^{o}C for 40 sec, 72^{o}C for 30 sec and 72^{o}C for 7 min.

Results

Diarrheagenic E. coli in small mammals

A total of 117 non-duplicated *E. coli* was identified from 231 small mammals captured in Vietnam and Indonesia. Of which, 79 AMR *E. coli* and 38 susceptible *E. coli* were described in chapter I and II. Then, these *E. coli* isolates were examined the multiplex PCR to detect the presence of virulence genes associated with ETEC, EPEC, STEC, EIEC and EAEC (Table 3.1). Two DEC were identified from 231 small mammals. The first DEC carried *ipa*H gene, which belongs to EIEC group, and was a non-AMR *E. coli* isolated from *R. norvegicus* in Giap Bat cargo station in Hanoi. Another isolate carried *aai*C gene, indicating the pathogen of EAEC group and was isolated from an *R. norvegicus* in Thanh Cong market in Vietnam. Resistance phenotype was ampicillin, nalidixic acid, sulfamethoxazole-trimethoprim, and tetracycline, indicating MDR, and AMR genotypes were observed in bla_{TEM}, *sul2*, *tet*(A). Other genes of ETEC, EPEC and STEC were not found.

Prevalence of Salmonella spp. in small mammals

There was no *Salmonella* isolate from rodents in Hanoi, Vietnam. In contrast, four *Salmonella* isolates were identified from rodents in Bogor, Indonesia. These isolates carried *hilA* gene detected by PCR methods and were classified into Enterica group. The susceptibility was observed in four isolates against all ten antimicrobials tested in this study (Table 3.2).

*Prevalence of S. aureus in small mammals and detection of mec*A *and PVL genes*

A total of 24 *S. aureus* were isolated from 231 captured small mammals in this study. Of which, eight and 16 *S. aureus* were detected from 144 rodents and 87 small mammals captured in Hanoi, Vietnam and Bogor, Indonesia, respectively. AMR phenotype of *S. aureus* isolated from rodents (*R. norvegicus*) in Hanoi was ciprofloxacin (37.5%: 3/8),

sulfamethoxazole-trimethoprim (37.5%: 3/8), followed by gentamycin (25%: 2/8), chloramphenicol (12.5%: 1/8), and cefoxitin (12.5%: 1/8). Other phenotypes were observed as susceptible to tetracycline and vancomycin (Table 3.3). Taken together, HN-76 and HN-133 isolates were identified as MDR *S. aureus*. AMR profile of *S. aureus* isolated from rodents (*R. norvegicus*) in Bogor, Indonesia, was observed in ciprofloxacin (18.8%: 3/16) and tetracycline (12.5%: 1/16). Susceptibility test was showed susceptible to cefoxitin, chloramphenicol, gentamycin, sulfamethoxazole and vancomycin (Table 3.4). There was no MDR *S. aureus* in isolates from Bogor, Indonesia.

The MRSA strain was determined by using cefoxitin as MIC > 4 µg/ ml regarding the guideline of CLSI 2011 [91] and detection of *mecA* gene. HN-133 was identified to MRSA, which was isolated from an *R. norvegicus* captured in Hanoi, Vietnam. As described above, HN-133 was also an MDR isolate, which had a phenotype resistant to cefoxitin, ciprofloxacin, chloramphenicol, gentamicin and sulfamethoxazole-trimethoprim. Other *S. aureus* isolates were identified as MSSA strains.

Twenty-four isolates were tested for the presence of *luk*S/F-PV gene. None of the isolates was positive for PVL toxin.

Discussion

Previous reports on AMR bacterial pathogens isolated from small mammals were focused on only one pathogen such as pathogenic *E. coli* [75], *Salmonella* [50, 57] or MRSA [71]. My study was the first report which monitoring AMR in three pathogens isolated from small mammals including DEC, *Salmonella* and *S. aureus*.

DEC has been reported in association with several foodborne outbreaks in developed and developing countries [125]. In the present study, *aai*C gene, which associated with the EAEC group, was found in an MDR *E. coli* isolated from an urban rodent. The other was *ipa*H gene, which associated with EIEC group, in a susceptible *E. coli*. The both of DEC were isolated from Vietnam and showed 1.4% among urban rodents in Vietnam. The prevalence of DEC isolated from rodents was varied between locations. In previous studies, those prevalence were 0% in Berlin, Germany [75], 3.8% in Vancouver, Canada [46], and 8.5% in Buenos Aires, Argentina [48]. The prevalence of DEC in rodents might reflect the DEC in the microbial communities in the environment geographically. The prevalence of DEC in this study was lower than those observed in calves (31.3%), chickens (10.9%) and healthy persons (6.7%) in Vietnam [142-144]. Although the contamination of DEC in the environment might be low, this study suggested that small mammals could acquire of DEC from the environment, including AMR isolate.

Rodents could be infected with *Salmonella* spp. with frequently without symptoms [49]. In this study, four *Salmonella* spp. (Enterica group) were found in rodents, accounted for 1.8% (4/223), not in house shrews (Table 3.2). All four of *Salmonella* spp., 4.6% (4/87) were isolated from rodents in Bogor, Indonesia. In contrast, *Salmonella* was not detected from rodents in Hanoi, Vietnam. Since rodents might gain *Salmonella* strains present in the environment, this study indicated that *Salmonella* spp. were disseminated in urban areas, particularly in markets, of Bogor city, Indonesia. Previous studies showed that the prevalence of *Salmonella* was often

higher among rodents living in nature (>19%) [55, 145] than living in urban areas (<5%) [46, 73, 116]. The prevalence was observed in Piraeus, Greece (4%) [73], Trinidad and Tobago (2%) [116] and Vancouver, Canada (0.5%) [46]. Similarly, this study showed the none or low prevalence of *Salmonella spp*. isolated from rodents in the city of Vietnam and Indonesia. However, a few exceptions were observed in the prevalence of *Salmonella* spp. isolated from rodents. For example, the prevalence of *Salmonella* spp. was found relatively high (15%) in urban rodents in Yokohama, Japan [51], and lower in rodents from farms (0%) in the United Kingdom and [146] and Netherland [52]. These results suggested that it is difficult to conclude the environmental load of *Salmonella* spp., and the accumulation of evidence should be required.

While laboratory mice have been reported that they are frequently colonized with *S. aureus* [147], the colonization in rodents remains unclear. My study suggested that rodents could acquire *S. aureus*, including MRSA strains [71]. The prevalence of rodents carried *S. aureus* was ranged from 7.1% to 41.9% in previous studies [61, 70, 148]. In this study, *S. aureus* was isolated from 24 out of 231 (10.4%) small mammals. None of *S. aureus* was detected in house shrews, likely due to the lower number of house shrews obtained. The AMR profile of MSSA strains isolated from rodents in Hanoi was resistant to ciprofloxacin, chloramphenicol, gentamicin, and sulfamethoxazole-trimethoprim. Those profiles observed in Bogor were resistant to ciprofloxacin and tetracycline. All the *S. aureus* isolates showed susceptible to vancomycin, which was similar to previous reports in MRSA strains isolated from small mammals [71, 72]. Previous studies in small mammals were largely focused on MRSA strains [71, 72]. The prevalence of MSSA isolated in small mammals was limited, and AMR profile of MSSA strains has never been revealed. The identification of MSSA and their AMR profiles in this study contributes to the knowledge accumulation of *S. aureus* associated with rodents in the city.

In this study, one and none of MRSA was isolated from an urban rodent in Hanoi, Vietnam and in Bogor, Indonesia, respectively. In Hanoi, the prevalence of MRSA in rodents (0.7%: 1/144) was lower in comparison to the community (7.9%: 80/1016) [149]. In comparison with MRSA isolated from rodents, the prevalence of MRSA in Hanoi was also lower than those in Vancouver city, Canada (3.5%) [71], Guangzhou city, China (5.2%), livestock farms in Netherland (11.6%) [70] and rural areas in Germany, France and the Czech Republic (15.3%) [61]. Except for MRSA isolated from rodents in Canada, most of MRSA was LA-MRSA. These results suggested that rodents might infect with LA-MRSA more than MRSA originating from humans. This study revealed that the contamination and distribution of *S. aureus* and MRSA in the environment were quite low in Hanoi, Vietnam and in Bogor, Indonesia.

PVL was not found in any *S. aureus* isolates in this study. PVL is a cytotoxin, which associated with a skin infection and pneumonia [150]. It is likely that *S. aureus* isolated from urban rodents were associated with other diseases. In this study, rodents gained *S. aureus* from markets and cargo station, and might excreted feces to the environment. Rodent feces contained *S. aureus* might contaminate feed and transmit *S. aureus* to everywhere, causing the various disease in humans and animals.

Previous studies have been primarily focused on rodents associated with zoonotic pathogens with natural reservoirs such as *Leptospira* [151], *Rickettsia* [152], *Hantavirus* [153], and etc. This study indicated that rodents might acquire bacteria, including foodborne and AMR pathogens from the environment and then, excrete feces containing these bacteria to the environment again. They might become both of the indicators of the bacteria present in the environment and the spreader of foodborne and AMR pathogens. To prevent the potential transmission of these pathogens, pest control and improvement of hygiene are required.

Figure 3.1

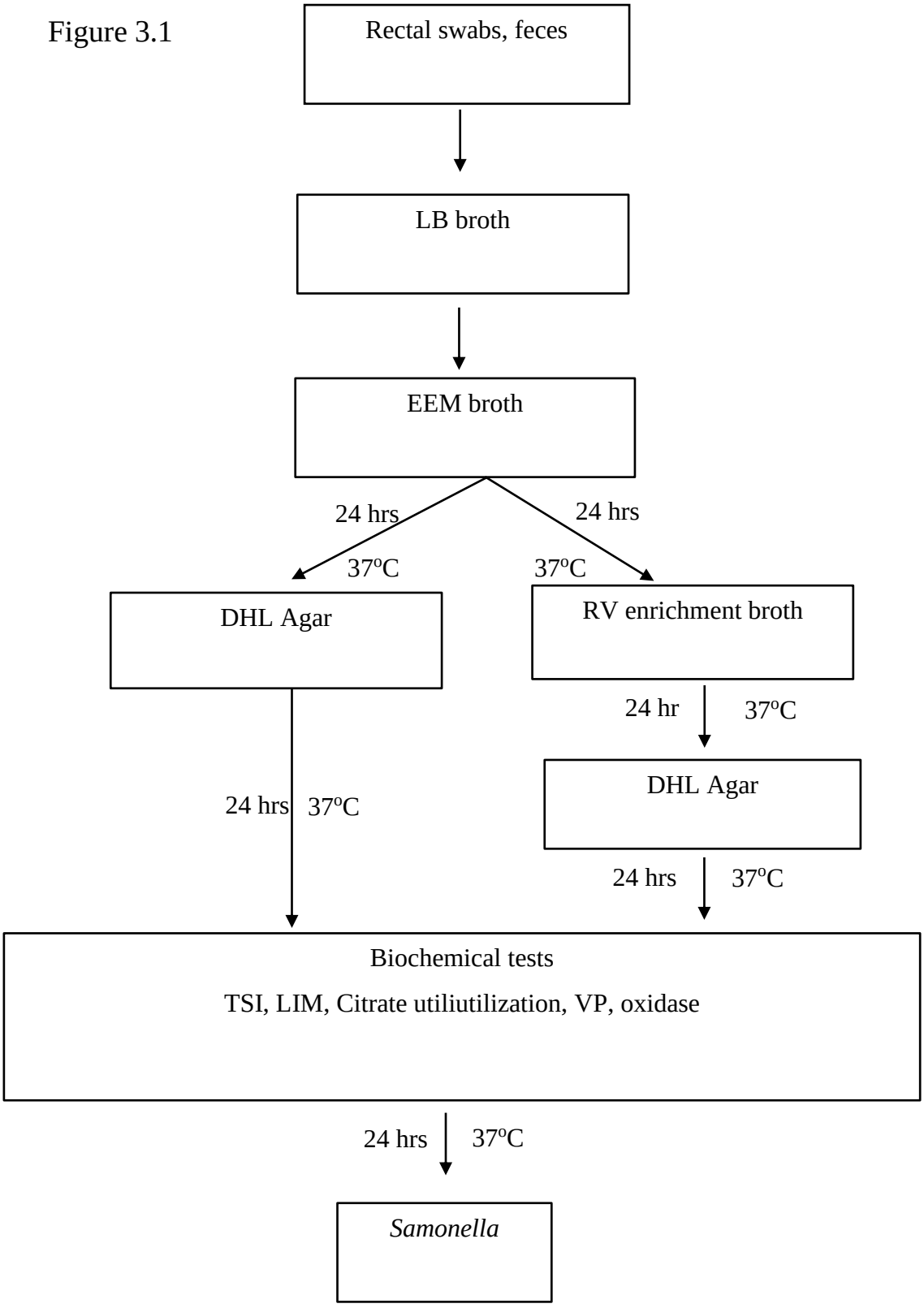

Figure 3.1. Flow diagram showing the identification procedures of *Salmonella* isolated from small mammals trapped in Hanoi, Vietnam, and Bogor, Indonesia.

Figure 3.2

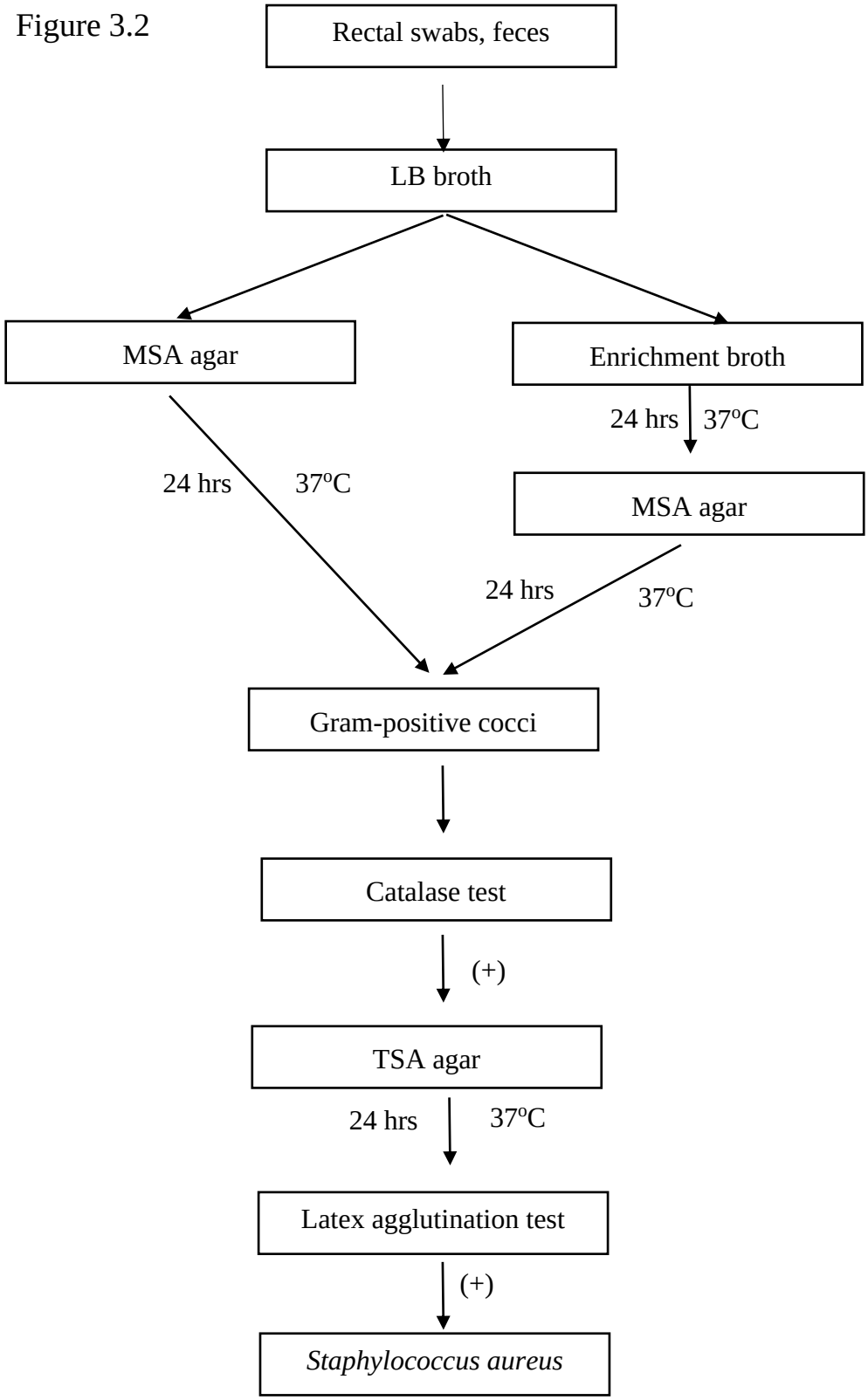

Figure 3.2. Flow diagram showing the identification procedures of *Staphylococcus aureus* isolated from small mammals trapped in Hanoi, Vietnam, and Bogor, Indonesia.

Table 3.1. List of primers for the identification of diarrheagenic *E. coli*

Pathogen[a)]	Primer name	Target gene	Primer sequence (5'-3')	Amplicon (bp)	Reference
ETEC	LT-F	*elt*	CACACGGAGCTCCTCAGTC	508	[154]
	LT-R		CCCCCAGCCTAGCTTAGTTT		
	ST-F	*est*	GCTAAACCAGTAGAGGTCTTCAAAA	147	[154]
	ST-R		CCCGGTACAGAGCAGGATTACAACA		
EPEC	BFPA-F	*bfpA*	GGAAGTCAAATTCATGGGGG	367	[154]
	BFPA-R		GGAATCAGACGCAGACTGGT		
	SK1	*eae*	CCCGAATTCGGCACAAGCATAAGC	881	[155]
	SK2		CCCGGATCCGTCTCGCCAGTATTCG		
STEC	VTcom-u	*stx*	GAGCGAAATAATTTATATGTG	518	[155]
	VTcom-d		TGATGATGGCAATTCAGTAT		
	SK1	*eae*	CCCGAATTCGGCACAAGCATAAGC	881	[155]
	SK2		CCCGGATCCGTCTCGCCAGTATTCG		
EIEC	ipaIII	*ipaH*	GTTCCTTGACCGCCTTTCCGATACCGTC	619	[155]
	ipaIV		GCCGGTCAGCCACCCTCTGAGAGTAC		
	CVD432F	*aatA*	CTGGCGAAAGACTGTATCAT	630	[154]
	CVD432R		CAATGTATAGAAATCCGCTGTT		
EAEC	AAIC F	*aaiC*	ATTGTCCTCAGGCATTTCAC	215	[154]
	AAIC R		ACGACACCCCTGATAAACAA		
	aggRks1	*aggR*	GTATACACAAAAGAAGGAAGC	254	[155]
	aggRks2		ACAGAATCGTCAGCATCAGC		

a) ETEC: Enterotoxigenic *E. coli*, EPEC: Enteropathogenic *E. coli*, STEC: Shiga toxin-producing *E. coli*, EIEC: Enteroinvasive *E. coli*; EAEC: Enteroaggregative *E. coli*

Table 3.2. Antimicrobial resistance patterns for *Salmonella* spp. isolates from rodents in Bogor, Indonesia

ID			BG-39	BG-47	BG-78	BG-79
Location			Anyar market	Anyar market	Bogor market	Bogor market
Antimicrobial agents[a)]	ABP	Beta-lactams	S[b)]	S	S	S
	ACV		S	S	S	S
	CDZ		S	S	S	S
	CTX		S	S	S	S
	CIP	Quinolone	S	S	S	S
	NA		S	S	S	S
	CP	Chloramphenicol	S	S	S	S
	GM	Aminoglycoside	S	S	S	S
	ST	Sulfonamide	S	S	S	S
	TC	Tetracycline	S	S	S	S

a) ABP: Ampicillin, ACV: Amoxicillin - Clavulanate, CDZ: Cefodizime, CTX: Cefotaxime, CIP: Ciprofloxacin, NA: Nalidixic acid, CP: Chloramphenicol, GM: Gentamicin, ST: Sulfamethoxazole - Trimethoprim, TC: Tetracycline

b) S: Susceptible

Table 3.3. Characteristics of *S. aureus* isolated from rodents in Hanoi, Vietnam

ID	Location	Species	PVL[a)]	*mecA*	Antimicrobial agents[b)]						
					CFX[c)]	CIP	CP	GM	TC	ST	VA[c)]
HN-61	Thanh Cong market	*Rattus norvegicus*	-	-	4	S	S	S	S	S	0.5
HN-70	Thanh Cong market	*Rattus norvegicus*	-	-	2	R	S	S	S	R	0.5
HN-73	Giap Bat cargo station	*Rattus norvegicus*	-	-	2	S	S	S	S	S	0.5
HN-74	Giap Bat cargo station	*Rattus norvegicus*	-	-	2	S	S	S	S	S	0.5
HN-76	Giap Bat cargo station	*Rattus norvegicus*	-	-	2	R	S	R	S	R	0.5
HN-128	Thai Ha market	*Rattus norvegicus*	-	-	<2	S	S	S	S	S	0.5
HN-133	Ha Dong market	*Rattus norvegicus*	-	+	≥16	R	R	R	S	R	1
HN-135	Phung Khoang market	*Rattus norvegicus*	-	-	<2	S	S	S	S	S	0.5

a) PVL: PantonValentine leukocidin toxin

b) CFX: Cefoxitin, CIP: Ciprofloxacin, CP: Chloramphenicol, GM: Gentamicin, ST: Sulfamethoxazole - Trimethoprim, TC: Tetracycline, VA: Vancomycin.

c) Minimum inhibitory concentration (µg/ml), CFX: S≤4, R>4, VA: S≤2, R≥16.

Table 3.4. Characteristics of *S. aureus* isolated from rodents in Bogor, Indonesia

ID	Location	Species	PVL[a)]	*mec*A	Antimicrobial agents[b)]						
					CFX[c)]	CIP	CP	GM	TC	ST	VA[c)]
BG-4	Bogor market	*Rattus norvegicus*	-	-	<2	S	S	S	S	S	0.5
BG-22	Anyar market	*Rattus norvegicus*	-	-	<2	S	S	S	S	S	0.5
BG-32	Anyar market	*Rattus norvegicus*	-	-	<2	S	S	S	S	S	0.5
BG-35	Anyar market	*Rattus norvegicus*	-	-	<2	S	S	S	S	S	0.5
BG-37	Anyar market	*Rattus norvegicus*	-	-	<2	R	S	S	R	S	0.5
BG-38	Anyar market	*Rattus norvegicus*	-	-	<2	R	S	S	S	S	0.5
BG-42	Anyar market	*Rattus norvegicus*	-	-	<2	S	S	S	S	S	1
BG-44	Anyar market	*Rattus norvegicus*	-	-	<2	R	S	S	S	S	0.5
BG-45	Anyar market	*Rattus norvegicus*	-	-	<2	S	S	S	S	S	0.5
BG-49	Anyar market	*Rattus norvegicus*	-	-	<2	S	S	S	R	S	0.5
BG-50	Anyar market	*Rattus norvegicus*	-	-	<2	S	S	S	S	S	0.5
BG-57	Anyar market	*Rattus norvegicus*	-	-	<2	S	S	S	S	S	0.5
BG-61	Anyar market	*Rattus norvegicus*	-	-	<2	S	S	S	S	S	0.5
BG-65	Merdeka market	*Rattus norvegicus*	-	-	<2	S	S	S	S	S	1
BG-71	Jambu Dua market	*Rattus norvegicus*	-	-	<2	S	S	S	S	S	0.5
B79	Bogor market	*Rattus norvegicus*	-	-	<2	S	S	S	S	S	0.5

a) PVL: PantonValentine leukocidin toxin

b) CFX: Cefoxitin, CIP: Ciprofloxacin, CP: Chloramphenicol, GM: Gentamicin, ST: Sulfamethoxazole - Trimethoprim, TC: Tetracycline, VA: Vancomycin.

c) Minimum inhibitory concentration (µg/ml), CFX: S≤4, R>4, VA: S≤2, R≥16.

General conclusion

AMR is a serious public health problem rapidly expanded across the world. Small mammals such as rodents and house shrews are not naturally colonized with AMR bacteria. They accidentally acquire AMR bacteria through their habitats. They may represent the presence of AMR *E. coli* in the environment, particularly ESBL-producing and colistin-resistant *E. coli*. Further, small mammals could also obtain pathogens such as *S. aureus* and foodborne pathogens including DEC and *Salmonella* spp. in the environment. Humans may ingest contaminated food or water, resulting in infection with various diseases. The identification of AMR bacteria in the environment is a crucial driver for implements the preventive methods for AMR transmission.

In chapter I, urban rodents in Hanoi were found as acquired AMR *E. coli* including ESBL-producing and colistin-resistant *E. coli* from the environment. The prevalence of *E. coli* resistant to tetracycline and ampicillin was relatively high. It may reflect the extensive use of these antimicrobials in humans [99, 156]. In this study, three and one ESBL-producing *E. coli* were isolated from rodents dwelling in a hospital and a nearby market, respectively. The prevalence of ESBL-producing *E. coli* isolated from rodents was much lower than the prevalence observed in hospital settings in Hanoi, Vietnam [157]. It is suggesting that rodents might receive ESBL-producing *E. coli* originating from hospitalized patients. In addition, rodents dwelling in hospital settings were found carried of colistin-resistant *E. coli*. It indicated that hospitals in Hanoi might become a source of ESBL-producing and colistin-resistant *E. coli*. Colistin-resistant *E. coli* was also found in rodents residing in markets. Although colistin-resistant *E. coli* has never been reported in humans living in the city, Vietnam, the prevalence of colistin-resistant *E. coli* in rural areas was relatively high in healthy individuals [85]. This study revealed that colistin-resistant *E. coli* have existed in Hanoi, assuming that humans may contribute to the spread of these bacteria in the city. This is the first study on AMR *E. coli* isolated from urban rodents in Vietnam, indicating that AMR *E. coli* presence in urban areas

were able to infect mammals from the environment. Since human facilities are heavily infested with rodents in the city, the potential risk of rodents carrying AMR *E. coli* to public health may higher than in rural areas.

In chapter II, the most common AMR phenotype of *E. coli* isolated from small mammals was against tetracycline and ampicillin in Bogor, Indonesia. In comparison to the report in 1988, both of the resistance to tetracycline and ampicillin increased in AMR *E. coli* isolated from small mammals. This result indicated a long-term usage of these antimicrobials as therapeutic and preventive use to humans and livestock in Indonesia. The prevalence of AMR *E. coli* isolated from small mammals was similar to those observed in hospital settings in this country [31]. This result indicated that AMR *E. coli* found in small mammals might be originated from humans. However, there was no candidate for ESBL-producing and colistin-resistant *E. coli* due to the sampling sites. In chapter I sampling in Hanoi, Vietnam, small mammals dwelling in hospital were likely acquired of ESBL-producing and colistin-resistant *E. coli*. On the other hand, the sampling sites were conducted only at markets in Indonesia. In this study, the prevalence of MDR *E. coli* was two times higher than the prevalence of MDR *E. coli* in a previous study conducted in 1988. It elucidated that AMR *E. coli* were increased in the environment in Bogor city, Indonesia, particularly in *E. coli* resistant to ampicillin and tetracycline. The overuse or misuse of antimicrobial agents in treatment and disease prevention for humans might cause the increase of AMR *E. coli* contaminated in the environment in Bogor, Indonesia.

In chapter III, small mammals infected with *S. aureus* and foodborne pathogens such as DEC and *Salmonella*. The prevalence of DEC isolated from urban rodents was lower in comparison to those isolated from livestock and humans in Vietnam [142-144]. There was no isolation of DEC in Indonesia. The prevalence of *Salmonella* was often higher from rodents residing in nature than living in urban areas [46, 55, 73, 116, 145]. None of *Salmonella* was

isolated in Vietnam. The prevalence of *Salmonella* isolated from rodents was lower than the previous study, those isolated from beef, fish, vegetable in the supermarkets in Bogor, Indonesia [158]. These results suggested that it is difficult to conclude the prevalence of DEC and *Salmonella* in the environment using small mammals due to the susceptibility to infection with those in small mammals. In this study, both MSSA and MRSA were detected in rodents in Hanoi and Bogor. AMR profiles of MSSA strains isolated from small mammals have never been revealed. The data of this study contributes to the knowledge accumulation of *S. aureus* associated with rodents in the city in Vietnam and Indonesia. The previous studies have been described that rodents were infected with both of LA-MRSA and CA-MRSA in the urban areas [71, 72]. An MRSA isolated in this study will be required to examine the identification into LA-MRSA and CA-MRSA in further study.

In general, the small mammals do not treat with antimicrobial agents. My study focused on the prevalence of AMR *E. coli*, including MDR, ESBL-producing and colistin-resistant *E. coli* in small mammals. These results indicated the widespread AMR bacteria in the environment and the potential risk of AMR bacteria transmission from environment to mammalian hosts including humans. Although the AMR *E. coli*, MRSA and foodborne pathogens were detected in the feces of small mammals in this study, the concentration of these bacteria and resistance genes were not determined. The concentration of these bacteria and their AMR genes would be valuable information for future studies. Hygiene practice including pest control in the urban areas is critical for preventing unexpected transmission of AMR *E. coli* and foodborne pathogens to humans.

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